Cryobiology xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Cryobiology

journal homepage: www.elsevier.com/locate/cryo

Transcriptome and proteome analyses to investigate the molecular underpinnings of cold response in the Colorado potato beetle, *Leptinotarsa decemlineata*

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ARTICLE INFO

Keywords:
Cold hardiness
Overwintering
Insect pest
RNA-Sequencing
Liquid chromatography

ABSTRACT

The Colorado potato beetle (*Leptinotarsa decemlineata* (Say)) is an insect that can cope with prolonged periods of low temperatures exposure. The molecular changes required to adapt to such conditions have not been thoroughly investigated in this insect. The current work aims at characterizing deregulated transcripts and proteins in adult *L. decemlineata* exposed to 15 °C and −5 °C using RNA-sequencing-based transcriptomics and liquid chromatography tandem mass spectrometry (LC-MS/MS)-based proteomics approaches, respectively. RNA-sequencing highlighted the differential expression of several transcripts, including ubiquilin-1 and ubiquitin carboxyl-terminal hydrolase 5, in insects submitted to low temperatures when compared with control insects. In addition, proteomics approach detected 2840 proteins in cold-exposed beetles including elevated levels for 409 proteins and reduced levels for 200 proteins. Cuticular proteins CP1, CP4, CP5 and CP7 as well as eukaryotic translation initiation factor 4B were notable proteins with elevated levels in cold insects. Functional analysis of targets modulated at low temperatures using DAVID indicated processes likely affected under cold conditions including select metabolic cascades and RNA-associated processes. Overall, this work presents molecular candidates impacted by low temperatures exposure in *L. decemlineata* and builds on the current knowledge associated with response to these conditions in this insect.

1. Introduction

The Colorado potato beetle, *Leptinotarsa decemlineata* (Say), is an insect pest that can damage potato crops and that affects ultimately the potato industry in multiple areas worldwide [54]. These insects can defoliate potato plants leading to significant reduction in potato yields [16]. Insecticides and strategies relying on biological control and plant resistance have been used to target *L. decemlineata* populations [1]. Nevertheless, this insect has demonstrated substantial adaptability and can resist treatment by numerous classes of insecticides [2,42]. It is interesting to note that the freeze-avoiding *L. decemlineata* can also cope with low temperatures and thus confront the unfavorable conditions associated with winter [17]. Low temperatures exotherms of −8.8 °C and −11.7 °C were recorded in active or diapausing beetles, respectively [6]. Their ability to deal successfully with long winters and spread to higher latitudes has been highlighted previously [25]. Several factors have been shown to influence *L. decemlineata* overwintering including soil depth at which they confront the cold season as well as lipid stores [36]. Nevertheless, and unlike with the response to insecticides, the molecular levers underlying cold response in *L. decemlineata* have not been investigated extensively.

Pioneering work on the topic reported the influence of environmental conditioning on differential expression of two transcripts associated with the heat shock protein 70 (Hsp70) family in *L. decemlineata* [58]. A subsequent study revealed elevated Hsp70 protein levels in beetles submitted to a cold shock [29]. Cold-associated differential expression of multiple microRNAs (miRNAs), small non-coding RNAs that can impact expression of a wide array of transcripts [22], was also recently reported in *L. decemlineata* maintained at −5 °C [32]. While these studies have provided important leads in the understanding of

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https://doi.org/10.1016/j.cryobiol.2019.03.011
Received 20 December 2018; Received in revised form 28 March 2019; Accepted 29 March 2019

cold response in *L. decemlineata*, a clear knowledge gap nevertheless exists regarding the molecular changes underlying response to low temperatures in this insect. Several studies undertaken in cold-hardy insects have leveraged high-throughput approaches to identify signatures of targets relevant to cold response. Proteomic profiling of adult *Drosophila melanogaster* exposed to three temperatures identified modulated proteins linked to processes such as carbohydrate metabolism and translation during acclimation periods [9]. Metabolomic- and microarray-based approaches were more recently used to explore the molecular levers underlying recovery from cold stress in the drosophilid fly larvae *Chymomyza costata* [45]. Similarly, metabolomics and transcriptomics approaches in warm- and cold-acclimated *D. melanogaster* highlighted a correlation between increased cold tolerance and proline and glutathione metabolism [31]. NMR-based metabolites profiling was undertaken on hemolymph collected from five drosophilid species exhibiting varying responses to cold exposure and revealed elevated levels of cryoprotectants such as trehalose and proline in the chill-tolerant insects [34]. Tissue-specific transcriptome profiling of warm- and cold-exposed *Gryllus pennsylvanicus* crickets showed a series of novel molecular leads with relevance to cold hardiness plasticity [10]. Overall, these studies support the potential of using high-throughput approaches to reveal molecular changes underlying cold response in cold-hardy insects.

The current study was performed to address the aforementioned gap using transcriptomics- and proteomics-based approaches in cold-exposed *L. decemlineata*. With the overarching hypothesis that adult *L. decemlineata* submitted to cold temperatures leverage a signature of transcripts and proteins similar to the one observed in other cold-hardy insects, this work highlighted a series of modulated transcript and protein targets in *L. decemlineata* confronted to sub-zero temperatures. Functional annotation of the differentially expressed targets in cold-exposed insects revealed processes relevant to cold response, including fatty acid metabolism and RNA-associated processes, that are aligned with work performed in models of insect cold hardiness. The overall impact of these changes on cold response is discussed.

2. Materials and methods

2.1. Insect collection

Adult Colorado potato beetles were sampled as described previously [32]. Insects were collected in August 2015 from a potato field not exposed to insecticides and located at the Fredericton Research and Development Centre (New Brunswick, Canada). Beetles (~50) were deposited in plastic containers with potato leaves. Containers were closed using a lid with insect screening for ventilation. Insects were carried to Moncton (New Brunswick, Canada) where they were acclimated for one week in an insect rearing tent containing potato plants and located in an incubator (Thermo Fisher Scientific, Waltham, MA, USA) set at 15 °C under a 16L:8D cycle. A group of 15 active beetles were rapidly sampled after this weeklong incubation by depositing them in liquid nitrogen and were used as controls. Remaining active insects were subsequently acclimated to 5 °C for 2 h and then brought to −5 °C where they were kept at this temperature for 2 h. A group of 15 insects was sampled as above. Insects submitted to identical experimental protocol and not sacrificed in liquid nitrogen were viable when placed on a counter top following low temperatures exposure. Control and −5 °C-exposed insects were stored at −80 °C.

2.2. Transcriptomic analysis

2.2.1. RNA isolation

MirVana miRNA Isolation Kit (Thermo Fisher Scientific) was used to isolate small and large RNA fractions from control (15 °C) and cold-exposed (−5 °C) *L. decemlineata* following manufacturer's protocol and as reported before [28]. Sex of beetles was not taken into consideration. Fractions were prepared in triplicates for both temperature groups using two beetles per replicate. Large RNA fractions were subsequently stored at −80 °C until shipment on dry ice.

