Metabolomic and transcriptomic responses of ticks during recovery from cold shock reveal mechanisms of survival

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ABSTRACT

Ticks are blood-feeding ectoparasites but spend most of their life off-host where they may have to tolerate low winter temperatures. Rapid cold-hardening (RCH) is a process commonly used by arthropods, including ticks, to improve survival of acute low temperature exposure. However, little is known about the underlying mechanisms in ticks associated with RCH, cold shock, and recovery from these stresses. In the present study, we investigated the extent to which RCH influences gene expression and metabolism during recovery from cold stress in *Dermacentor variabilis,* the American dog tick, using a combined transcriptomics and metabolomics approach. Following recovery from RCH, 1,860 genes were differentially expressed in ticks, whereas only 99 genes responded during recovery to direct cold shock. Recovery from RCH resulted in an upregulation of

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various pathways associated with ion binding, transport, metabolism, and cellular structures seen in the response of other arthropods to cold. The accumulation of various metabolites, including several amino acids and betaine, corresponded to transcriptional shifts in the pathways associated with these molecules, suggesting congruent metabolome and transcriptome changes. Ticks receiving exogenous betaine and valine demonstrated enhanced cold tolerance, suggesting cryoprotective effects of these metabolites. Overall, many of the responses during recovery from cold shock in ticks were similar to those observed in other arthropods, but several adjustments may be distinct from other currently examined taxa.

INTRODUCTION

Ticks are hematophagous ectoparasites and are vectors for a myriad of disease-causing pathogens in vertebrate hosts throughout their ranges (Sonenshine and Roe, 2013). The American dog tick, *Dermacentor variabilis*, is one of the most widely distributed ticks in North America and is a prolific vector for Rocky Mountain spotted fever and tularemia (de la Fuente et al., 2008; Goethert and Telford, 2009). In recent years, the geographic distribution of *D. variabilis* has changed, including an expansion of this species northward (Dergousoff et al., 2013). This augmentation of geographic range is occurring in nearly all ixodid ticks (Daniel et al., 2003; Lindgren et al., 2000; Raghavan et al., 2019; Sonenshine, 2018), and is likely increasing the risk of tick-borne diseases due to altered human and animal interaction with ticks.

There are a multitude of factors that influence the distribution of ticks; however, low temperatures in winter are one factor that limits the latitudinal and altitudinal distribution of various tick species (Daniel et al., 2003; Dantas-Torres and Otranto, 2011; Dergousoff et al., 2013; Lindgren et al., 2000). *D. variabilis* overwinters in any of its life stages particularly at the northern limits of its range (Belozerov et al., 2002; Burg, 2001; Smith and Cole, 1941; Sonenshine, 1993). To enhance overwintering survival, ticks employ a variety of strategies including accumulation of cryoprotectants (Neelakanta et al., 2010; Yu et al., 2014), entering a diapause-like state (Yoder et al., 2016), and seeking sheltered hibernacula

(Burks et al., 1996; Rosendale et al., 2016a). These approaches likely improve survival by reducing the direct and/or indirect effects of low temperature as *D. variabilis*, like most tick species, are chill-susceptible and experience mortality above their super-cooling points. (Burks et al., 1996; Dautel and Knülle, 1996; Needham et al., 1996; Rosendale et al., 2016a; Yu et al., 2014). Another potential strategy for survival of sub-zero temperatures is employing the rapid cold-hardening response, which has been shown to occur in several tick species, including *D. variabilis* (Rosendale et al., 2016a; Wang et al., 2017; Yu et al., 2014).

During rapid-cold hardening (RCH), arthropods can dramatically improve their cold hardiness after a brief exposure to low temperatures (Lee et al., 1987). In contrast to long-term cold acclimation, which occurs over days to weeks, RCH occurs in minutes to hours and has been documented in a wide range of arthropods (Elnitsky and Lee, 2010). RCH is a critical component of ectotherm survival when daily fluctuating temperatures swing between above and below freezing. The mechanisms of RCH are incompletely understood; however, various physiological changes have been implicated in the process. RCH is regulated by several signaling events, including MAP kinase, apoptosis, and calcium signaling (Teets and Denlinger, 2013). These signaling pathways, among others, contribute to the accumulation of various cryoprotectants, cause changes in the fatty acid composition of cell membranes, inhibit apoptotic pathways, and cause changes in chaperone protein abundance (Teets and Denlinger, 2013). However, there is still much unknown about the underlying mechanisms of RCH, with even less known about this phenomenon in non-insect arthropods.

The role that transcriptional changes play in the RCH response is unclear due to discrepancies among species and study design (Teets et al., 2020). Although an upregulation of several genes has been observed during RCH (reviewed in Teets et al., 2020), most studies indicated that the RCH response occurs with little to no change in transcript expression (Sinclair et al., 2007; Teets et al., 2012; Vesala et al., 2011). However, the transcriptional profile of flies recovering from RCH and cold-shock is distinct from untreated controls (Teets et al., 2012). Similarly, the accumulation of cryoprotectants during RCH exposure is minimal (Teets et al., 2020), whereas a recovery period following

RCH and cold-shock can dramatically impact the metabolic profile (Teets et al., 2012). These transcriptional and metabolomic adjustments during recovery from RCH and cold shock may contribute to cold injury repair and/or prepare the organism for subsequent cold exposure.

In ticks, there is a paucity of information on the physiological and molecular responses to low temperatures, including recovery from cold exposure. It is also unclear how important the RCH response is in tick overwintering. *D. variabilis, Haemaphysalis longicornis*, and *D. silvarum* undergo RCH (Rosendale et al., 2016a; Wang et al., 2017; Yu et al., 2014), but *I. scapularis* seems to lack this response (Vandyk et al., 1996). The bush tick, *H. longicornis*, responds to short-term cold acclimation through changes in water content and protein levels, but not glycerol; however, these alterations seem to be life-stage specific (Yu et al., 2014). The actual mechanisms of cold hardiness in ticks remains largely unknown; therefore, the purpose of this study was to examine the physiological and molecular responses of *D. variabilis* during recovery from cold and RCH exposure using a combined transcriptomic and metabolomic approach followed by targeted functional studies. Previous reports suggest that a recovery period is required to elicit transcriptional changes following RCH (reviewed in Teets et al., 2020); therefore, this study included a recovery period. With this experimental design, the hypothesis that gene expression during recovery from cold shock is modulated by an RCH pretreatment can be tested.

MATERIALS AND METHODS

Ticks and experimental treatments

Unfed *D. variabilis* and *Amblyomma maculatum* were acquired from the Oklahoma State University (OSU) Tick Rearing Facility (Stillwater, OK, USA). The laboratory colonies at OSU are kept under constant conditions (14:10 h, light:dark (L:D), 97% relative humidity (RH), and 25±1°C) year round. Upon arrival, groups of 10-15 ticks were transferred to 15 cm^3 mesh-covered vials and placed in closed chambers containing a supersaturated solution of potassium nitrate, providing 93% RH (Winston and Bates, 1960). Ticks were kept in these chambers at 26±1°C and 15:9 h L:D until used in experiments, usually within one month.

For treatments, ticks were randomly chosen from multiple rearing batches for each replicate.

To examine the effect of rapid cold hardening on cold tolerance, cold-shock survival was determined using a 2 h exposure to subzero temperatures. Groups of 10 ticks (N = 7 groups) were placed in 1.5 cm^3 mesh-covered tubes and these tubes were placed in 50 ml vials, which were plugged with insulating foam then suspended in a chilled water: ethylene glycol (40:60) solution. Temperatures were maintained (± 0.1°C) using a programmable refrigerated bath (Arctic A25; Thermo Scientific, Pittsburgh, PA, USA). The temperature inside the tick-containing tubes equilibrated with the bath temperature within approximately 10 min, so an incubation time of 2 h 10 min was used for all trails. Cold-shocked ticks were transferred directly from 26°C to the subzero temperature, whereas RCH ticks were exposed to 4°C for 2 h prior to the subzero temperature. Following treatment, ticks were returned to rearing conditions and assessed for survival after 48 h recovery and were considered alive if they could self-right and/or move several body lengths spontaneously or in response to being handled.

For RNA-sequencing and metabolomics analyses, female *D. variabilis* were individually transferred to 1.5 cm^3 mesh-covered tubes and exposed to the following temperature treatments using the same general method described above: control (C), 26°C for 6 h; cold shock (CS), 26°C for 2 h followed by -5°C for 2 h, and a 2 h recovery at 26°C; rapid cold hardening (RCH), 4°C for 2 h, followed by -5°C for 2 h, and a 2 h recovery at 26°C. At the end of the treatment, ticks were flash frozen and stored at -80°C. Following low-temperature exposure, some ticks show signs of life after 2 h of recovery, only to succumb to death several hours later. Therefore, a temperature that resulted in no mortality (-5°C) was chosen for RNA-seq treatments to prevent the confounding effects on transcriptional changes in dying ticks.

Sequencing and de novo assembly

To obtain adequate RNA concentrations and reduce individual variation, each RNA sample consisted of 8 ticks that were removed from -80°C and immediately homogenized together. Ticks were manually cut into small pieces, placed in 1 ml of chilled TRIzol® reagent

(Invitrogen, Carlsbad, CA, USA), and homogenized using a BeadBlaster 24 microtube homogenizer (Benchmark Scientific, Edison, NJ, USA). Total RNA was extracted in TRIzol by following the manufacturer's protocol and then treated with DNase I (Thermo Scientific, Pittsburgh, PA, USA) to eliminate potential genomic DNA contamination. RNA was concentrated using the GeneJET RNA Cleanup and Concentration Micro Kit (Thermo Scientific), and the final RNA concentration and purity were determined with a Nanodrop (Thermo Fisher Scientific, Waltham, MA, USA).

The DNA Sequencing and Genotyping Core at the Cincinnati Children's Hospital Medical Center (CCHMC) constructed the poly(A) library and performed the sequencing. RNA was quantified using the Qubit 3.0 Fluorometer (Life Technologies, Carlsbad, CA, USA), and 150 to 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). Samples were 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. Reads were single-end and 75 bases in length with approximately 30 million high-quality reads being generated per sample. Raw RNA-seq data were uploaded to the National Center for Biotechnology Information's (NCBI) Sequence Read Archive: Bio-project PRJNA657863.

Sequences generated by Illumina sequencing were trimmed for ambiguities (0 ambiguities were allowed) and quality (using a Phred quality score limit of 0.05). The 5′ and 3′ termini were trimmed to remove 5 and 8 nucleotides from the sequence, respectively, and resulting sequences less than 40 nucleotides in length were discarded. The quality of resulting cleaned sequences was verified with the FastQC package (Andrews, 2010).

Transcripts were *de novo* assembled using three separate assembly programs: Trinity (Grabherr et al., 2011), Velvet-Oases (Schulz et al., 2012), and CLC Genomics Workbench (Qiagen, Redwood City, CA, USA), with a minimum contig length of 200. Contigs generated from these programs were combined into a single assembly and redundant contigs were removed by analyzing the assembly with the CD-HIT-EST (Huang et al., 2010) clustering algorithm using an 95% similarity threshold. Quality of the assembly was assessed by the

identification of orthrologs based on BUSCO and CEGMA gene sets (Parra et al., 2007; Simão et al., 2015).

Differential expression and enrichment analyses

Reads were mapped to the *de novo* assembly using CLC Genomics with at least 70% of the read having at least 80% identity with the reference and a mismatch cost of 2. Reads were allowed to align to no more than 10 different places on the reference assembly. Expression values were measured as reads per kilobase of transcript, per million mapped reads (RPKM). Significant changes in expression among samples were analyzed using the 'differential expression for RNA-Seq tool' in CLC Genomics. This analysis utilizes a negative binomial Generalized Linear Model and differences between treatment groups are determined with a Wald test. Transcripts were considered differentially expressed when the FDR *P*-value was ≤ 0.05 and the fold change was ≥ |2|.

