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Global and comparative proteomic profiling of overwintering and developing mountain pine beetle, *Dendroctonus ponderosae* (Coleoptera: Curculionidae), larvae

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ABSTRACT

Background: Mountain pine beetles, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae), are native to western North America, but have recently begun to expand their range across the Canadian Rocky Mountains. The requirement for larvae to withstand extremely cold winter temperatures and potentially toxic host secondary metabolites in the midst of their ongoing development makes this a critical period of their lives.

Results: We have uncovered global protein profiles for overwintering mountain pine beetle larvae. We have also quantitatively compared the proteomes for overwintering larvae sampled during autumn cooling and spring warming using iTRAQ methods. We identified 1507 unique proteins across all samples. In total, 33 proteins exhibited differential expression (FDR < 0.05) when compared between larvae before and after a cold snap in the autumn; and 473 proteins exhibited differential expression in the spring when measured before and after a steady incline in mean daily temperature. Eighteen proteins showed significant changes in both autumn and spring samples.

Conclusions: These first proteomic data for mountain pine beetle larvae show evidence of the involvement of trehalose, 2-deoxyglucose, and antioxidant enzymes in overwintering physiology; confirm and expand upon previous work implicating glycerol in cold tolerance in this insect; and provide new, detailed information on developmental processes in beetles. These results and associated data will be an invaluable resource for future targeted research on cold tolerance mechanisms in the mountain pine beetle and developmental biology in coleopterans.

1. Introduction

Populations of mountain pine beetles, *Dendroctonus ponderosae* Hopkins (Coleoptera: Curculionidae), have increased rapidly in recent years and have had major impacts on forests throughout western North America. A general warming trend in temperatures across western Canada is one factor that has influenced the spread of this outbreak (Stahl et al., 2006; Carroll et al., 2003). The massive infestations have caused large-scale economic, social, and environmental changes in the region and on a global scale (Safranyik and Wilson, 2006; Kurz et al., 2008). Populations of the insects are now spreading to forests outside of their historical range (Nealis and Peter, 2008) and into a new host species (jack pines, *Pinus banksiana*) east of the Canadian Rocky Mountains (Cullingham et al., 2011).

Mountain pine beetles spend the majority of their one-year life cycle as larvae under the bark of host trees, and must endure prolonged cold exposure in that location during winter. Cold temperatures, particularly before the larval beetles have developed resistance to freezing, have been suggested to cause significant mortality in overwintering populations (Stahl et al., 2006; Cole, 1981; Safranyik, 1978). Though temperatures regularly fall below their bodily fluid freezing point, larvae avoid internal ice formation by shifting energy from developmental and basal metabolism to the biosynthesis of cryoprotectants, mainly glycerol, to attain a supercooled state (Bentz and Mullins, 1999; Fraser, 2011; Joanisse and Storey, 1994c).

Larvae develop through four instars (Safranyik and Wilson, 2006) and alternate daily between states of quiescence and further development (Powell and Logan, 2005). Cold tolerance

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increases with successive larval instars, with third and fourth instar larvae, the usual overwintering stages, not experiencing high mortality until temperatures reach −30 to −40 °C for several hours (Wygant, 1940). If gradually allowed to cold harden and produce sufficient glycerol, late-instar larvae can survive for extended periods of time at temperatures near −40 °C (Safranyik and Wilson, 2006; Wygant, 1940). Until recently, there was no evidence to suggest that mountain pine beetles undergo diapause, a longer and distinctly regulated period of suspended development, during any life stage. However, Lester and Irwin (2012) suggest that overwintering adults that survive under the bark through the winter months may undergo a period of facultative diapause.

While occupying a large latitudinal and elevational range throughout western North America, the most limiting factor in further range expansion of the mountain pine beetle is unfavorable climatic conditions (Carroll et al., 2003). The continued eastward shift of the leading edges of the mountain pine beetle infestation into the very cold northern boreal forest will be dependent, in part, upon the physiology that underlies this insect's cold tolerance mechanisms.

The recent development of mountain pine beetle genomic resources – including an extensive EST database (Keeling et al., 2012) – has expanded our ability to identify genes and their protein products associated with processes of successful insect outbreaks. Because measures of transcript accumulation alone are not always a reliable indicator of protein abundance (Greenbaum et al., 2003; Maier et al., 2009), we conducted a quantitative comparison of protein profiles in larval mountain pine beetles that were field-sampled in the autumn, before and during a cold spell, and in early- and late-spring as ambient temperatures increased.