2.2.2. Library preparation

Transcriptome sequencing and analysis was performed by PrimBio Research Institute (Exton, PA, USA). cDNA libraries were constructed using Ion Total RNA-Seq Kit v2 (Thermo Fisher Scientific) following manufacturer's protocol for preparation of whole transcriptome libraries. Briefly, 100 ng of large RNA fractions was fragmented for 3 min with RNAase III. Fragmented RNA was purified using nucleic acid binding beads and following manufacturer's protocol from the Magnetic Bead Cleanup Module cleanup kit (Thermo Fisher Scientific). Purified samples were run on an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to assess yield and size distribution of the fragmented RNA. An amount of 50 ng fragmented total RNA was hybridized with Ion Adapters in a thermocycler for 10 min at 65 °C and 5 min at 30 °C. Hybridized fragmented total RNA was then incubated for 1 h at 30 °C with ligase. Hybridized samples were mixed with reverse transcriptase and incubated at 70 °C for 10 min. Samples were snap cooled and incubated at 42 °C for 30 min to generate cDNA libraries. Libraries were purified with nucleic acid binding beads and standard protocol as above. PCR-based amplification of cDNA libraries was performed using Platinum PCR SuperMix High Fidelity and Ion Xpress Barcode reverse and forward primers (Thermo Fisher Scientific). Agilent High Sensitivity DNA Kit was used to determine the yield and size distribution of each cDNA library.

2.2.3. Enrichment and sequencing

Pooled and barcoded libraries were used for templating using the Ion Chef system and Ion Chef 200 kit (Thermo Fischer Scientific). Briefly, 100 pM of pooled libraries were combined and 70 µl of each sample was loaded on the system. Samples were subsequently loaded onto a PI chip (Thermo Fischer Scientific). Quality control was performed on samples 15 h into the run. Beads were isolated and quality assessment was performed on Qubit Fluorometer (Thermo Fisher Scientific). Runs were subsequently resumed and samples loaded onto a PI chip. Loaded PI chip was placed into the Ion Proton System for transcriptome sequencing using standard run parameters.

2.2.4. Alignment and data analysis

FASTQ files obtained were processed using the Strand NGS software (Strand Life Sciences, Bangalore, India) and initial alignment was performed using the *L. decemlineata* [23] transcriptome or the red flour beetle *Tribolium castaneum* reference genome [51]. Processed FASTQ files were also subsequently aligned to the recently reported *L. decemlineata* reference genome [40]. Data sets were deposited in the Sequence Read Archive (SRA) of NCBI under accession number SRP155214. RNA-sequencing analysis was performed using the aligned sequence alignment map (SAM) files. Reads were filtered based on read metrics with parameters including alignment score > 90, no “N” allowed in reads and number of multiple matches allowed ≤1. Filtered raw reads for each sample were quantified, normalized and baseline-transformed by the DESeq algorithm available in R/Bioconductor package [3]. Normalized reads between analyzed samples were used as expression values. Moderated *t*-test [44] was used to determine significant differentially expressed transcripts between conditions ($P < 0.05$). Fold-changes were obtained between conditions for each target.

2.3. Proteomic analysis

2.3.1. Protein isolation

Protein isolation was performed on control (15 °C) and cold-exposed (−5 °C) *L. decemlineata*. Isolates were prepared in triplicates for both temperature groups using a single beetle per replicate. Sex of beetles

was not taken into consideration. Briefly, protein extracts were prepared in 1 ml of homogenizing buffer containing 20 mM HEPES, 200 mM NaCl, 0.1 mM EDTA, 10 mM NaF, 1 mM Na_3VO_4 and 10 mM β-glycerophosphate, adjusted at pH 7.0, with phenylmethylsulfonyl fluoride added immediately before homogenization. Tissues were homogenized for 2 min using a TissueLyser (Qiagen, Hilden, Germany) at a rate of 25 movements/s using one 3 mm tungsten carbide bead per tube. Samples were centrifuged at 1500 × *g* and the pellet was kept. Protein extracts were prepared using a previously reported chloroform/methanol protocol [20]. Samples were stored at −80 °C and ultimately shipped on dry ice for mass spectrometry analysis.

2.3.2. Sample preparation and mass spectrometry

Sample preparation and mass spectrometry analysis were conducted at Dalhousie University's Proteomics and Mass Spectrometry Core Facility (Halifax, NS, Canada). Protein lysates concentration was determined using the Bradford method according to manufacturer's protocol (Bio-Rad, Hercules, CA, USA). A solution containing 200 µg of proteins in 200 µl of 0.4 M triethylammonium bicarbonate (TEAB) and 8 M urea was subsequently prepared. Samples were reduced by adding 10 µl of 0.5 M dithiothreitol and incubating them for 30 min at 56 °C. After reduction, samples were alkylated by adding 20 µl of 0.7 M iodoacetamide and then incubated in the dark for 30 min with occasional shaking. Samples were diluted 1:10 with H_2O and digested overnight at 37 °C with 20 µg of MS grade trypsin (Thermo Fisher Scientific). Each sample was subsequently desalted using Oasis HLB Extraction Cartridges (#WAT094226, Waters, Milford, MA, USA) and labeled using the tandem mass tag (TMT) 10plex system (Thermo Fisher Scientific) following manufacturer's recommendations. Samples were combined, desalted using the same Oasis HLB cartridges as above, resuspended in 200 µl of a 5% acetonitrile, 10 mM ammonium formate pH 8.0 solution and further collected into 0.6 ml fractions using high pH Reverse-phase high-performance (Hi-pH RP) liquid chromatography performed on an AKTA Pure system (GE Healthcare Life Sciences, Mississauga, ON, Canada). Hi-pH RP chromatography was performed on a 100 × 4.6 mm Onyx Monolithic column (Phenomenex, Torrance, CA, USA) at a 0.5 m/min flow rate across a 40 min gradient from 5% to 95% acetonitrile in 10 mM ammonium formate pH 8.0. The collected fractions were further concatenated into 20 fractions as previously reported [57]. Each fraction was dried by vacuum centrifugation and resuspended in 3% acetonitrile with 0.1% formic acid. Eluting peptides were analyzed by LC-MS/MS operated in triple-stage mass spectrometry (MS3) triggered Data Dependent Acquisition (DDA) mode [50] on an UltiMate 3000 RSLCnano nano-flow chromatograph coupled to a Velos PRO Orbitrap tandem mass spectrometer (Thermo Fisher Scientific). Chromatographic separation was performed on manually packed 50 cm × 75 µm PicoFrit columns (PicoFrit PicoTip Emitter #PF360-75-10-N-5, New Objective, Woburn, MA, USA) using a Jupiter 4 µm 90 Å Proteo (Phenomenex, Torrance, CA, USA) stationary phase.

2.3.3. Data analysis

Collected data was initially queried using either *L. decemlineata* [23] transcriptome or *T. castaneum* as references [51] containing 851 and 42,247 annotated proteins, respectively. Data was subsequently aligned using *L. decemlineata* NCBI protein database comprising the recently reported *L. decemlineata* genome [40] and containing 20,048 annotated proteins. Protein identification and quantification was performed using Proteome Discoverer v2.1.1.21 (Thermo Fisher Scientific). Peptide production settings was set at maximum missed cleavage of 2, minimum peptide length of 8 and maximum length of 25 amino acid residues. Error windows were set to 20 ppm for the precursor mass tolerance and 0.8 Da for the fragment mass tolerance. Moderated *t*-test was performed on the generated data using the Linear Models for Microarray Data and RNA-seq Data (LIMMA) tool [44]. Differentially expressed proteins ($P < 0.1$) were identified in cold-exposed versus control *L. decemlineata*.

2.4. Functional assessment of differentially expressed targets

Functional assessment of differentially expressed transcripts identified by high-throughput sequencing using *T. castaneum* genome was performed with the Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatics resources v6.8 [19]. A total of 439 differentially expressed transcripts were linked to a *T. castaneum* gene ID. KEGG pathways, GO terms and COG clusters associated with these targets were subsequently generated. For functional assessment of differentially expressed proteins in cold-exposed beetles as determined by LC-MS/MS, relevant gene IDs were identified for each modulated protein using National Center for Biotechnology Information (NCBI) database. A list of 115 differentially expressed proteins with unique identifiers was obtained. Functional annotation of these proteins was performed as above.