To identify differentially expressed contigs, sequences were searched (BLASTx) with an expectation value (e-value) threshold of 0.001 against NCBI's arthropod, non-redundant (nr) protein database, the SwissProt protein database, and the reference protein sequences (RefSeq) for fly (*Drosophila melanogaster*) and the black-legged tick *Ixodes scapularis*. The highest scoring blast hit in each search was used to assign a gene ID to each contig. Enrichment of functional pathways was identified by submitting hits from the *I. scapularis* search to the PANTHER Gene List Analysis (Mi et al., 2017), DAVID functional annotation database (Huang et al., 2009) and g:Profiler Gene Group Functional Profiling (Reimand et al., 2016). GO terms were analyzed for over-representation, and functional annotations were considered significantly enriched when the corrected *P*-value was ≤ 0.05. A full list of GO terms that were enriched in two or more of the analyses was generated for each treatment and the number of significant GO terms was reduced by using REVIGO (Supek et al., 2011) to eliminate redundant terms. Lastly, specific unidentified contigs were searched (BLASTx) with an expectation value (e-value) threshold of 0.001 against the recently completed tick genomes (Jia et al. 2020) to identify tick-specific and *Dermacentor*-specific contigs.

qPCR validation

To validate the RNA-seq results, qPCR validation was performed as described in (Rosendale et al., 2016b) with a separate group of ticks (*N*=5-6) exposed to cold shock or RCH treatments with recovery periods as described for RNA-seq. Ticks were individually homogenized in 1 ml of chilled TRIzol® reagent and RNA was extracted and concentrated as described for RNA-seq. Complementary DNA (cDNA) was made from approximately 300 ng RNA the using the DyNAmo cDNA Synthesis Kit (Thermo Scientific). qPCR reactions consisted of KiCqStart SYBR Green qPCR ReadyMix (Sigma Aldrich, St. Louis, MO, USA), 300 nM forward and reverse primers, cDNA diluted 1:50, and nuclease free water. Primers were designed based on sequences obtained from the RNA-seq analysis (Table S1). These contigs were chosen as their expression levels in RNA-seq were neither particularly high nor low, and their fold changes did not exceed the median fold change for differentially expressed contigs. qPCR reactions were analyzed using an MX3005p Real-time PCR System (Agilent Technologies). PCR consisted of activation for 3 min at 95°C followed by 40 cycles of denaturation (10 sec at 95°C), annealing/extension (30 sec at 55°C), and denaturation (10 sec at 95°C). Following amplification, a melt curve analysis was performed from 55°C to 95°C with 0.5°C increments every 15 sec**.** Samples were run in triplicate and the average C_q value was determined. The $\Delta\Delta C_q$ method (Schmittgen and Livak, 2008) was used to determine expression levels, using β-actin to normalize genes of interest. Actin was chosen as the reference gene as it has been used successfully as a reference gene in other tick-stress studies (Rosendale el a., 2016b); additionally, there was no difference in expression of this gene among our samples (C vs. CS FDR *P*-value = 0.99; C vs RCH FDR *P*-value = 0.92). The fold change in these genes 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

A nuclear magnetic resonance (NMR)-based metabolomics approach was used to identify metabolites present in ticks. NMR experiments were performed at the NMR-based Metabolomics Core at CCHMC. A total of 15 ticks per sample were lyophilized overnight

and the dried samples were weighted into 2 ml standard tubes containing 2.8 mm metal beads (Bertin Corp, Rockville, MD, USA). Ticks were homogenized 3 times for 30 s at 4000 rpm with a Minilys homogenizer (Bertin Corp) and 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 (methanol: chloroform: water ratio was 2:2:1.8). The mixture was vortexed, incubated on ice for 10 min, and centrifuged at 2000 x *g* for 5 min. The polar phase was transferred into a 1.5 ml tube and dried by vacuum centrifugation for 2-3 h at room temperature. The dried metabolites were re-suspended in 0.6 ml of NMR buffer containing 100 mM phosphate buffer (pH 7.3), 1 mM TMSP (3-Trimethylsilyl 2,2,3,3-d4 propionate), and 1 mg/ml 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) for NMR data acquisition. Data were acquired using one-dimensional ^{1}H Nuclear Overhauser Effect Spectroscopy (NOESY) NMR experiments on a 600 MHz INOVA NMR spectrometer (Agilent Technologies, Santa Clara, CA, USA). Representative samples were analyzed with two-dimensional ^{1}H-^{13}C heteronuclear single quantum correlations (HSQC) and ^{1}H-^{1}H Total Correlation Spectroscopy (TOCSY) for metabolite annotation. Spectral data were phase corrected, baseline corrected, and aligned to the internal reference standard peak, trimethylsilyl propanoic acid (TMSP), using TopSpin 3.1(Bruker, Billerica, MA, USA).

Metabolite identity 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). Metabolite concentrations were determined using Chenomx NMR Suite integration tool and normalized to the dry mass of the ticks. Normalized metabolite concentrations were compared using a one-way ANOVA with Tukey post-hoc tests.

Metabolite loading

To evaluate their cryoprotective effects, several of the metabolites that were found to be elevated following recovery from RCH were injected into female *D. variabilis* and ticks were subsequently exposed to subzero temperatures. *D. variabilis* (*N*=10 ticks per group) were injected with either insect Ringer's solution (187 mM NaCl, 21 mM KCl, 7 mM $CaCl_2$, and 1 mM $MgCl_2$) or insect Ringer's solution with 1, 0.1, or 0.01 M alanine, betaine, glycine, or valine. Additionally, male and female *D. variabilis* and female *A. maculatum* were injected with a Ringer's or 1 M betaine solution to allow for a sex and species comparison, respectively. The cuticle was pierced with a 28.5-gauge needle on the ventral idiosoma just posterior to the fourth coxa, 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 2 h at 26°C and 100% RH and were then exposed to subzero temperatures (-13.5°C for *D. variabilis* and -12.0°C for *A. maculatum*). Following cold shock, ticks were returned to 26°C and 100% RH and survival was assessed 48 h post-treatment. Survival was compared among Ringer's and metabolite-injected ticks using a Student's *t*-test or ANOVA.

RESULTS

Effect of RCH on cold hardiness

To determine if adult *D. variabilis* demonstrate a typical RCH response, survival of several subzero temperatures was examined. There was no difference ($F_{2,15} = 0.18$, $P = 0.84$) in the dry mass of ticks in the control, cold-shock, or RCH groups (3.11 ± 0.09, 3.07 ± 0.16, 3.17 ± 0.11 mg, respectively), nor did treatments affect ($F_{2,15} = 0.25$, $P = 0.78$) water content of the ticks (approximately 1.3 mg water mg^{-1} dry mass, 57%, for all groups). Survival was high (100%) for both CS and RCH groups exposed to -5°C; however, survival of the CS group dropped below 50% at temperatures lower than -8°C (Fig. 1). Overall, survival of RCH ticks was greater ($F_{1,72} = 48.4$, $P < 0.0001$) than that of CS ticks, with a 2 h pre-exposure to 4°C improving survival of -8 to -12°C by 50-30%.

Transcriptome analysis

Nine cDNA libraries were generated from the total homogenates of control ticks and those recovered from cold shock or RCH (3 per group). These libraries were sequenced using Illumina HiSeq technology, resulting in 391,281,556 cleaned reads. Sequences from all samples were used in the *de novo* transcriptome assembly using the Trinity, Velvet-Oasis, and CLC Genomics Workbench assembly programs. Contigs from each of these three assemblies were combined into a single assembly, and duplicate contigs were removed with CD-HIT-EST (Table 1). Contig sizes ranged from 200 to ~14,000 bases with the number of contigs decreasing with increasing contig length. Contigs were annotated by searching (BLASTx) NCBI's arthropod nr and SwissProt databases as well as RefSeq sequences from *D. melanogaster* and the *Ixodes* genus. The majority of the matches were to the black-legged tick, *I. scapularis* (Fig. 2). BUSCO and CEGMA scores were comparable to previous *D. variabilis* (Rosendale et al. 2016; 2018), where over 96% of BUSCO genes from *Ixodes scapularis* predicted gene set were present in our assembly.

Differential expression of transcripts was analyzed among the control ticks and those recovered from cold shock or RCH. Seventy-three percent (62-80%) of the reads from control ticks, 80% (78-84%) from cold-shocked, and 78% (77-79%) of reads from RCH ticks mapped to the reference assembly. We found 99, 1,860, and 1,376 genes with expression levels significantly different between control and CS, control and RCH, and CS and RCH, respectively (Table 2). When compared to control ticks, most (~65%) of the genes differentially expressed in CS ticks also showed expression changes when the cold shock was preceded with RCH (Fig. 3). The full set of differentially regulated genes is presented in Table S2. To validate our RNA-Seq results, qPCR was used to measure the expression of several genes, normalized to β-actin. qPCR and RNA-Seq results were similar based on the Pearson correlation coefficient (r=0.952; Fig. S1).

In ticks exposed to cold shock, there was an upregulation of several genes related to glucose metabolism, chaperone & repair; with most genes being salivary gland proteins. When analyzed for GO terms, only carbohydrate biosynthetic process and monosaccharide biosynthetic process where enriched in the upregulated genes of CS ticks . There was no GO term enrichment in the downregulated genes of CS ticks. In contrast to the CS group, ticks

exposed to the RCH conditions showed a large number of differentially expressed genes. For genes upregulated during recovery from RCH and a subsequent cold shock, there was an enrichment of a variety of GO terms (Figs. 4, S2). GO terms that were enriched in RCH ticks included biological processes such as protein metabolic process, transport, and oxidation-reduction process; molecular functions such as catalytic activity and binding; and cellular components such as ribosomes and cytoskeleton. For genes that were downregulated in response to RCH, there was an enrichment of various GO terms including metabolic processes, binding, and organelles. The full list of GO terms that were differentially expressed in two or more analyses (see methods) for all comparisons is shown in Table S3.

Metabolome analysis

NMR-based metabolomics was used to identify differences in metabolite concentrations among control ticks and those recovered from cold shock or RCH. Using spectra from ^{1}H NMR, the concentrations of 29 metabolites were identified (Table S4), of which six showed significant difference between the control and RCH groups (Table 3). There were no differences in metabolite concentration between the cold-shock group and the other two groups.

Functional study

Based on our combined metabolomic and transcriptomic results, several metabolites were identified that accumulated during the RCH-recovery process. To determine if these metabolites have cryoprotective effects, ticks were injected with several concentrations (1, 0.1, or 0.01 M) of alanine, betaine, glycine, or valine solutions and subjected to subzero temperatures. Injection of 0.5 µL of these solutions would theoretically increase the concentration of these metabolites by approximately 150, 15, or 1.5 nmol/mg dry mass; these lower concentrations are within the range of observed increases of these metabolites (Table 3). Survival of exposure to -13.5°C was significantly ($P < 0.05$) affected by injection of betaine or valine as compared to ticks injected with insect Ringer's (Fig. 5A-C). However, there was no effect of concentration of these metabolites as *D. variabilis* injected with

either betaine or valine at 1, 0.1, and 0.01 M showed no statistical difference in survival (Fig. 5A-C). In *D. variabilis*, female survival was ~4-fold higher (t_{20} = 5.07, P < 0.0001) in the betaine-injected groups, while in males survival was ~2-fold higher (t_{22} = 2.26, P = 0.034) (Fig. 5D). *A. maculatum* exposed to -12°C showed a classic RCH response, and cold hardiness was also improved through injection of betaine (Fig. 5E). *A. maculatum* receiving RCH treatment showed an 8-fold higher (P < 0.01) survival than the cold-shock group, whereas betaine injection improved survival 2.7-fold (P < 0.05) over the Ringer's solution group.

DISCUSSION

The American dog tick can transmit various diseases, including Rocky Mountain spotted fever, and has a very wide geographic distribution, making it an important disease vector in North America (de la Fuente et al., 2008). Augmentation of the geographic range of *D. variabilis* suggests an increase in the impact of this species as a vector (Dergousoff et al., 2013), and changes in distribution are occurring in a variety of tick species (Sonenshine, 2018). The geographic range of ticks is often limited by climate, including the ability to survive low winter temperatures (Dantas-Torres and Otranto, 2011; Dautel et al., 2016; Randolph, 2004); therefore, understanding winter survival may be important in predicting population dynamics. However, little is known about the mechanisms underlying low temperature survival in ticks. In the present study, we examined the response of ticks to cold-shock and rapid-cold hardening and found changes at the transcriptome and metabolome levels that revealed multiple biological pathways that are likely important in the recovery from these conditions. We also demonstrated the ability of betaine to improve cold hardiness in ticks.