Our investigation to identify the proteins that are present in overwintering and developing larvae, and which proteins seasonally shift in abundance, will provide a rich source of information to confirm existing and identify novel gene candidates for further study.

2. Materials and methods

2.1. Collection of larval specimens

The sampling sites contained lodgepole pines (*Pinus contorta*) that were naturally infested with mountain pine beetle and were located near Tête Jaune Cache, British Columbia, Canada (N 53° 3′ 36.00″, W 119° 36′ 54.00″ and N 52° 55′ 4.00″, W 119° 21′ 23.00″). Eleven freshly attacked lodgepole pines were each fitted with three iButton temperature dataloggers (Maxim, Sunnyvale, CA, USA) that recorded ambient temperature every 30 min throughout the study period. Temperatures were monitored at the base of each tree, and at 1.3 m above the forest floor on both the north and south sides of tree boles. Overwintering mountain pine beetle larvae were excised and live-collected from under the bark, flash frozen in individual vials, immediately transported back to the lab covered in dry ice, and stored at −80 °C until protein extractions were conducted. Protein extractions were performed on insects collected on 26 September and 7 November 2008 (early- and late-autumn) and on 25 March and 27 May 2009 (early- and late-spring).

2.2. Protein extraction

Eight frozen larvae from each of the four collection dates, chosen randomly from the eleven trees, were used per extraction and each extraction was replicated four times. Larvae were thawed on ice for 5 min and were then homogenized in 500 μL TCA buffer containing 15% trichloroacetic acid (Sigma–Aldrich) and 1% dithiothreitol (Fisher Scientific) by weight (protocol adapted from L.J. Foster, personal communication). Samples were homogenized six times for 1 min at 1500 rpm on a GenoGrinder 2000 (SpexCertiprep, USA) with 3 min incubation on ice in between. The homogenate was transferred to a new tube and incubated on ice for 30 min. Samples were then centrifuged at 18,000×*g* for 10 min at 4 °C and the supernatant was removed. The pellet was resuspended in 1 mL of ice-cold acetone and incubated on ice for 5 min. This acetone wash was repeated four times. Pellets were air dried for 10 min to allow the acetone to evaporate and were then solubilized in 1 mL urea/thiourea buffer containing 6 M urea (Fisher Scientific) and 1 M thiourea (Fisher Scientific) in 100 mM Tris–Cl (pH 8.0; Ultrapure, Invitrogen). A final centrifugation at 18,000×*g* was conducted for 10 min at room temperature to pellet any remaining insoluble debris. The supernatant containing total insect proteins was collected and stored at −80 °C for subsequent iTRAQ analysis. Protein concentration and quality was confirmed by a Bradford Protein Assay Kit (Fisher Scientific, Ottawa, ON) and Experion Pro260 Chip analysis (Biorad, Hercules, CA).

2.3. Experimental design and iTRAQ analysis

Four biological replicates (each containing protein from eight pooled larvae) from each of the two autumn sampling dates were analyzed in one eight-plex run, while four biological replicates from each of the two spring sampling dates were analyzed in a separate eight-plex run. We compared protein abundances between 7 November 2008 and 26 September 2008, and between 27 May 2009 and 25 March 2009.

Protein extracts were analyzed by eight-plex isobaric tags for relative and absolute quantification (iTRAQ) at the University of Victoria Genome British Columbia Proteomics Centre (Victoria, British Columbia, Canada) as per Lippert et al. (2009) and Ohlund et al. (2011). All data were analyzed with ProteinPilot™ Software v3.0 (Applied Biosystems) using the Paragonô algorithm (Shilov et al., 2007) against a translated database of MPB transcriptome sequences (Keeling et al., 2012). The following analytical parameters were applied: Cys alkylation: MMTS; digestion: trypsin; instrument: QSTAR ESI; and an Unused ProtScore threshold of >1.3 (95% protein confidence).