2.5. qRT-PCR-based quantification of differentially expressed targets

L. decemlineata primers were conceived based on reference genome [40]. Primer sequences and efficiencies are displayed in Table S1. Target transcripts were subsequently amplified on novel *L. decemlineata* RNA isolates via RT-PCR following a previously reported approach [32]. These RNA isolates were isolated as described above on adult insects collected in June 2016 from a potato field located at the Fredericton Research and Development Centre (New Brunswick). PCR products were sequenced at the Université Laval (Quebec City, Qc) and confirmed as correct products using BLAST. Initial qRT-PCR runs that amplified targets in cDNA dilutions and using different annealing temperatures were performed to evaluate primer efficiencies. Optimal annealing temperatures were subsequently used in qRT-PCR reactions. Reactions were initiated as described before [27]. Amplification protocol consisted of an initial denaturing step at 95 °C for 5 min, followed by 39 cycles at 95 °C for 15 s and optimal annealing temperature for 30 s 18S rRNA transcripts, used as a housekeeping control, were amplified at an annealing temperature of 58.0 °C and with a 99.6% efficiency.

3. Results

3.1. Quantitative transcriptome analysis

To identify differentially expressed transcripts in *L. decemlineata* submitted to cold temperatures, high-throughput sequencing was initially conducted. Levels of 2326 transcripts exhibited significant changes in cold-exposed versus control beetles using *L. decemlineata* [23] transcriptome and *T. castaneum* genome. A total of 1496 transcripts were up-regulated while 830 transcripts displayed down-regulation in cold-exposed *L. decemlineata*. Transcripts with absolute log2 fold-changes above 2.0 are shown in Table S2 and Table S3. Results obtained from high-throughput sequencing were also analyzed using the recently reported *L. decemlineata* reference genome [40]. This approach yielded 394 deregulated transcripts. Increased levels in −5 °C-exposed *L. decemlineata* were observed for 251 transcripts while decreased levels were observed for 143 transcripts. Transcripts with absolute log2 fold-changes greater than 2.0 are shown in Table S4 and Table S5.

When both approaches were combined and uncharacterized transcripts were not taken into account, transcripts that displayed the strongest up-regulation in cold-exposed *L. decemlineata* included cytosolic iron-sulfur protein assembly protein CIAO1 (Ld_c71829), cytochrome P450 18a1 (Lde_c011286), ubiquitin carboxyl-terminal hydrolase 5 (UBP5) (Ld_rep_c72260), transmembrane protease serine 9 (LOC100142246), esterase FE4-like (Lde_c014543) and chloride channel protein 2-like (Ld_c289). Transcripts that showed the strongest reduction in expression levels in *L. decemlineata* maintained at low temperatures were associated with ribosomal protein L31

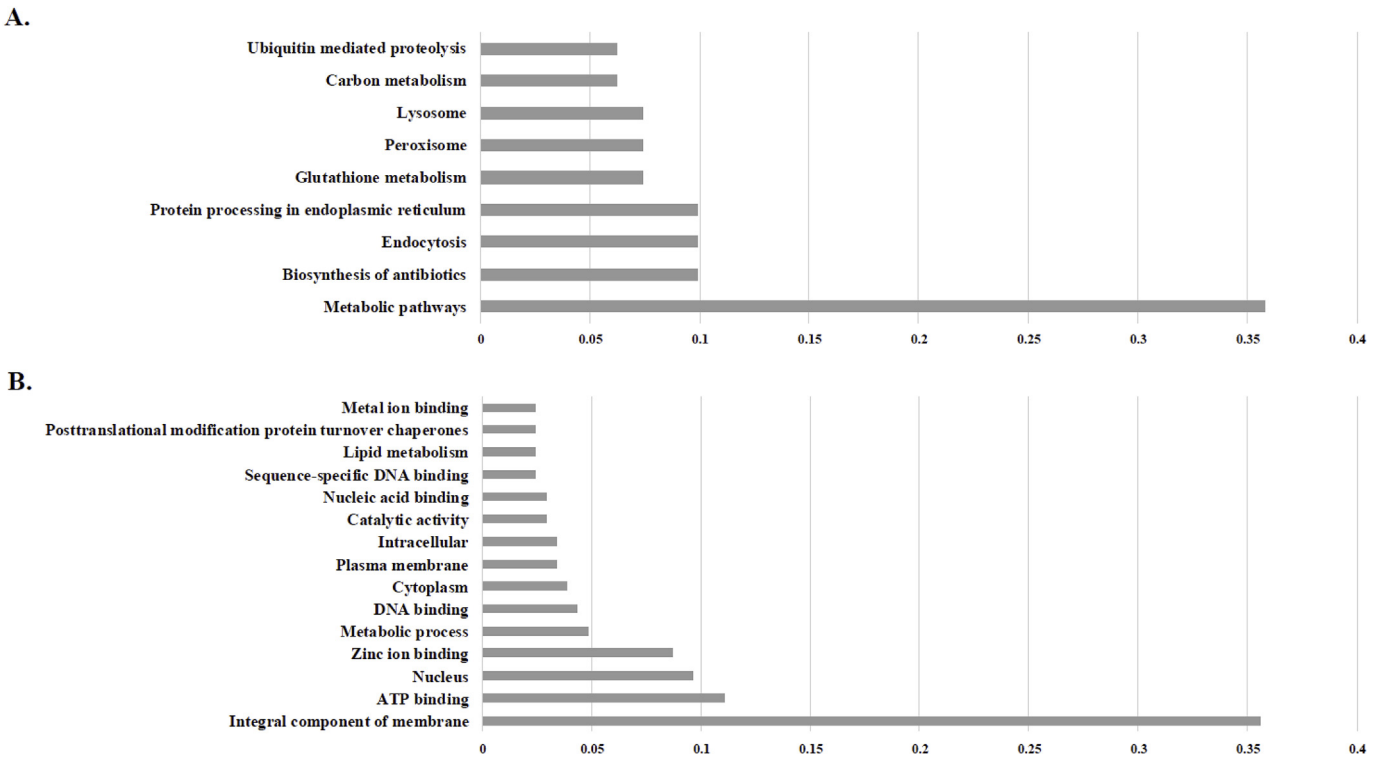

Fig. 1. Biological processes associated with deregulated targets observed in cold-exposed *L. decemlineata* as identified with the Database for Annotation, Visualization and Integrated Discovery (DAVID) tool. Panels **A** (KEGG) and **B** (GO/COG) present processes associated with differentially expressed transcripts. Values are percentages of target candidates associated with listed processes.

(Ld_rep_c33523), t-complex protein 1 subunit beta (TCP-1β) (Ld_rep_c36033), heat shock 70 kDa protein cognate 5-like (Ld_rep_c34306), succinate dehydrogenase (Ld_rep_c47258) and phospholipase A2 (Lde_c019150). Multiple deregulated transcripts coding for ubiquitin-associated targets were observed in cold insects including UBP5, ubiquilin-1 (LOC659758), e3 ubiquitin-protein ligase SHPRH (Ld_c11078) and ubiquitin-protein ligase E3A (Ld_c2612) with log2 fold-changes of 2.53, −2.54, −1.80 and −1.41, respectively.

Relevant processes pertaining to these differentially expressed transcripts identified in *L. decemlineata* following cold exposure were identified using DAVID and are presented in Fig. 1. The primary processes generated using this approach consisted of integral components of membranes (GO:0016,021), metabolic pathways (01100) and ATP binding (GO:0005524). Various additional processes were linked to protein homeostasis including protein processing in endoplasmic reticulum (04141), posttranslational modification protein turnover chaperones and ubiquitin mediated proteolysis (04120).