Rapid-cold hardening

By exposing ticks to a brief (2 h) period at 4°C prior to exposure to subzero temperatures, cold hardiness was dramatically improved. Adult *D. variabilis* displayed the classic rapid-cold hardening response, which is common in arthropods and has been documented in

multiple classes (Teets et al., 2020). Although RCH has been most thoroughly examined in insects, it has been documented in Acari in both mites (Broufas and Koveos, 2001; Ghazy and Amano, 2014) and ticks (Rosendale et al., 2016a; Wang et al., 2017; Yu et al., 2014). However, in ticks, the RCH response does not seem to be ubiquitous. *Ixodes scapularis* seems to lack a RCH response (Vandyk et al., 1996), whereas *Haemaphysalis longicornis* (Yu et al., 2014) and *Amblyomma maculatum* (present study) undergo RCH. Even within a genus (*Dermacentor*) there is variation in the RCH response; *D. variabilis* (present study; Rosendale et al., 2016) and *D. silvarum* (Wang et al., 2017) have both been shown to undergo RCH, whereas RCH is not found in *D. albipictus* (Holmes et al., 2018). This discrepancy may be an artifact of methodologies used in these various studies as certain acclimation temperatures and/or durations are more effective at eliciting a RCH response in ticks than others and physiological state of ticks can greatly impact stress tolerance (Rosendale et al., 2016a; Rosendale et al., 2017; Wang et al., 2017; Yu et al., 2014). In *H. longicornis*, RCH results in increased water content (Yu et al., 2014); however, in adult *D. variabilis* (present study) and *D. silvarum* (Wang et al., 2017) water content does not change, suggesting that alterations in water content is not a critical component of the RCH response in ticks.

Transcriptional changes following recovery from RCH

Following recovery from cold shock and RCH, a suite of genes that likely contribute to cold hardiness was differentially regulated. For the CS ticks, a relatively small number of genes (99) were differentially regulated as compared to the much larger number that changed in the RCH group (1,860), and there were many differences between CS and RCH ticks (1,376 genes). This is different from what was observed in flesh flies exposed to similar cold-shock and RCH treatments (Teets et al., 2012). In Teets et al. (2012), gene expression profiles after 2 h recovery were nearly identical between flies directly exposed to a cold shock and those given RCH prior to cold shock. Due to our experimental design, it is impossible to determine if the transcriptomic changes in the RCH group occurred during the brief acclimation period or during the recovery phase; however, studies on flies suggest that it is the recovery period that is critical for the changes in gene expression (Sinclair et al., 2007;

Teets et al., 2012). Although much of the changes may occur during recovery, our data indicate that in ticks, a brief acclimation period triggers a much more robust transcriptomic response than cold shock alone. The actual mechanism that accounts for this more robust response remains unclear. It is possible that the RCH acclimation is necessary to trigger a transcriptomic response following recovery from cold. Alternatively, the RCH treatment may allow ticks to more quickly restore physiological functioning to alter gene expression following cold exposure, whereas ticks directly exposed to cold shock may require a longer recovery period.

This transcriptomic response included an upregulation of 728 genes that contained a variety of genes and/or GO terms that likely contribute to enhanced cold hardiness (Fig. 6). Many of these genes were categorized as binding, including calcium binding and iron ion binding, both of which are important mechanisms in the RCH response (Gerken et al., 2015; Teets et al., 2020). Adjustments to membrane transport properties is important in both seasonal acclimation and RCH (Armstrong et al., 2012; Koštál et al., 2006; Teets and Denlinger, 2013) and long-term acclimation leads to an upregulation of transport-related genes (Enriquez and Colinet, 2019). In RCH recovered ticks, 20 genes related to transporter activity were upregulated, including several specifically related to ion transport, which could be important in restoring ion homeostasis during cold-shock recovery (MacMillan et al., 2012; Teets and Denlinger, 2013). Structural components of the cell, including the plasma membranes and cytoskeleton, are vulnerable to damage from cold, and an important RCH mechanism is modifications to these structures (Kim et al., 2006; Li and Denlinger, 2008; Teets and Denlinger, 2013). In our RCH recovered ticks, several GO terms related to the cytoskeleton were enriched in the upregulated genes and 44 genes related to membrane were upregulated. In RCH recovered ticks, several transcripts with similarity to heat-shock protein 70 (Hsp70) were upregulated. Hsps act as molecular chaperones to protect proteins, contributing to cold tolerance, and Hsp70 is specifically important in surviving low temperature (Rinehart et al., 2007).

Among the groups of ticks recovered from RCH and CS, there were 56 common transcripts that were upregulated; for these genes, the GO terms carbohydrate biosynthetic process and gluconeogenesis were enriched. Genes of note include glucose-6-phosphatase (G6P) and phosphoenolpyruvate carboxykinase (PEPCK). Although accumulation of carbohydrate-based cryoprotectants is a hallmark response to long-term cold acclimation, RCH does not always result in increased cryoprotectant levels (Teets et al., 2012). Glycerol has been suggested as a cryoprotectant in ticks (Wang et al., 2017; Yu et al., 2014); however, neither glycerol nor glucose (another common cryoprotectant) changed levels in our experiments (discussed below). Alternatively, this pathway could be a common stress response in ticks as PEPCK is upregulated in dehydrated ticks (Rosendale et al., 2016b) and contributes to tolerance of oxidative stress in tick cells (Della Noce et al., 2019).
In overwintering *D. silvarum*, molecular functions such as catalytic activity and binding, cellular components including membrane, and biological processes such as oxidation-reduction process and metabolic process were highly enriched GO terms (as compared to summer ticks) (Yu et al., 2020). For most of the GO terms enriched in winter *D. silvarum* there were genes that were upregulated in our RCH recovery ticks that matched those categories (Fig. 7). Enrichment of these terms in response to both long- and short-term cold exposure suggests an importance for these pathways in survival of low temperature.

Metabolomics and functional analysis

Accumulation of various molecules during the cold-hardening process can serve a protective role against cold injury (Teets and Denlinger, 2013). *D. variabilis* showed several changes to the metabolome during recovery from RCH that were supported by the transcriptome data. Levels of several amino acids, including alanine, glycine, and valine, were significantly increased following recovery from RCH. This augmentation of amino acid levels was potentially the result of increased protein catabolism, as there was an upregulation of proteolysis genes. Amino acids such as proline can act as potent cryoprotectants when they are accumulated prior to cold exposure (Koštál et al., 2011). Neither alanine nor glycine impacted cold-shock survival of *D. variabilis* in our

experiments; however, when valine was exogenously supplied prior to cold-shock treatments, survival improved.

Valine has been suggested as one of multiple amino acids to improve seasonal cold hardiness in insects (Feng et al., 2016), and our data support the cryoprotective nature of this amino acid. Increases in valine concentrations in arthropods have been noted in response to both long-term, low-temperature acclimation (Storey et al., 1986) and seasonally in overwintering insects (Feng et al., 2016, Qiang et al., 2012). Valine has been suggested to improve cold hardiness by contributing to the lower super-cooling point in winter-acclimated lepidopteran (Qiang et al., 2012). However, while proline has been extensively studied in cold and freeze tolerance (Koštál et al., 2011), less is known about the specific cryoprotective mechanisms linked to other amino acids, like valine. Teets et al. (2012) noted that RCH treatment alone was not enough to elicit an accumulation of valine in flies. If the valine in ticks is accumulated during the recovery period, it may serve a role in the restoration of homeostasis and/or may contribute to survival of subsequent cold exposure. Alternatively, it is possible that the protein breakdown is due to a disruption in homeostasis caused by cold-shock damage, or proteolysis may be a common stress response in ticks as dehydrated *D. variabilis* (Rosendale et al., 2016b) and overwintering *D. silvarum* (Yu et al., 2020) also show an upregulation of this pathway.

The accumulation of betaine in ticks that recovered from our RCH treatment prompted investigation of this molecule as a potential cryoprotectant in ticks. Betaine was investigated more thoroughly than valine for a cryoprotective role as it has not been described as a cryoprotectant in arthropods, whereas some amino acids are known to have a cryoprotective effect (Teets and Denlinger, 2013). The increase in concentration of betaine was coordinated with an upregulation of several transcripts with high similarity to betaine-related genes, including a betaine-aldehyde dehydrogenase and a sodium- and chloride-dependent betaine transporter. Betaine is a common compatible osmolyte that has a protective role during osmotic stress by stabilizing protein structure and membrane integrity (Yancey, 2005). In addition to being an osmoprotectant, betaine also has cryoprotective properties in plants (Xing and Rajashekar, 2001). In the freeze-tolerant Alaskan beetle, *Upis ceramboides*, a betaine-like substance accumulated under winter

conditions, although its direct role as a cryoprotectant was not established (Walters et al., 2009). The positive effect of exogenously supplied betaine over a 100-fold range of concentration on the cold hardiness of *D. variabilis* suggests that this molecule can have a cryoprotective effect if accumulated prior to cold exposure. It has been suggested that betaine acts as a cryoprotectant through both colligative and non-colligative mechanisms in plants (Xing and Rajashekar, 2001). Further study is needed to confirm that the levels of betaine accumulation observed in this study are high enough to have colligative effects and what, if any, non-colligative benefits betaine may provide to cold-hardiness in ticks. Administration of exogenous valine and betaine improved the cold-hardiness of *D. variabilis* 2- to 3-fold as compared to controls. Neither of these metabolites were as efficacious in improving low-temperature survival as RCH, even at relatively high doses, indicating that a multifaceted response is necessary during RCH. There are several possible explanations for the lack of a concentration effect in our treatments. The cryoprotective effect of metabolites, including amino acids, can have non-colligative mechanisms (Toxopeus et al., 2019), and it is possible this is the case for valine and betaine in these ticks. Alternatively, valine and betaine may show colligative effects on cold-hardiness, but the lowest concentration administered (similar to physiological levels) in our experiments represent the maximal colligatively induced improvements in tick cold-hardiness. Further study is needed to elucidate the cryoprotective mechanisms of valine and betaine.

Survival of low temperatures by ticks seems to be related to the accumulation of molecules such as glycerol (Wang et al., 2017; Yu et al., 2014); however, we did not observe a significant change in glycerol levels. There was also no change in glucose, another common cryoprotectant (Overgaard et al., 2007; Teets and Denlinger, 2013). It is possible that glycerol plays a role in the cold hardiness of *D. variablilis* and no changes in the levels of this molecule were detected due to the treatment methods. In *H. longicornis*, glycerol levels were not affected by 2 h at 0°C but did increase after 10 d at low temperature (Yu et al., 2014). Similarly, *D. silvarum* showed no change in glycerol levels after 2 h at 0°C, and only females increased glycerol in response to 2 h at -3°C (Wang et al., 2017).

Conclusions

Ticks spend the vast majority of their lives off-host, and establishment of a population is dependent on overwintering survival (Dergousoff et al., 2013; Gray et al., 2009). Rapid-cold hardening substantially improves the ability of *D. variabilis* to survive cold shock and likely contributes to survival of ticks under ecologically relevant conditions. We found a suite of molecular and biochemical changes in *D. variabilis* that received an RCH treatment prior to cold shock and a recovery period, and these changes were distinct from those in ticks that experienced cold shock and recovery only. These changes include an upregulation of genes related to cell structure, transport, signaling, and metabolism that are common across a wide range of arthropods (Teets et al., 2020). Due to our experimental design, it is unclear if these changes occurred during the brief acclimation or during the recovery period. If these changes occurred prior to cold shock, then they likely contributed to cold-hardiness; however, if the recovery period is required, then these changes potentially contribute to subsequent cold exposures and/or contribute to repair and re-establishment of homeostasis. Enhanced cold hardiness in *D. variabilis* is facilitated by the supplementation valine and betaine, which improved cold survival in both *D. variabilis* and *A. maculatum*. Further research is warranted to determine the cryoprotective mechanisms of physiological levels of betaine. Overall, ticks respond to RCH conditions by activating various pathways to reduce cold-induced damage, some of which directly contribute to survival of cold exposure while others may prepare ticks for subsequent chilling events.

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Competing interests

The authors declare no competing or financial interests.