3.2. Quantitative proteome analysis

LC-MS/MS-based proteomics approach was undertaken to identify differentially expressed proteins in −5 °C- versus 15 °C-exposed *L. decemlineata*. Initially, 1733 proteins were detected using information available for *L. decemlineata* [23] and for *T. castaneum*. A total of 445 proteins having more than two unique peptides identified were shown to be deregulated between both conditions. Elevated levels in cold-exposed insects were observed for 204 proteins while reduced expression was measured for 241 proteins. Over- and under-expressed proteins with absolute log2 fold-changes above 1.25 are presented in Table 1 and Table 2, respectively.

Primary proteins that exhibited elevated levels in *L. decemlineata* maintained at −5 °C when compared to control consisted of putative cuticle protein CP5 (ABZ04122.1), microtubule-associated protein 2 isoform X1 (XP_015840744.1), putative cuticle protein CP7 (ABZ04124.1) and protein Lsm14 homolog B (XP_008198980.1). Proteins displaying the strongest reduction in expression levels were histone H3 (XP_975,418.1), endopolygalacturonase (ADU33363.2) and 3-hydroxy-3-methylglutaryl coenzyme A reductase 2 (Hmgcr2) (AKO63319.1). Elevated levels of multiple proteins associated with stress granules formation and translational regulation were observed in cold-exposed *L. decemlineata* including the eukaryotic translation initiation factor 4B (eIF4B) (ALE20570.1), vigilin (EFA10280.1) and Lsm14. Several cuticular proteins also displayed increased levels in cold-exposed insects such as cuticle proteins CP1 (ABW74141.1), CP4 (ABW74144.1), CP5 and CP7. Additional proteins relevant to insect stress adaptation were overexpressed including diapause protein 1 (CAA53691.1) and a cytochrome P450 (AGT57845.1).

Functional analysis of these differentially expressed proteins was performed using DAVID to identify processes associated with the deregulated protein targets. Primary KEGG as well as GO and COG processes observed using this approach are presented in Fig. 2. Processes associated with metabolism were obtained including metabolic pathways (01100), oxidative phosphorylation (00190), purine metabolism (00230) and fatty acid metabolism (01212). Multiple RNA-associated processes were also observed such as RNA transport (03013), RNA binding (GO:0003723) and mRNA surveillance pathway (03015).

Data collected from this LC-MS/MS-based proteomics approach was further queried against the recently reported *L. decemlineata* reference genome [40]. A total of 2840 proteins were detected and are presented in a volcano plot in Fig. 3. Increased levels in cold-exposed insects were measured for 409 proteins and decreased expression was observed for 200 proteins. Up- and down-regulated proteins with absolute log2 fold-changes above 2.5 are presented in Table 3 and Table 4, respectively.

Proteins that showed increased expression in cold-exposed *L. decemlineata* using this approach notably consisted of dentin sialophosphoprotein-like (XP_023027757.1), host cell factor-1 (XP_023027834.1) and thymosin beta isoform X2 (XP_023014866.1). Proteins showing the most substantial reduction in expression levels

Table 1
Proteins displaying elevated levels in *L. decemlineata* submitted to low temperatures as determined by LC-MS/MS using *L. decemlineata* [23] transcriptome and *T. castaneum* genome as references. [a]Scores associated with protein identification. [b]Overlap ratio of protein sequence. [c]Unique peptide mapped to the protein.

Log2 fold-change					
Accession	Description	−5 °C/Control	Score[a]	Coverage[b]	Peptide[c]
ABZ04122.1	Putative cuticle protein CP5 [Leptinotarsa decemlineata]	3,90	10,97	24	4
XP_015840744.1	PREDICTED: microtubule-associated protein 2 isoform X1 [Tribolium castaneum]	3,32	7,81	3	2
ABZ04124.1	Putative cuticle protein CP7, partial [Leptinotarsa decemlineata]	2,93	13,77	28	3
XP_008198980.1	PREDICTED: protein LSM14 homolog B isoform X1 [Tribolium castaneum]	2,92	13,20	6	3
ABW74141.1	Cuticular protein Ld-CP1v2 [Leptinotarsa decemlineata]	2,69	11,36	34	6
XP_008193884.1	PREDICTED: uncharacterized protein F13E6.1 isoform X1 [Tribolium castaneum]	2,57	17,43	20	6
XP_008195148.1	PREDICTED: far upstream element-binding protein 3 isoform X1 [Tribolium castaneum]	2,49	11,64	3	3
ALE20570.1	EIF4B [Leptinotarsa decemlineata]	2,44	23,23	13	7
ABW74144.1	Cuticular protein Ld-CP4 [Leptinotarsa decemlineata]	2,37	20,08	40	5
XP_975,440.2	PREDICTED: polyadenylate-binding protein 2 isoform X2 [Tribolium castaneum]	2,27	4,19	5	3
XP_015840759.1	PREDICTED: troponin T isoform X3 [Tribolium castaneum]	2,14	33,43	18	2
CAA53691.1	Diapause protein 1, partial [Leptinotarsa decemlineata]	2,07	26,56	15	9
EFA00923.1	Fidgetin-like protein 1 [Tribolium castaneum]	1,99	8,36	10	4
EFA00109.1	Muscle-specific protein 20-like Protein [Tribolium castaneum]	1,97	25,65	34	5
EFA06590.1	LIM and SH3 domain protein Lasp-like Protein [Tribolium castaneum]	1,97	8,04	11	3
XP_966,594.1	PREDICTED: Y-box factor homolog isoform X1 [Tribolium castaneum]	1,96	19,82	11	5
XP_015840550.1	PREDICTED: protein lethal(2)essential for life isoform X1 [Tribolium castaneum]	1,91	17,72	28	6
XP_008193543.1	PREDICTED: coiled-coil domain-containing protein 6 isoform X1 [Tribolium castaneum]	1,62	17,77	17	6
EFA08785.1	RNA-binding protein lark-like Protein [Tribolium castaneum]	1,55	9,01	12	4
XP_008193830.1	PREDICTED: histone H1B-like [Tribolium castaneum]	1,54	6,77	12	3
XP_008201493.1	PREDICTED: histone H1.3 [Tribolium castaneum]	1,54	6,77	10	3
XP_008195798.2	PREDICTED: late histone H1, partial [Tribolium castaneum]	1,54	6,77	7	3
XP_015833244.1	PREDICTED: ELAV-like protein 4 isoform X4 [Tribolium castaneum]	1,50	3,45	5	2
EEZ99985.1	Nuclear valosin-containing protein-like [Tribolium castaneum]	1,49	6,01	4	3
AGX25154.1	Calumenin precursor, partial [Leptinotarsa decemlineata]	1,40	16,01	28	3
EFA10280.1	Vigilin-like Protein [Tribolium castaneum]	1,40	9,64	5	6
XP_970,719.1	PREDICTED: paramyosin, long form [Tribolium castaneum]	1,34	82,99	22	24
XP_008193884.1	PREDICTED: uncharacterized protein F13E6.1 isoform X1 [Tribolium castaneum]	1,31	17,43	20	6
XP_008197362.1	PREDICTED: eukaryotic peptide chain release factor GTP-binding subunit ERF3A isoform X1 [Tribolium castaneum]	1,25	26,86	12	5

were cathepsin L-like proteinase isoform X1 (XP_023029676.1), esterase (AIY68358.1) and histone H3 (XP_023030471.1).