Data accessibility

Transcriptome data can be accessed through the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov) Sequence Read Archive: Bio-project PRJNA657863. Raw data is available via Dryad (https://doi.org/10.5061/dryad.jsxksn08g).

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Figures

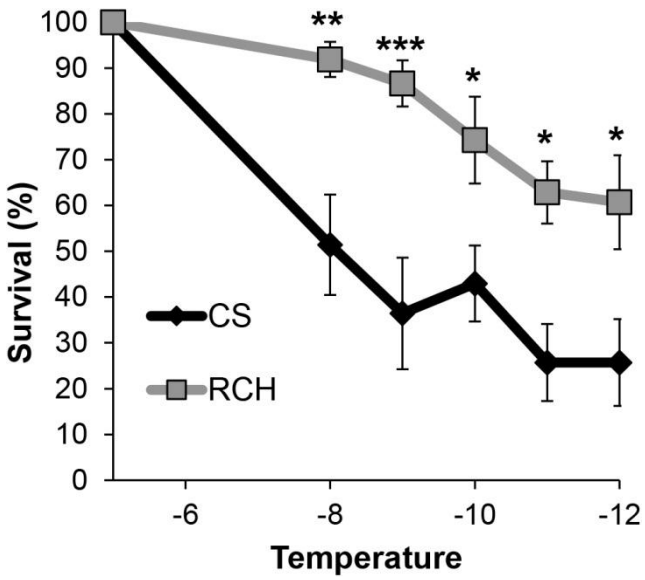


Figure 1. Effect of rapid cold-hardening (RCH) on the cold tolerance of female *D. variabilis*. Cold-shocked ticks (CS) were directly transferred from rearing conditions to test temperatures for 2 h, whereas RCH ticks were first exposed to 4°C for 2 h prior to being transferred to the test temperature. Survival, determined by normal mobility and locomotor behavior, was assessed 48 h post-treatment. Asterisks indicate mean values (± s.e.m., *N*=7 groups of 10 ticks) that differed between CS and RCH groups (ANOVA, Bonferonni, * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

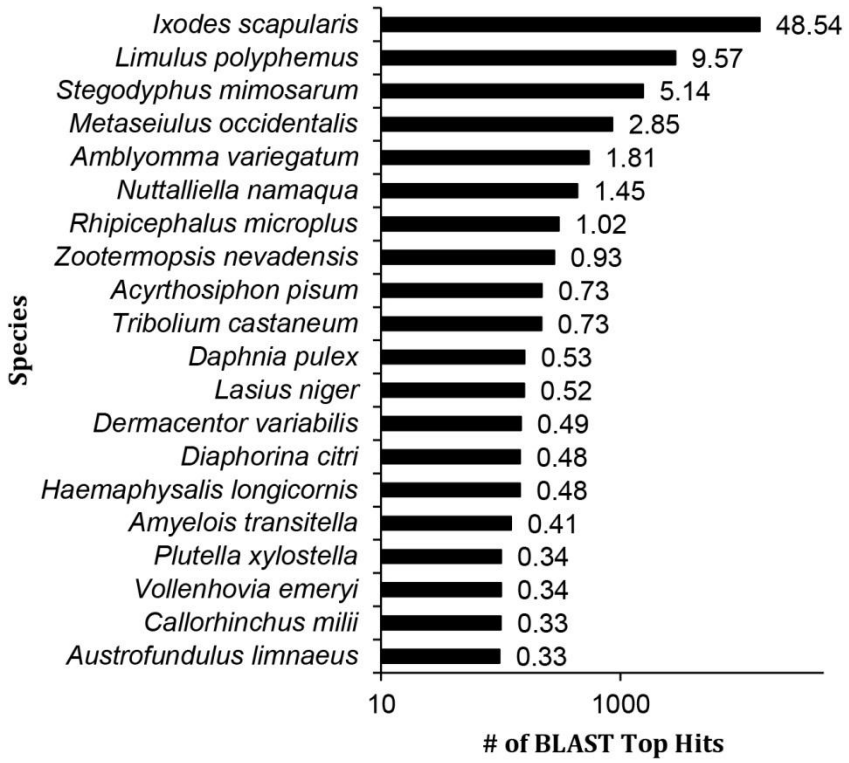


Figure 2. Species distribution of the top blast hits for the contigs of the transcriptome of *D. variabilis.* The 20 species with the most blast hits are shown. A cut-off e-value of 0.001 was used. Numbers beside bars represent the percentage of contigs represented by each species out of the total number of contigs with blast hits.

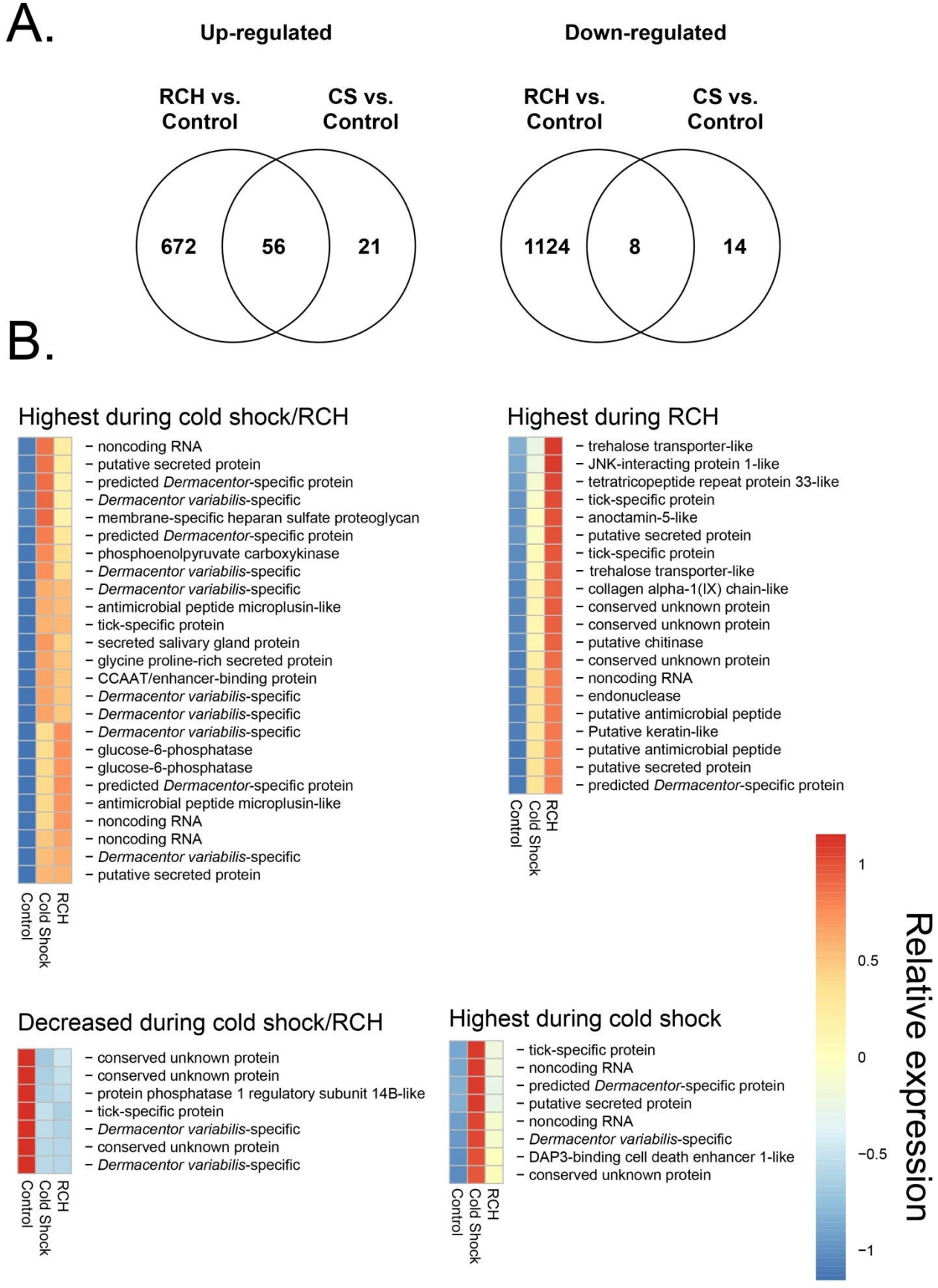
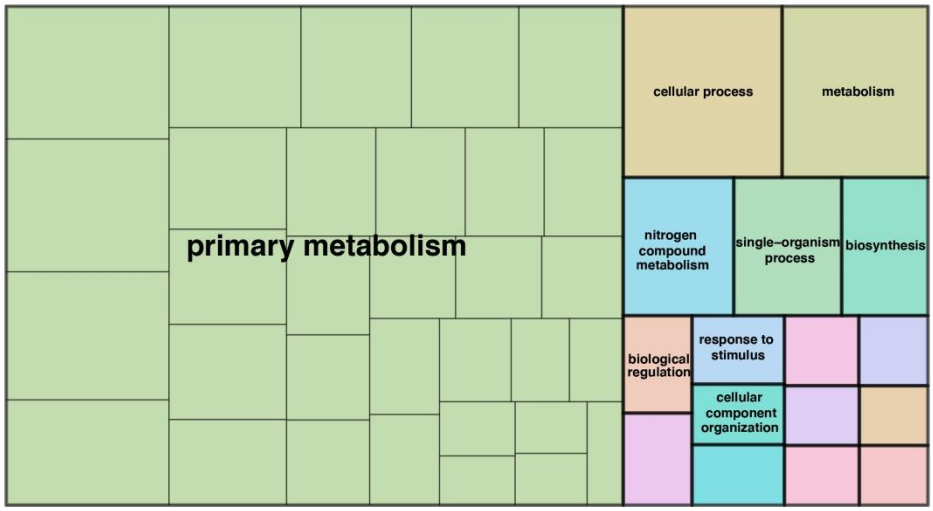
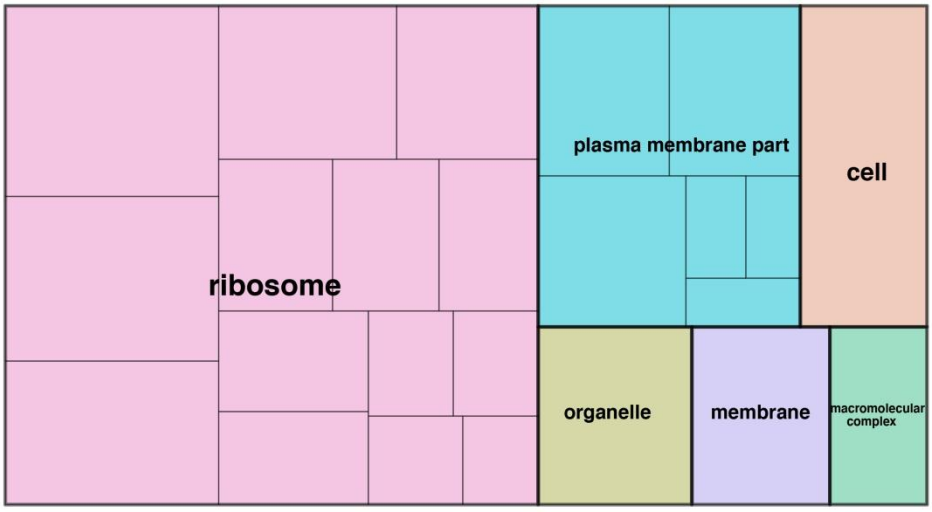


Figure 3. Comparison of differentially expressed transcripts. (A) Number of unique and shared transcripts up and downregulated in ticks recovered from rapid cold-hardening (RCH) or cold-shock (CS) conditions as compared to control ticks. (B) Heatmap of relative expression values for transcripts shared between CS and RCH ticks. Significance was based on a false detection rate ($P < 0.05$) and two-fold differential expression. For contigs with no functional assignments, tick-specific indicates that the contig is found in other tick species, *Dermacentor*-specific indicates the contig is found in *Dermacentor silvarum, Dermacentor variabilis*-specific was not present in any other tick species based on comparison to tick genomes (Jia et al. 2020).