3.3. Expression levels of select targets in cold-exposed *L. decemlineata*

Levels of select cold-associated transcripts identified by RNA-sequencing were quantified in control and −5 °C-exposed insects using qRT-PCR. Transcript levels of ABAT, ABCC4, BMP10, CG8306, Cyp18a1, DGKE, FER, GlcAT-P, Gld, GRP-1, HIBCH, JV, PGBD2, PLA2, SgAbd-8 and ZNF502 are presented in Fig. 4. Six transcript targets were either up-regulated (CG8306, Cyp18a1, GlcAT-P and JV) or down-regulated (FER and GRP-1) when measured by both RNA-sequencing- and qRT-PCR-based approaches. Other transcript targets showed opposing expression status when measured using the two experimental

Table 2
Proteins exhibiting reduced expression in cold-exposed versus control *L. decemlineata* as determined by LC-MS/MS using *L. decemlineata* [23] transcriptome and *T. castaneum* genome as references. [a]Scores associated with protein identification. [b]Overlap ratio of protein sequence. [c]Unique peptide mapped to the protein.

Log2 fold-change					
Accession	Description	−5 °C/Control	Score[a]	Coverage[b]	Peptide[c]
XP_975,418.1	PREDICTED: histone H3 [Tribolium castaneum]	−2,58	4,35	7	2
ADU33363.2	Endopolygalacturonase [Leptinotarsa decemlineata]	−2,25	28,59	21	6
AKO63319.1	3-hydroxy-3-methylglutaryl coenzyme A reductase 2 [Leptinotarsa decemlineata]	−1,90	16,85	10	6
AGT57845.1	Cytochrome P450 334e3. partial [Leptinotarsa decemlineata]	−1,79	20,79	19	8
AIY68358.1	Esterase [Leptinotarsa decemlineata]	−1,70	25,13	18	8
ADU33347.1	Endo-beta-1.4-glucanase [Leptinotarsa decemlineata]	−1,65	17,62	23	4
AIY68368.1	Esterase [Leptinotarsa decemlineata]	−1,60	14,81	16	6
ADU33352.1	Glycoside hydrolase family protein 48 [Leptinotarsa decemlineata]	−1,52	72,17	31	16
AIY68344.1	Esterase. partial [Leptinotarsa decemlineata]	−1,50	9,42	5	2
ABM55480.1	Digestive cysteine protease intestain [Leptinotarsa decemlineata]	−1,49	37,34	27	7
EEZ98647.1	Cytosol aminopeptidase-like Protein [Tribolium castaneum]	−1,49	15,37	11	5
AEX93415.1	Serine protease S1A-1 [Leptinotarsa decemlineata]	−1,45	14,20	23	5
AKO63322.1	Diphosphomevalonate decarboxylase [Leptinotarsa decemlineata]	−1,42	18,37	17	10
XP_015834189.1	PREDICTED: titin isoform X1 [Tribolium castaneum]	−1,42	132,79	3	51
ADU33357.1	Endopolygalacturonase [Leptinotarsa decemlineata]	−1,41	12,74	17	5
XP_015833280.1	PREDICTED: outer dense fiber protein 2 isoform X1 [Tribolium castaneum]	−1,37	4,15	1	2
XP_015834189.1	PREDICTED: titin isoform X1 [Tribolium castaneum]	−1,34	132,79	3	51
AAG01177.2	Inducible heat shock 70 kDa protein. partial [Leptinotarsa decemlineata]	−1,31	32,68	21	9
AIY68354.1	Esterase [Leptinotarsa decemlineata]	−1,26	20,16	15	8
AAN77410.1	Digestive cysteine protease intestain. partial [Leptinotarsa decemlineata]	−1,26	29,24	24	7
ADU33361.2	Endopolygalacturonase [Leptinotarsa decemlineata]	−1,25	19,19	26	8

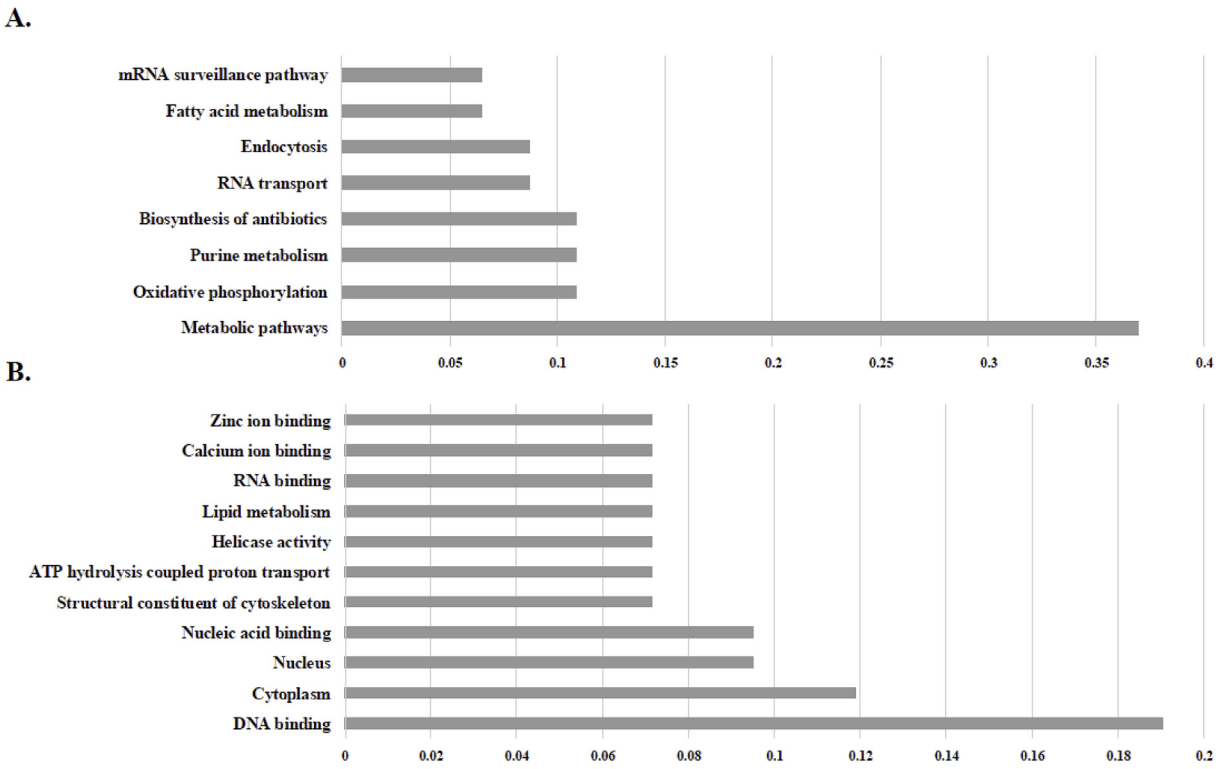

Fig. 2. Processes linked with cold-associated protein targets in *L. decemlineata* as identified with DAVID. Panels **A** (KEGG) and **B** (GO/COG) depict processes linked with modulated proteins. Values are percentages of target candidates pertaining to the listed processes.