A. Biological Process



B. Cell component



C. Molecular function

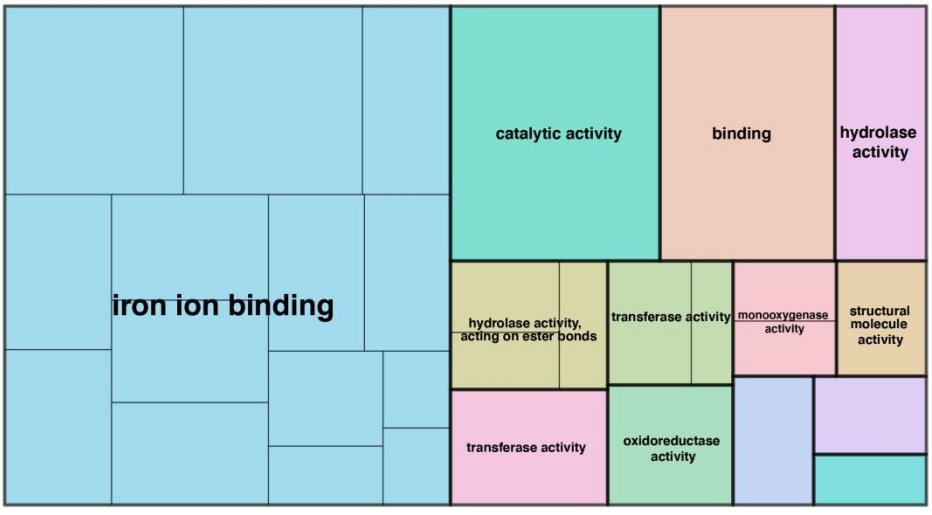


Figure 4. Gene ontology (GO) analysis of genes upregulated following recovery from rapid-cold hardening, as compared to control ticks. Genes associated with (A) biological process (B) cellular component or (C) molecular function. GO conducted with DAVID (Huang et al., 2009) and visualized with REVIGO (Supek et al., 2011).

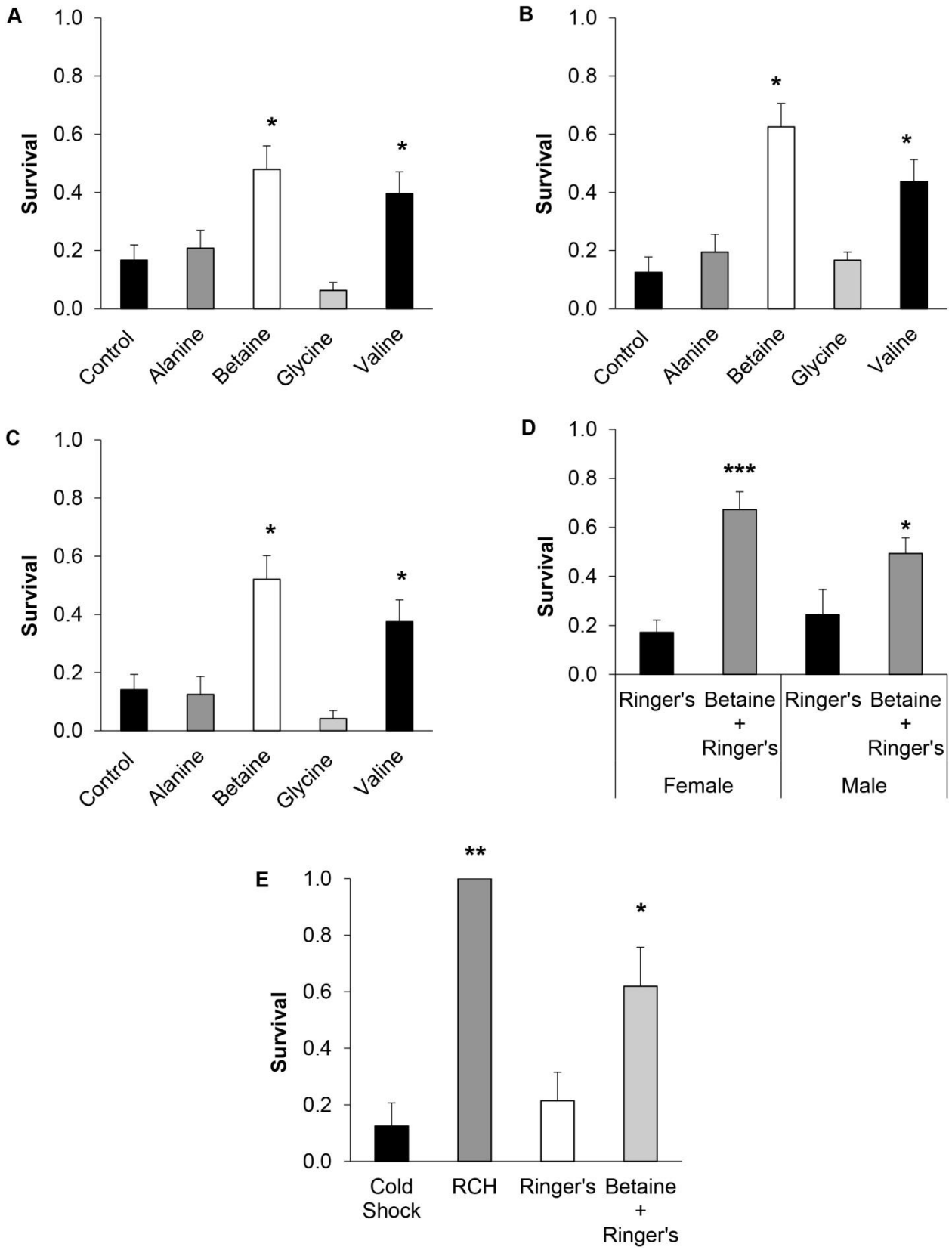
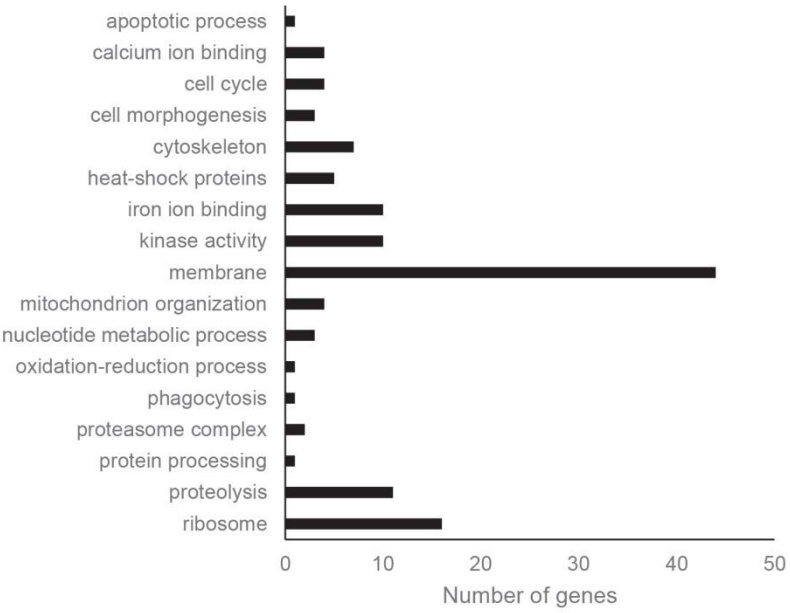
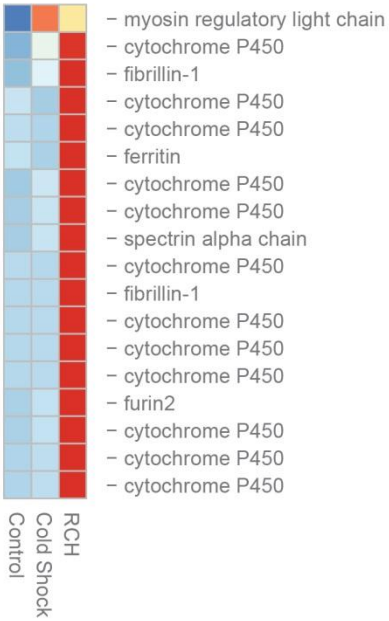


Figure 5. Administration of exogenous betaine and valine improves cold hardiness of ticks. *D. variabilis* female, *N*=6 groups of 10 ticks) were injected with insect Ringer's (control) or Ringer's containing alanine, betaine, glycine, or valine then exposed to -13.5°C. Metabolite concentrations were 1 M (A), 0.1 M (B), or 0.01 M (C). (D) Effect of 1 M betaine in Male (*N*=12 groups of 10 ticks) and female (*N*=11 groups of 10 ticks) *D. variablis*. (E) *A. maculatum* (female, *N*=8 groups of 10 ticks) were either directly exposed (cold shock), first exposed to 4°C for 2 h (RCH), or received an insect Ringer's or betaine solution(1 M) injection prior to exposure to -12°C. Survival, determined by normal mobility and locomotor behavior, was assessed 48 h post-treatment. Effect of metabolite type and concentration was examine in *D. variablis* with a two-way ANOVA. *D. variabilis* were examined within a sex using a Student's *t-test* whereas *A. maculatum* were analyzed with an ANOVA and Bonferonni post-hoc test (cold shock-RCH or Ringer's-betaine). Asterisks indicate mean values (± s.e.m.) that differed between comparisons (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

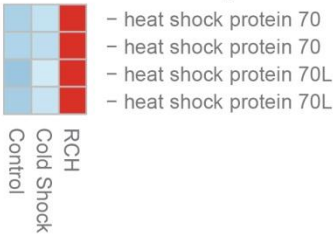
A.



B. Iron/calcium binding



Heat shock proteins



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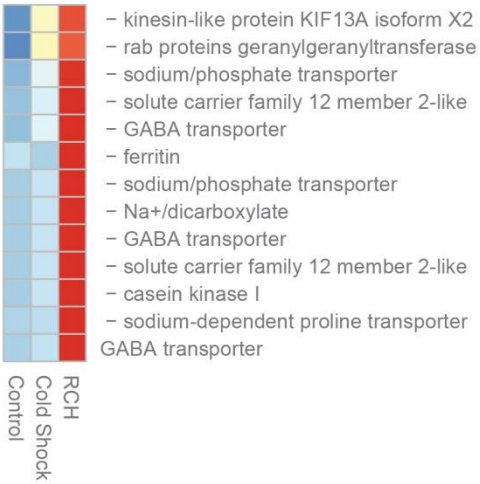


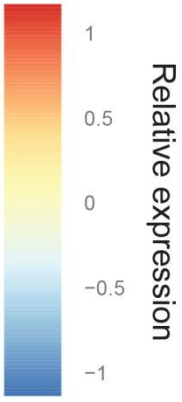


Figure 6. Expression of RCH-relevant genes. (A) Number of genes significantly upregulated in rapid-cold hardening (RCH) recovered ticks that belong to gene ontology (GO) categories or pathways that are important in the RCH response (Teets et al., 2020). (B) Heatmap of relative expression values for several important GO categories. For contigs with no functional assignments, tick-specific indicates that the contig is found in other tick species, Dermacentor-specific indicates the contig is found in Dermacentor silvarum, and Dermacentor variabilis-specific was not present in any other tick species based on comparison to tick genomes (Jia et al. 2020).