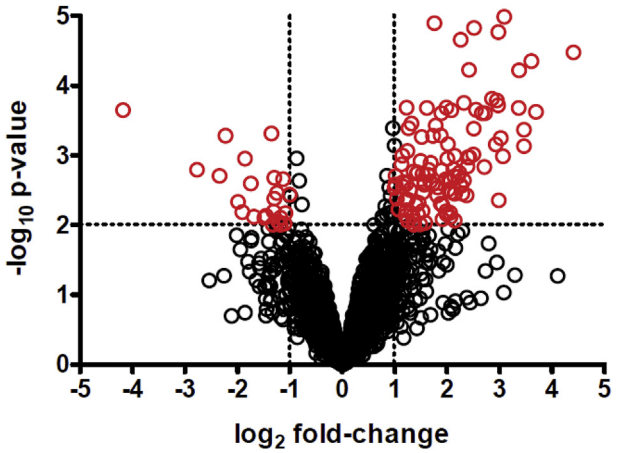

Fig. 3. Volcano plot depicting the proteomics data collected in cold-exposed *L. decemlineata*. Absolute log2 fold-changes and protein expression ratios in −5 °C versus 15 °C insects are plotted on the y-axis and x-axis, respectively. Horizontal dotted line presents *P* values of 0.05 cut-off position while the vertical dotted lines discriminate between proteins having absolute log2 fold-changes of 1. Red dots represent proteins with $P < 0.05$ and absolute log2 fold-changes above 1. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

approaches.

4. Discussion

The molecular adaptations underlying the ability of *L. decemlineata* to cope with sub-zero temperatures are not well-characterized. Multiple studies have relied on omics-based approaches to decipher the molecular underpinnings of cold adaptation in many insect species including the freeze-avoiding Alaskan beetle *Cucujus clavipes puniceus* [8], the Asian corn borer *Ostrinia furnacalis* [43] and a New Zealand alpine stick insect [11] to name a few. A similar approach has not yet been performed in *L. decemlineata*. This work reports differentially expressed transcripts and proteins in cold-exposed *L. decemlineata* using transcriptomics- and proteomics-based experimental tools.

Initial investigation of transcripts differentially expressed in cold-exposed *L. decemlineata* by RNA-sequencing notably highlighted deregulation of several transcripts associated with the ubiquitin-proteasome system (UPS). Even though activity of this pathway has not been thoroughly explored in models of insect cold hardiness, examples exist of UPS-associated targets expression and activity deregulation following cold exposure including the E3 ubiquitin-protein ligase in the cold-hardy beetle *Harmonia axyridis* [48] and the proteasome in shrimps submitted to freeze-thaw cycles [15]. Such cycles were also associated with elevated levels of ubiquitin and ubiquitin conjugates levels in cold-exposed liver samples of the freeze-tolerant wood frog *Rana sylvatica* [56]. These studies support the occurrence of protein turnover in response to low temperatures challenges. The present RNA-sequencing approach also revealed up-regulation of transcripts coding for serine protease inhibitors (serpins) including a serpin-like protein (Ld_rep_c38819) and the serpin peptidase inhibitor 20 (Ld_rep_c68571). Several members of this family have displayed elevated levels in models of cold adaptation. Mammalian hibernation-associated protein HP55, member of a complex that displays significant expression in blood samples of hibernating chipmunks, exhibits substantial homology to the serpin Alpha-1-antitrypsin [33]. Microarray-based approaches also lead to the identification of up-regulated serpins C1, E2 and F2 during hibernation [46,47]. This variation was postulated to underlie the inflammation and immune responses depression observed in mammalian hibernators. Follow-up work to better characterize the expression and functions of select members of the serpin family in cold-exposed models is envisioned. In addition, in-depth analysis of the generated transcriptomics data by leveraging recently disseminated information on the Colorado potato beetle genome [40] might also yield valuable transcripts associated with low temperatures response.

Table 3
Proteins showing increased levels in *L. decemlineata* exposed to low temperatures as determined by LC-MS/MS using *L. decemlineata* [40] as reference. [a]Scores associated with protein identification. [b]Overlap ratio of protein sequence. [c]Unique peptide mapped to the protein.

Accession	Description	−5 °C/Control	Score[a]	Coverage[b]	Peptide[c]
XP_023027757.1	Dentin sialophosphoprotein-like [Leptinotarsa decemlineata]	4,41	4,96	2	2
XP_023020459.1	Uncharacterized protein LOC111509022, partial [Leptinotarsa decemlineata]	4,11	63,92	48	9
XP_023027834.1	Host cell factor 1-like, partial [Leptinotarsa decemlineata]	3,69	25,84	20	8
XP_023014866.1	Thymosin beta isoform X2 [Leptinotarsa decemlineata]	3,61	25,10	50	3
XP_023021423.1	Endocuticle structural glycoprotein SgAbd-2-like [Leptinotarsa decemlineata]	3,47	44,60	37	6
XP_023027311.1	ADP-ribosylation factor GTPase-activating protein 2-like [Leptinotarsa decemlineata]	3,47	14,17	10	5
XP_023030369.1	Glutamic acid-rich protein-like [Leptinotarsa decemlineata]	3,37	27,53	21	7
XP_023029885.1	Acaloleptin A-like [Leptinotarsa decemlineata]	3,29	5,44	26	2
XP_023017419.1	Uncharacterized protein LOC111506545 isoform X1 [Leptinotarsa decemlineata]	3,09	9,62	17	3
XP_023024657.1	Glucose dehydrogenase [FAD, quinone]-like, partial [Leptinotarsa decemlineata]	3,08	4,11	4	2
ABW74140.1	Cuticular protein Ld-CP1v1 [Leptinotarsa decemlineata]	3,06	19,29	35	7
XP_023022706.1	Glycine-rich cell wall structural protein 1.8-like isoform X1 [Leptinotarsa decemlineata]	3,02	38,88	9	3
XP_023019133.1	Death-associated protein 1 [Leptinotarsa decemlineata]	2,98	13,60	39	3
ABZ04124.1	Putative cuticle protein CP7, partial [Leptinotarsa decemlineata]	2,97	19,88	28	3
XP_023029262.1	Uncharacterized protein LOC111517359 [Leptinotarsa decemlineata]	2,97	19,37	11	4
XP_023025857.1	Protein LSM14 homolog B, partial [Leptinotarsa decemlineata]	2,95	57,70	32	8
XP_023015702.1	DNA ligase 1-like [Leptinotarsa decemlineata]	2,95	8,97	12	2
XP_023014867.1	Thymosin beta isoform X3 [Leptinotarsa decemlineata]	2,93	18,61	42	2
XP_023013693.1	Uncharacterized protein LOC111503583 isoform X1 [Leptinotarsa decemlineata]	2,86	11,55	7	4
XP_023027974.1	Protein amnionless [Leptinotarsa decemlineata]	2,79	7,03	7	2
XP_023024610.1	Uncharacterized protein LOC111512697 [Leptinotarsa decemlineata]	2,74	17,66	16	2
XP_023012739.1	Lysosomal aspartic protease-like [Leptinotarsa decemlineata]	2,72	48,01	15	6
XP_023020610.1	rRNA methyltransferase 3, mitochondrial [Leptinotarsa decemlineata]	2,71	8,74	10	4
XP_023013883.1	Acidic leucine-rich nuclear phosphoprotein 32 family member B [Leptinotarsa decemlineata]	2,66	12,40	23	3
XP_023019726.1	Sorting nexin-2 [Leptinotarsa decemlineata]	2,55	23,98	12	2
XP_023023783.1	Uncharacterized abhydrolase domain-containing protein DDB_G0269086, partial [Leptinotarsa decemlineata]	2,51	177,97	62	38
XP_023021426.1	Endocuticle structural glycoprotein SgAbd-2-like isoform X1 [Leptinotarsa decemlineata]	2,51	29,04	23	2