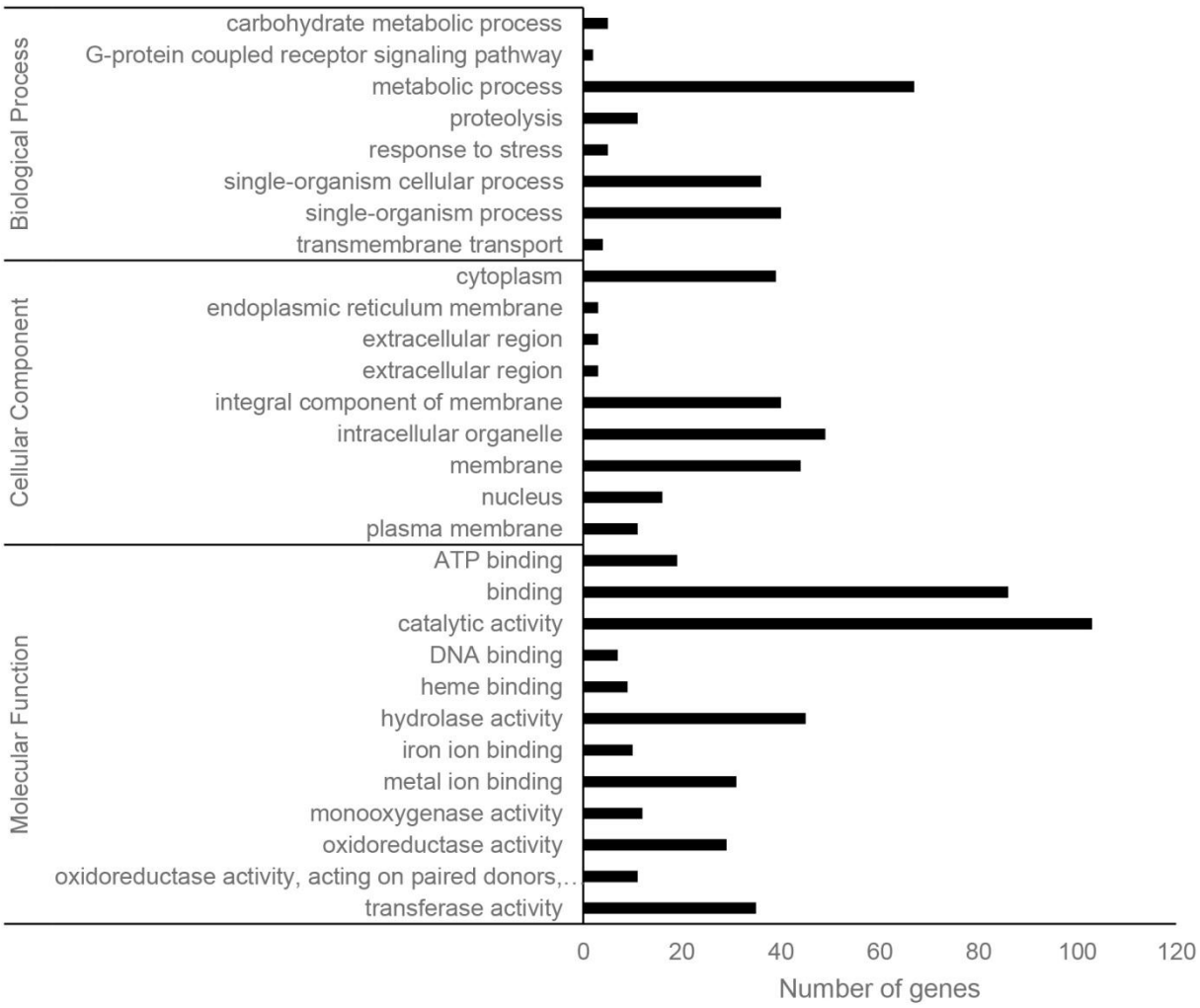


Figure 7. Number of genes significantly upregulated in *D. variabilis* following recovery from rapid-cold hardened (RCH) that belong to gene ontology (GO) categories or pathways that are important in overwintering *D. silvarum* (Yu et al., 2020).

Table 1. Summary of the de novo assembly of the transcriptome of *D. variabilis*

Total number of contigs	152,689
N50	1263
Average contig length (bp)	712
Maximum contig length (bp)	13,987
Total number of bases	108,732,649
[a]Percentage Arthropod BUSCO genes that aligned to assembly, tBLASTn (10^{-5})	92
Percentage *Ixodes* BUSCO genes that aligned to assembly, tBLASTn (10^{-5})	96
[b]Percentage *D. melanogaster* CORE genes that aligned to assembly, tBLASTn (10^{-5})	67
[c]Percentage mapped back	77 ± 2

N50, shortest sequence length at 50% of the transcriptome.

[a]The arthropod set of Benchmarking sets of Universal Single-Copy Orthologs (BUSCO) were downloaded from OrthoDB (Simão et al., 2015).

[b]Core eukaryotic genes data set for *D. melanogaster* were acquired from the CEGMA database (Parra et al., 2007).

[c]Mean percentage of reads from all six RNA-Seq samples that mapped onto the assembled transcriptome.

Table 2. Summary of the number of differentially expressed contigs in pairwise comparisons.

Comparison	FDR <0.05	Up	Down
Control vs CS	99	77	22
Control vs RCH	1,860	728	1,132
CS vs RCH	1,376	365	1,011

Contigs with a false discovery rate (FDR) <0.05 and with ≥2-fold difference were considered significantly up- or downregulated.

Table 3. Concentration of metabolites in whole-tick homogenates of *D. variabilis* that were significantly different among control ticks and those recovered from cold shock or rapid cold hardened (RCH).

Metabolite	Control	Cold Shock	RCH
Alanine	22.99±0.88[a]	24.56±1.04[ab]	25.75±1.42[b]
Betaine	16.01±0.52[a]	17.04±0.62[ab]	17.85±0.20[b]
Glutamate	17.10±0.61[a]	18.86±0.90[ab]	19.60±0.51[b]
Glycine	15.58±0.72[a]	16.37±0.90[ab]	17.72±1.25[b]
Lactate	13.43±0.57[a]	14.89±0.68[ab]	15.50±0.51[b]
Valine	8.78±0.44[a]	10.17±0.40[ab]	11.01±0.40[b]

Groups of 15 ticks were pooled for analysis and values were determined from N=6-11 samples. Mean ± sem values (nmol mg-1 dry mass) were compared with ANOVA followed by Tukey post-hoc test. Values that do not share a letter are significantly different.

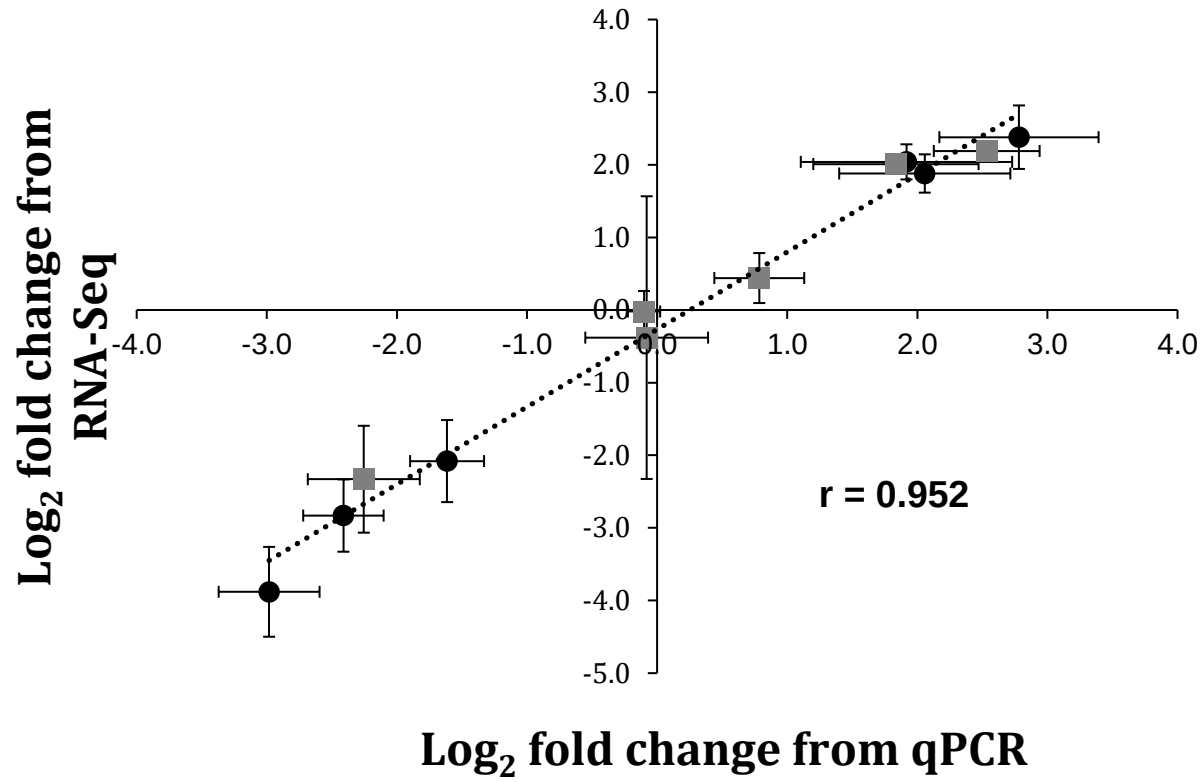
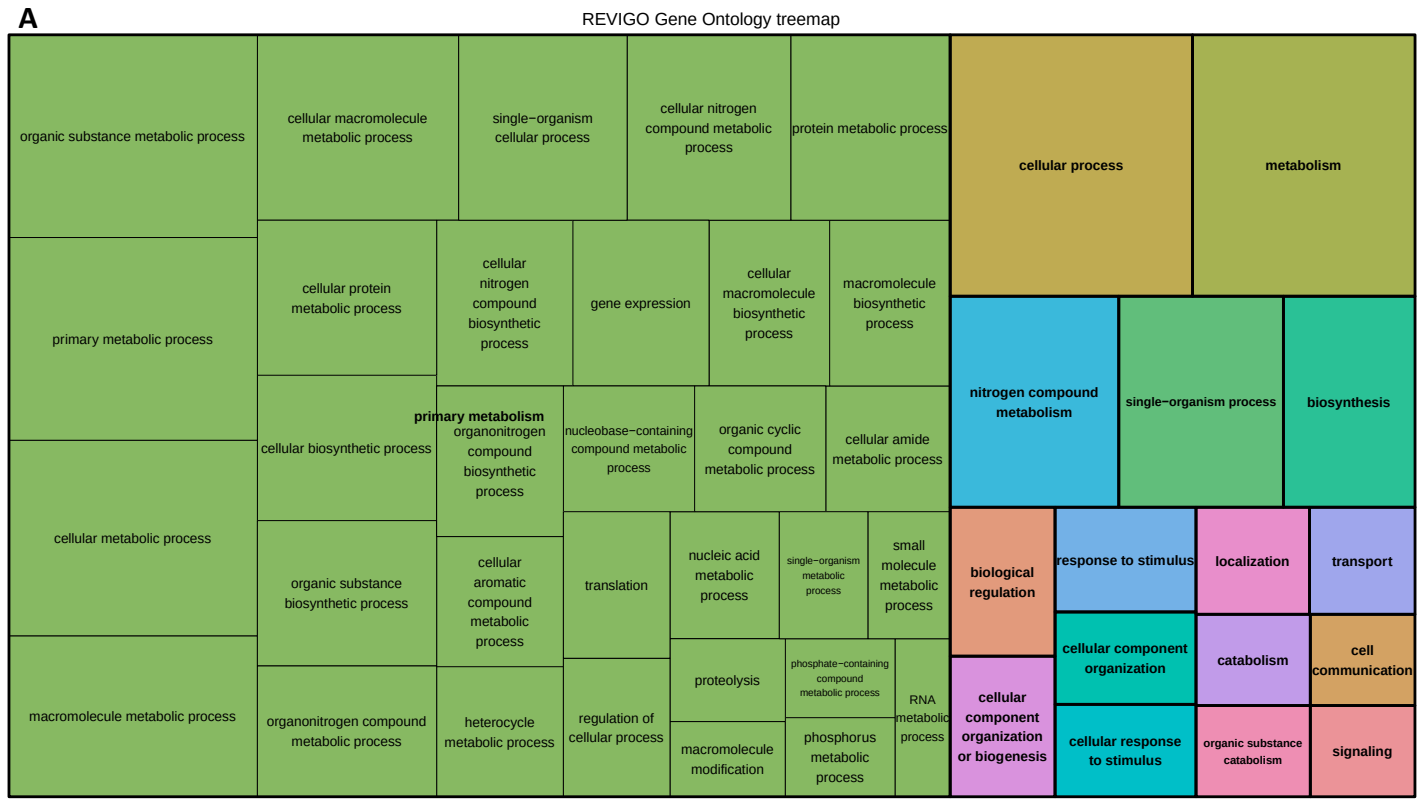
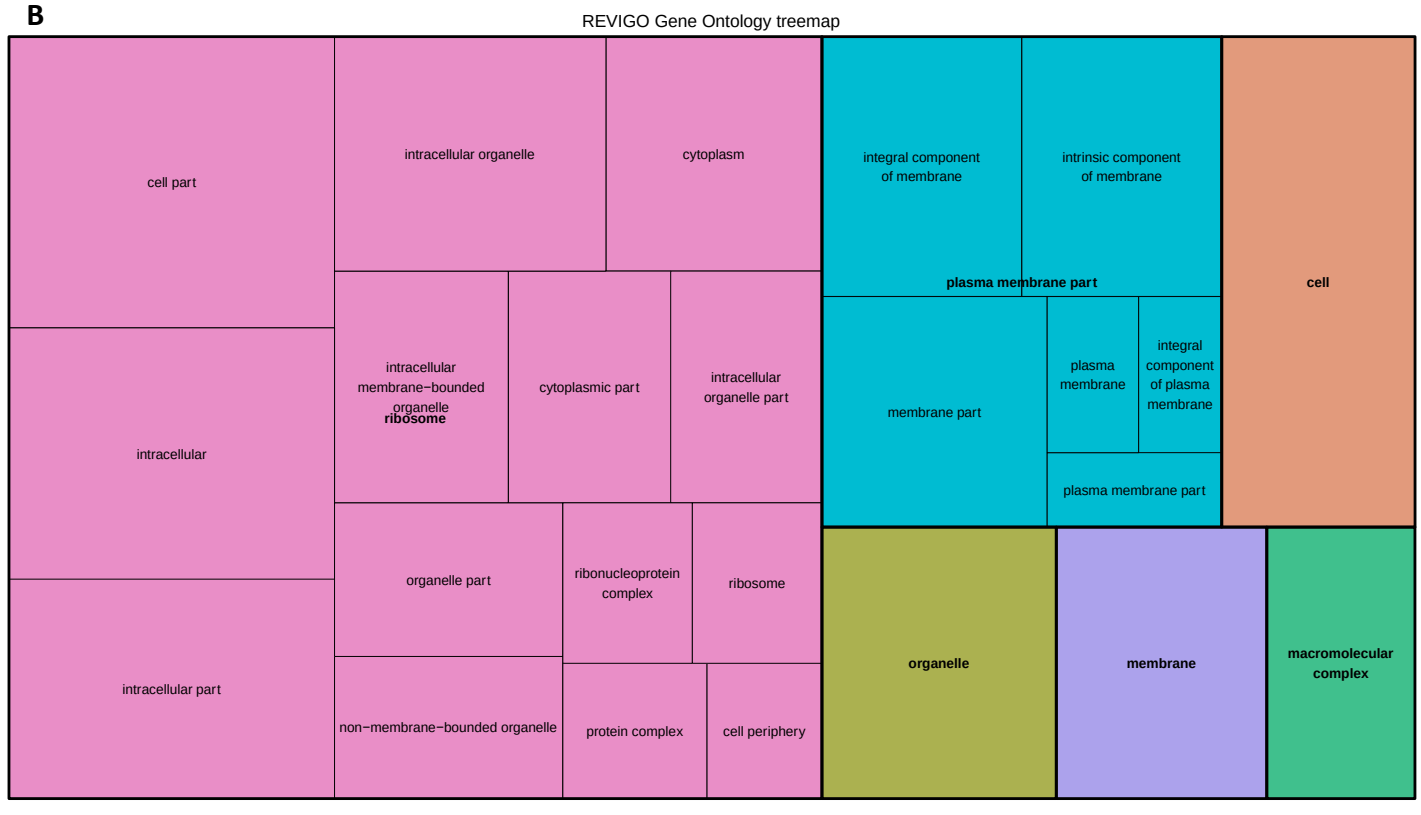