Subsequent proteomics-based approach in cold-exposed *L. decemlineata* also revealed proteins, such as lethal(2)essential for life (l(2)efl) (XP_015840550.1) and multiple cuticular proteins, previously linked with response to environmental stresses in various models. Elevated protein levels of l(2)efl, a small heat shock protein also known as CryAB, were observed in *L. decemlineata* exposed to low temperatures. Previous work reported elevated l(2)efl transcript levels in *D. melanogaster* exposed to a wide range of stresses including heat shock, hyperoxia and ionizing radiation [24]. L(2)efl expression has also been shown to be under the control of the fruit fly FOXO (dFOXO) transcription factor [13,53]. It is noteworthy to point out that DAF-16, the FOXO ortholog in *Caenorhabditis elegans*, has been associated with cold temperature resilience in long-lived nematodes [39]. While no information is available regarding l(2)efl expression in cold-exposed insects, the results gathered here provide another condition following which modulation of this target is observed and supports an investigation of the role of l(2)efl in insect cold hardiness. Multiple cuticular proteins, including CP1, CP4, CP5 and CP7, were differentially expressed in cold-exposed Colorado potato beetles. qRT-PCR-based quantification of CP7 transcript levels also revealed elevated levels in cold-exposed insects. Cuticular proteins are key components of insect cuticles and previous work reported differential expression of transcripts coding for such proteins in *L. decemlineata* exposed to various environmental stresses [61]. Two microarray-based studies showed elevated transcript levels of cuticular genes in the Antarctic collembolan *Cryptopygus antarcticus* that had been either cold-acclimated or that displayed low supercooling points [7,37] supporting the

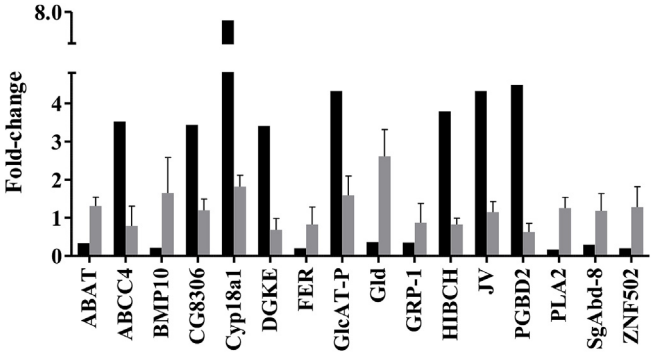

Fig. 4. Relative expression of select targets as quantified by qRT-PCR in cold-exposed versus control *L. decemlineata*. Expression levels determined by next-generation sequencing are presented in dark. Expression levels assessed by qRT-PCR are presented in gray. qRT-PCR shows normalized transcript levels (mean ± SEM, n = 3–4).

Table 4
Proteins showing decreased levels in *L. decemlineata* submitted to low temperatures as determined by LC-MS/MS using *L. decemlineata* [40] as reference. [a]Scores associated with protein identification. [b]Overlap ratio of protein sequence. [c]Unique peptide mapped to the protein.

Log2 fold-change					
Accession	Description	−5 °C/Control	Score[a]	Coverage[b]	Peptide[c]
XP_023029676.1	Cathepsin L-like proteinase isoform X1 [Leptinotarsa decemlineata]	−4,19	51,08	29	10
AIY68358.1	Esterase [Leptinotarsa decemlineata]	−2,77	47,32	21	2
XP_023030471.1	Histone H3 [Leptinotarsa decemlineata]	−2,54	10,40	14	2

potential involvement of cuticular rearrangements for cold hardiness in select Antarctic arthropods [49]. The expression data gathered on cuticular proteins in cold-exposed *L. decemlineata* appears aligned with this theme. It is interesting to note that miR-92a, a miRNA known to regulate the expression of cuticular protein CpCPR4 in *Culex pipiens pallens* [30], displayed substantial down-regulation in cold-exposed *L. decemlineata* [32]. Similarly, reduced levels of miR-282–5p [32] and the elevated expression of a predicted target CG14435, a protein with ubiquitin transferase activity, in cold-exposed *L. decemlineata* provide an additional link between miRNAs and targets with potential relevance to cold adaptation. The current work also revealed divergences in eIF4B transcript and protein levels in cold-exposed insects as measured by mass spectrometry and qRT-PCR suggesting the involvement of post-transcriptional regulatory mechanisms of target expression. Furthermore, while the most deregulated targets observed by RNA-sequencing and proteomics data gather in this work do not perfectly overlap, multiple studies have shown such a discrepancy and have notably attributed these variations to divergences in mRNA and protein half-lives [14,41]. Overall, these observations all support further investigation of miRNA-mediated regulation of cold-associated targets in this insect. It is also important to point out that while measured transcript levels quantified by RNA-sequencing and qRT-PCR were aligned for certain targets, discrepancies in levels obtained via both methods were observed for several others. This phenomenon has been reported previously [12]. In addition, qRT-PCR validation was realized using RNA isolates prepared from different insects and collected at different time-points during the summer. The seasonal effect could explain the divergent results of RNA-sequencing and qRT-PCR as observed by others. For instance, RNA-sequencing profiling performed in early versus late summer adult European corn borer moth *Ostrinia nubilalis* demonstrated differential expression of several gene targets between the two groups [52]. Follow-up work will be required to better delineate the molecular mechanisms underlying potential seasonal changes in *L. decemlineata*. The current study also highlighted several up-regulated proteins, including vigilin, associated with stress granules and processing bodies (P-bodies) in cold-exposed beetles. Stress granules and P-bodies are well-characterized RNA granules that can impact mRNA translation and degradation [5]. Previous work reported stress granules formation following low temperatures exposure in various models and linked their formation with translational arrest [4,18]. A study conducted on vigilin in yeast revealed its presence in stress granules and discussed its role during thermal stress response [55]. Deregulation of vigilin observed in cold-exposed *L. decemlineata* supports a more in-depth investigation of additional stress granules and P-bodies components in models of insect cold hardiness to better characterize the relevance of these structures, if at all, during cold exposure. Finally, it is also of interest to point out that transcript levels of select cold-associated targets identified by the current proteomics-based approach, including diapause protein-1 [60] as well as endo-polygalacturonase and digestive cysteine proteinase [59], were previously linked to diapausing *L. decemlineata* warranting closer investigation of a common signature, if any, of cold- and diapause-associated molecular changes in this insect.

Functional relevance of transcripts and proteins displaying significant changes in expression between cold-exposed and control *L. decemlineata* was conducted. This approach notably revealed several deregulated proteins associated with metabolic cascades such as oxidative phosphorylation and fatty acid metabolism. An overview of important metabolic pathways containing differentially expressed transcripts and proteins generated in the current work is presented in Fig. 5. Numerous studies focused on the metabolic impact of low temperatures exposure in insects have reported noteworthy metabolic cascades modulation. Pioneering work performed on the effect of cold temperatures on membrane lipids homeostasis showed that rapid cold hardening influenced the composition of phospholipid-associated fatty acids in *D. melanogaster* cellular membranes [35]. Dynamic changes associated with membrane lipids composition were also observed in cold-acclimated *D. melanogaster* larvae [21]. Seasonal changes were linked to variations in fatty acid unsaturation and membrane lipids composition in larvae of the codling moth, *Cydia pomonella* [38]. The results gathered in the current work appear aligned with these findings and reinforce the relevance of fatty acid homeostasis in cold-exposed *L. decemlineata*. Oxidative phosphorylation was also a metabolic process highlighted following functional annotation of proteins that displayed differential expression in *L. decemlineata* submitted to low temperatures. Recent work in the cold-exposed ghost moth *Hepialus xiaojinensis* positioned the tricarboxylic acid (TCA) cycle and oxidative phosphorylation as key cascades underlying cold resiliency in this insect [62]. The likely importance of the TCA cycle pathway was also highlighted in the cold-tolerant sycamore lace bug *Corythucha ciliate* using RNA-sequencing and comparative metabolomics approaches [26]. While predicted processes impacted by low temperatures revealed in cold-exposed *L. decemlineata* are in line with other studies, additional work to investigate the expression and activity status of specific molecular players regulating the identified metabolic cascades is envisioned.