Fig. S1. Results of qPCR validation experiment. Log fold changes obtained by RNA-seq and qPCR for each gene plotted with the best-fit regression line. Fold changes are relative to control for RCH (black circles) and cold-shock (grey squares). For qPCR, fold changes were determined using the ΔΔCq method with β-actin as a reference gene. Pearson correlation coefficient (r) is shown.

A



B



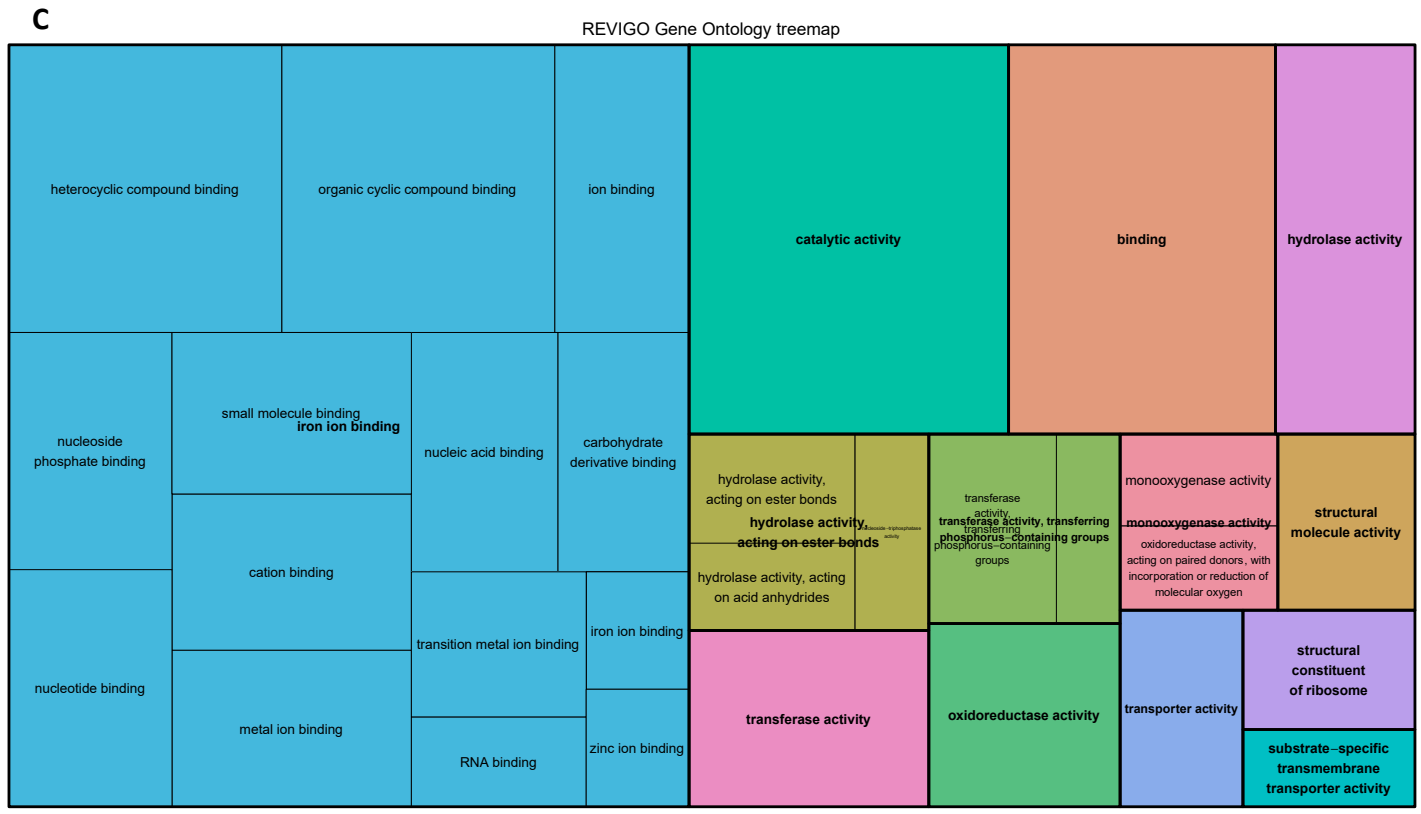


Fig. S2. Detailed view of gene ontology (GO) analysis of genes upregulated in rapid-cold hardening ticks, as compared to control ticks. Genes associated with (A) biological process (B) cellular component or (C) molecular function. GO conducted with DAVID (Huang et al., 2009) and visualized with REVIGO (Supek et al., 2011).

Table S1. Primers used for qPCR gene expression analysis

Contig	Gene ID	Sequence
comp209008_c0_seq1	*Leukocyte elastase inhibitor (XP_03756929)*	Forward 5- TGTTCGAAACTTCCACAGGTT -3 Reverse 5- CCGCAAGACCAACGAGAT-3
comp195726_c0_seq2	*actin (XP_029850485)*	Forward 5-TCCACGAGACCACCTACAA -3 Reverse 5-TGCATACGGTCGGCAATA C -3
comp206499_c0_seq1	*Unknown protein (XP_037573670)*	Forward 5- GCCTCGACTTCTTGAGGTATG-3 Reverse 5- AAATCGCATCGCCTTCTCT-3
comp202435_c0_seq1	*Cysteine-rich structural protein (XP_037523285)*	Forward 5- CCTACGAAAGGACAAGAGAGAATG-3 Reverse 5- GGCACTCCTTGTATTCCTGATG-3
comp201487_c0_seq1	*Uncharacterized protein (XP_037575908)*	Forward 5- GTCGGAGTTCAGCAAGAGATAC-3 Reverse 5- GTTAAAGTTCTTGTCCGCACATC-3
comp209830_c0_seq1	*Glucose-6-phosphatase (XP_037516723)*	Forward 5- CTGATGGATACGCTCTACGATAATG-3 Reverse 5- GAAGTTGGAGATCGCGAAGAA-3
comp216048_c0_seq2	*Uncharacterized protein (XP_037582455)*	Forward 5- GTTACGCACATCGGAAAGAATG-3 Reverse 5- CGTAGTTCCTTCGGTGAGAAAT-3

Contig Sequences:

comp209008_c0_seq1
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GGAGCCCAGCTTGACAAGTTCGTACCCCTTCGCCGCGAAACGAGCGGAATGCAGGAAGACTACATTGGCAGCTGGAAGCCGGAGAG
CCACGCTGTGAACTACTTCGACTTCGAAGAGGAAGACAGGCTGGACGAGACGTTCGTGTGGAACTTCGACATCAGGGACTGCGCAG
TAAGCAAGGACAGCAAGAGCTACCTGGACCTACTCTTCATAAGCGACAAGAACGTGAGCCAGCGAGCCCTGTGGTCCAACCGGGAC
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CCGGGCGAAAAGATGAAGCACGTCAGCGAGGTGCCATCGCAGGAGGAGTGCGCCGAAAAGTGCTTCAACGACGCTGGCTGCATGAC
GTTCATCTACTGCGACCACGGCTGCTTCTTCTCGCCCAGTAACCGGCCCATCGACTGGGGGAGACCCATGGCCATGAGCGGATGCCG
CCGCTACACCAAGGCAGATGCCGACATTAAGGCGACGTTCAGAAACAACTCCGAGGTTGTCAAGATGCTCAAGGACGAGGTCGACA
ACGCGCGGTTCGTGCTCAAGGTTGCCAGCGATGAACTCTCGAGTGCGGCAACATACACTGCCGTTAGGCTTAACACGGACGCCAGCC
CATTCAATGCGAACCACGCGGGCGGCCTGTACGACGTGGCGTTCCCCGAAAAGGTCCTGTCCATGGACGCGGACACGACGATGAAGG
CCATCATGCTGGACGGCAGCTCGGAGATCACGTCGGTGCAGCAGTGCTACCGCAACTGCATCACCGAGAGCGGCATGGAGTGCGCCT
CGTTCTCGTACTGCGCGGCCACCAAGGACTGCCGGCTGTCGTCCATCTACATCCGGGACGACGACGCGGCCGCCAAGAAGAGCCTGG
CCACCGACACGCGATGCGACGTCTACGCACGCTCCTACATCAACATGTACGAGAAGTTCCCGGGCACGGTGAGCCGGGTGGGCGGAG
CCCTGTCCAAGGACAACGTGCCCGACGAGAACCAGTGCGCGCAGCTGTGCAAGCACGACAAGCTCGTCCGGTGCCGCGGGTTCGAGT
ACTGCCCGGGCACCAAGCACTGCGTGCTGCACGAGAAGCACTTCCTCGACATCCGGCCCGAGGACGTCGTCAACGACACCGAGGCCT
GCTCCCACTACGCCCAAAAGTTCTCGGCCGACTACTGGGAGGGCGCCAAAGGGGAGCTGCTGGCCCCGGACTCGGTCACCGTGACCA
TCATCAGCTTCGAGGACTGCGCCAAGCAGTGCACCTTGGACCCGAGCGGGCGCTGCAGGTCGTTCAACTTCTGTCCCGCCAATGACG
GGCCCGACCGCAAGTGCGTGCTTAACGCGCACTCCATCGACGAAATGGAGGCCAACAAGGACGTCAAGTTCCAGCCGAGCAAGGACT
GCTTTTTCTACTTCCAGCAAGACCACATCAACGACTTCGTCCACTTCAACAAGAAGCAGGCGCTTTCTGCACAAGTCACCGGATACA
CCTCAGGAGGACTCGGTGGCTTGCTCTTCGGCATGCTCATCCTCGGTCTCGTGCTCGGCGCAGCAGGCTACACCGCGTTCAACTACT
TCCGGTCACGCCGAGGCGGGGCGGACAACACCACCGTTCGCTTCATGCGTCACGAAGACGCCAGTTAGGACTACGTCGGACATCCTT
CGTCGCGTCTTCGTCACATGCTGGATGTGAATGACATTTAGTGAGCAAAATGAGTTTTATCGGGGTGATTTAGCACCTTCAAAAAC
ACCTATTCCGCATGACGTCACCAAACATTACCCGAAAGGTTTTTGCAGAAGCAGCTTTTCAACATCATCGTCACCAACCTACTTTTA
GTTTGATAAACACAATGGTGATGAAGTTGAAGACAGTTTCTAAAAAAATAGCTTTCTTACTTTCATGACACCAAATGTCATACAAT
TAAGGTTGTGACTGAAAACTACATAGCAGTCAAGATGATTAGGACAGTGAACAGGGGTTTCAGATTTGAAACCGATGAAGCCATTC
GTAAAGAGCATATAAGTGTTAAAGACCACACAAAAGTACAATGTACGTATCCCACCCATTGGTGCTTCTATAGTTAAGAGACAAAG
ACCACGAACAATTCCGGCAGGTCACAAAGAAACAAGAAATACTGACGAAACAAAGTGCTGAAAAGTGAAACAAGAGCTCTTTTTTT
CTCTCAAACCGCACATGCGCCTTCTTCGTGCAACAAAAGCAATGATCCCCTTCCTTTCCTCTTCCGATTTTATTTATTGCAATGTCA
CGCTGCGGGAGCACTGTTCATTTGCCAAACATGTGAAGAAATAATAAATCCTATATGCCATCTGTACAAATAC

Table S2. Expression statistics and BLAST results for contigs that differentially expressed were not significantly different among control, cold shocked, and rapid cold hardened (RCH) ticks

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Table S3. GO terms enriched in upregulated or downregulated genes

Upregulated genes				
RCH vs Control	**CS vs Control**	**RCH Only**	**CS Only**	**RCH CS Shared**
Biological Process				
bio. p. (9.42E-06) cellular amide met. p. (2.48E-06) cellular bio. p. (3.70E-05) cellular macromolecule bio. p. (4.50E-06) cellular macromolecule met. p. (7.20E-03) cellular met. p. (1.41E-06) cellular nitrogen compound bio. p. (5.39E-07) cellular nitrogen compound met. p. (3.60E-04) cellular process (5.56E-08) cellular protein met. p. (1.36E-05) gene expression (1.87E-04) macromolecule bio. p. (5.34E-06) macromolecule met. p. (3.39E-04) met. p. (1.28E-10) nitrogen compound met. p. (4.23E-05) organic substance bio. p. (5.02E-06) organic substance met. p. (5.39E-06) organonitrogen compound bio. p. (3.80E-06) organonitrogen compound met. p. (7.89E-05) oxidation-reduction process (1.62E-04) positive regulation of phosphorus met. p. (1.30E-02) primary met. p. (6.43E-06) protein catabolic process (6.60E-03) protein met. p. (2.20E-05) Proteolysis (2.00E-02) Translation (5.38E-07)	carbohydrate bio. p. (5.30E-04) monosaccharide bio. p. (4.60E-05)	carboxylic acid transmembrane transport (4.15E-02) cellular macromolecule catabolic process (1.00E-02) cellular macromolecule met. p. (4.07E-03) cellular met. p. (9E-05) cellular process (7.19E-05) cellular protein met. p. (8.70E-03) D-amino acid met. p. (4.10E-03) macromolecule met. p. (1.65E-02) met. p. (1.93E-10) nitrogen compound met. p. (1.77E-03) organic substance bio. p. (4.48E-02) organic substance met. p. (1.41E-04) oxidation-reduction process (3.38E-03) positive regulation of cyclin-dependent protein serine/threonine kinase act. (2.40E-03) positive regulation of RNA met. p. (1.60E-02) primary met. p. (8.35E-05 protein met. p. (4.60E-03) Proteolysis (1.80E-02)	none	carbohydrate bio. p. (3.90E-04) Gluconeogenesis (8.75E-03)
Molecular Function				
anion bind. (4.15E-06) aromatase act. 7.30E-3) bind. (3.60E-09) carbohydrate derivative bind. (2.63E-04) catalytic act. (2.14E-09) cation bind. (7.45E-04) DNA polymerase act. (2.40E-04 endonuclease act. (2.00E-04) flavin adenine dinucleotide bind. (7.50E-03)	none	anion bind. (2.00E-07) bind. (1.37E-07) carbohydrate derivative bind. (3.44E-06) catalytic act. (4.99E-10) cation bind. (1.11E-02) DNA polymerase act. (3.00E-04) drug bind. (2.12E-04) heterocyclic compound bind. (1.26E-07) hydrolase act. (4.49E-05)	lipid transporter act. (2.00E-04)	none

heme bind. (3.31E-02) heterocyclic compound bind. (6.15E-09) hydrolase act. (1.64E-03) ion bind. (3.80E-11) metal ion bind. (7.27E-04) monooxygenase act. (3.74E-03) nucleoside phosphate bind. (6.14E-06) nucleotide bind. (6.70E-06) nucleotidyltransferase act. (1.40E-03) organic cyclic compound bind. (7.19E-09) oxidoreductase act. (2.71E-04) small molecule bind. (7.25E-06) structural constituent of ribosome (4.59E-11) structural molecule act. (5.45E-07) tetrapyrrole bind. (3.40E-02) transferase act., transferring phosphorus-containing groups (3.30E-02) transition metal ion bind. (3.10E-02)		hydrolase act., acting on acid anhydrides (2.74E-03) ion bind. (1.27E-10) metal ion bind. (1.09E-02) nucleoside phosphate bind. (1.25E-07) nucleoside-triphosphatase act. (1.58E-03) nucleotide bind. (1.43E-07) nucleotidyltransferase act. (6.80E-04) organic cyclic compound bind. (1.06E-07) oxidoreductase act. (1.77E-03) oxidoreductase act., acting on the CH-NH2 group of donors (5.50E-04) small molecule bind. (3.97E-07) transferase act., transferring phosphorus-containing groups (2.84E-03)		

Downregulated genes

RCH	CS	RCH Only	CS Only	RCH CS Shared
Biological Process				
bio. p. (1.59E-24) cellular amide met. p. (1.76E-37) cellular bio. p. (2.09E-20) cellular component biogenesis (6.28E-09) cellular component organization or biogenesis (5.63E-05) cellular macromolecule bio. p. (7.88E-27) cellular macromolecule met. p. (6.27E-15) cellular met. p. (1.15E-24) cellular nitrogen compound bio. p. (3.54E-28) cellular nitrogen compound met. p. (1.98E-25) cellular process (5.22E-25) cellular protein met. p. (1.18E-19) electron transport chain (7.07E-03) gene expression (4.51E-33) generation of precursor metabolites and energy (4.33E-05) macromolecular complex subunit organization (1.20E-04) macromolecule bio. p. (2.09E-26) macromolecule met. p. (1.52E-17) met. p. (1.53E-23) negative regulation of catalytic act. (3.40E-03) negative regulation of cellular protein	none	cellular amide met. p. (6.66E-08) cellular met. p. (2.01E-02) cellular nitrogen compound met. p. (1.52E-04) cellular protein met. p. (1.60E-02) gene expression (1.44E-06) met. p. (8.15E-05) nitrogen compound met. p. (2.70E-02) organonitrogen compound bio. p. (5.65E-05) organonitrogen compound met. p. (7.60E-07) peptide met. p. (2.75E-09) protein folding (2.10E-02)	spliceosomal snRNP assembly (2.92E-05)	none

met. p. (1.37E-02) nitrogen compound met. p. (1.62E-18) organelle assembly (3.30E-04) organic substance bio. p. (3.91E-20) organic substance met. p. (5.04E-18) organonitrogen compound bio. p. (2.51E-29) organonitrogen compound met. p. (1.90E-23) oxidation-reduction process (4.31E-05) primary met. p. (8.01E-16) protein met. p. (2.25E-15) regulation of protein met. p. (3.31E-02) ribonucleoprotein complex subunit organization (1.3612E-06) ribosomal small subunit assembly (1.10E-03) ribosome biogenesis (4.70E-14) RNA processing (9.10172E-05) Translation (2.54E-43)				
Molecular Function				
Binding (3.72E-11) cytochrome-c oxidase act. (3.40E-06) electron carrier act. (3.10E-05) endopeptidase inhibitor act. (1.30E-04) extracellular matrix structural constituent (1.80E-05) heme-copper terminal oxidase act. (4.00E-06) heterocyclic compound bind. (3.63E-08) nucleic acid bind. (2.27E-09) organic cyclic compound bind. (4.37E-08) oxidoreductase act. (3.30E-04) oxidoreductase act., acting on a heme group of donors (4.00E-06) retinal dehydrogenase act. (3.40E-04) RNA bind. (2.58E-23) rRNA bind. (4.00E-05) structural constituent of ribosome (4.08E-56) structural molecule act. (6.19E-44)	none	RNA bind. (9.06-05) structural constituent of ribosome (7.00E-10) structural molecule act. (7.33E-09)	citryl-CoA lyase act. (8.79E-04)	none

Act. – activity, bind. – binding, Bio. – biosynthetic, met. - metabolic p. – process
Lowest *p*-values from the DAVID, Panther, and g:Profiler shown.

Table s4. Concentration of metabolites in whole-tick homogenates of D. variabilis that were not significantly different among control, cold shocked, and rapid cold hardened (RCH) ticks

	Control	Cold Shock	RCH
4-Aminobutyrate	6.76±0.36	6.85±0.45	7.35±0.52
Acetate	2.56±0.12	2.56±0.13	2.80±0.17
Acetoacetate	0.83±0.05	0.89±0.07	0.91±0.04
Acetone	1.69±0.14	1.77±0.09	1.94±0.11
Adenine	2.75±0.13	2.97±0.14	3.21±0.22
Asparagine	0.88±0.12	0.98±0.07	1.14±0.14
Aspartate	1.49±0.06	1.63±0.12	1.69±0.09
Choline	8.44±0.59	9.09±0.35	9.47±0.73
Formate	0.16±0.01	0.20±0.03	0.23±0.02
Fumarate	0.09±0.01	0.08±0.01	0.08±0.01
Glucose	20.57±1.04	19.28±1.68	20.21±1.41
Glycerol	17.57±1.22	19.08±1.38	19.33±1.67
Glycerophosphocholine	2.54±0.20	2.83±0.24	2.99±0.17
Isoleucine	1.88±0.09	2.02±0.11	2.28±0.08
Leucine	4.49±0.23	4.95±0.33	5.31±0.19
Methionine	1.55±0.06	1.58±0.08	1.47±0.07
Ornithine	2.76±0.09	2.99±0.19	3.22±0.20
Phenylalanine	2.02±0.08	2.18±0.11	2.34±0.14
Proline	5.92±0.42	6.51±0.30	6.58±0.44
Succinate	3.17±0.13	3.38±0.23	3.65±0.25
Threonine	4.25±0.16	4.45±0.13	4.58±0.30
Tyrosine	2.47±0.10	2.63±0.14	2.81±0.18
Uracil	1.74±0.06	1.84±0.10	1.90±0.13

Groups of 15 ticks were pooled for analysis and values were determined from N=6-11 samples. Mean ± sem values (nmol mg-1 dry mass) were compared with ANOVA followed by Tukey post-hoc test.