In conclusion, this work has leveraged omics-based approaches to reveal multiple transcript and protein targets modulated by low temperatures in cold-exposed *L. decemlineata*. Functional assessment of the differentially expressed targets notably revealed key processes affected by cold exposure in this insect. Future work is planned, including RNAi-based approaches aimed at such targets, to better characterize the impact that select molecular players have on *L. decemlineata* cold response and survival at low temperatures. Assessment of identified leads in *L. decemlineata* maintained at low temperatures for a longer duration, as well as exploring the transcriptional changes observed in insects maintained at different temperatures prior to sub-zero exposure, would strengthen the molecular signatures reported in the current work. Investigation of differentially expressed targets in males versus females *L. decemlineata* exposed to cold would also allow exploration of sex-biased expression of select gene candidates, if any, which was not considered in this work. Overall, this study further contributes to the growing knowledge associated with the molecular changes linked to cold exposure in *L. decemlineata*.

Author contributions

Experimental design: L.G., S.B., A.C., S.G.L. and P.J.M.; Experimental work: L.G., M.D.M., J.J.F. and A.C.; Students supervision: A.C., S.G.L. and P.J.M.; Manuscript writing: L.G. and P.J.M. All authors have revised and approved the final manuscript.

Competing interests

The authors declare no competing or financial interests.

Funding

P.J.M. and S.G.L. are supported by Discovery Grants from the Natural Sciences and Engineering Research Council of Canada (RGPIN 2018-05974 and 435638-2013, respectively). P.J.M. is also supported by a Research Grant (EARI 15-007) via the Enabling Agricultural and Research Innovation (EARI) program under the Canada/New Brunswick Growing Forward 2 initiative.

Acknowledgements

We thank Zahidur Abedin for his assistance with the transcriptomics experiment and data analysis. We also thank Catherine Clark for her ongoing technical support with Colorado potato beetles.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://

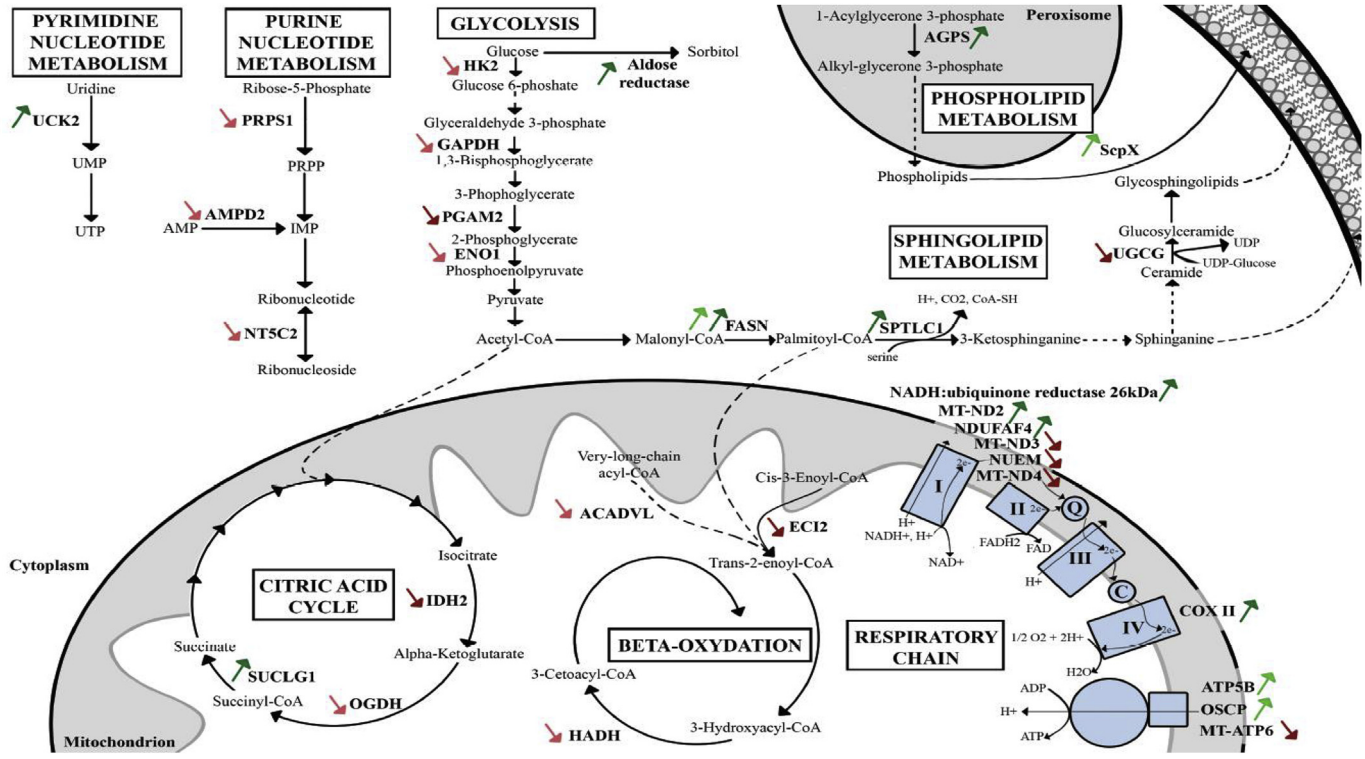

Fig. 5. An overview of key differentially expressed transcript and protein targets with relevance to select metabolic cascades in cold-exposed *L. decemlineata*. Green and red arrows represent up- or down-regulated targets, respectively. Darker and lighter arrows represent transcript and protein targets, respectively. Abbreviations: ACADVL: Acyl-CoA dehydrogenase very long chain; AGPS: Alkylglycerone phosphate synthase; AMPD2: Adenosine monophosphate deaminase 2; ATP5B: ATP synthase subunit beta, mitochondrial; COX11: Cytochrome *c* oxidase copper chaperone COX11; ECI2: Enoyl-CoA delta isomerase 2; ENO1: Enolase 1; FASN: Fatty acid synthase; GAPDH: Glyceraldehyde-3-phosphate dehydrogenase; HADH: Hydroxyacyl-CoA dehydrogenase; HK2: Hexokinase; IDH2: Isocitrate dehydrogenase 2; MT-ATP6: Mitochondrially encoded ATP synthase 6; MT-ND2: mitochondrially encoded NADH dehydrogenase 2; MT-ND3: Mitochondrially encoded NADH dehydrogenase 3; MT-ND4: Mitochondrially encoded NADH dehydrogenase 4; NDUFAF4: NADH:ubiquinone oxidoreductase complex assembly factor 4; NT5C2: 5′-nucleotidase, cytosolic II; NUEM: NADH-ubiquinone oxidoreductase 39 kDa subunit; OGDH: 2-oxoglutarate dehydrogenase, mitochondrial; OSCP: Oligomycin sensitivity-conferring protein; PGAM2: Phosphoglycerate mutase 2; PRPS1: Phosphoribosyl pyrophosphate synthetase 1; ScpX: Sterol carrier protein X-related thiolase; STPLC1: Serine palmitoyltransferase 1; SUCLG1: Succinate-CoA ligase alpha subunit; UCK2: Uridine-cytidine kinase; and UGCG: UDP-glucose ceramide glucosyltransferase. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

doi.org/10.1016/j.cryobiol.2019.03.011.

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