2.4. Protein quantification and statistical analysis

Protein quantities from all autumn samples were normalized to one of the replicates from 26 September 2008, assigning this replicate a quantity of 1. Likewise, spring quantities were normalized to one of the replicates from 25 March 2009. These normalized protein ratios were then $\log_{10}$ transformed to normally distribute the data. A two-tailed *t*-test was performed on the transformed ratio data to compare the two sample groups within each eight-plex. The Benjamini–Hochberg correction was applied to control for false discovery (Benjamini and Hochberg, 1995; Hakimov et al., 2009) by ranking proteins from smallest to largest *p*-value, multiplying each *p*-value by the total number of proteins originally quantified by iTRAQ, and dividing by its rank. Proteins with a *q*-value (BH-corrected *p*-value) <0.05 are considered significantly differentially expressed with a 5% false discovery rate (FDR < 0.05). Average fold changes, representing the change in protein abundance, were calculated for the later date, relative to the earlier date, from the untransformed iTRAQ ratio data.

Proteins were not filtered by their fold change values, as proteomics experiments have followed the general trend of microarray experiments in selecting fold change cutoffs that are arbitrary (Seshi, 2006), and the validity of these cutoffs has not been well assessed (Yang et al., 2002). For the purposes of presenting these data for the first time, all proteins that pass the FDR cutoff are

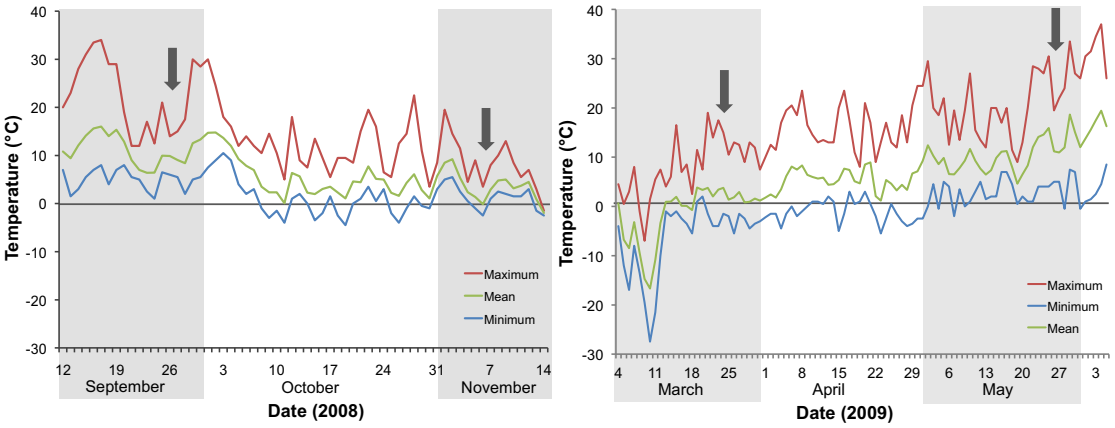

Fig. 1. Maximum, minimum, and mean daily temperatures logged at the sampling trees near Tête Jaune Cache, BC, Canada during autumn 2008 (left) and spring 2009 (right). Arrows indicate the four sampling dates for which mountain pine beetle larval proteomics data were acquired: 26 September 2008, 7 November 2008, 25 March 2009, and 27 May 2009.

included, and fold changes should be considered when regarding the importance of individual proteins. The majority of proteins that passed the FDR cutoff do have higher fold changes than the generally used cutoff of 1.2.

2.5. Protein identification and classification

Proteins were identified with the closest BLASTx hits of their corresponding assembled EST sequence to other insect proteins available on NCBI. All proteins were manually annotated using UniProtKB (The UniProt Consortium, 2011) and subsequently grouped into broad functional classes for comparison.

3. Results and discussion

3.1. Temperature data

Temperature data were compiled and maximum, minimum, and mean daily temperatures throughout the sampling period were determined (Fig. 1). Due to technical difficulties, 17 h of temperature data were lost between 9:36 a.m., 1 April, 2009 and 2:36 a.m., 2 April, 2009. Daily minimum, mean and maximum temperatures for 1 April, 2009 were estimated by averaging daily minimum, mean and maximum temperatures for 31 March, 2009 and 2 April, 2009. While these temperature data provide a good picture of weekly trends in ambient temperature variation at the sampling site, it is important to note that temperatures under the bark, where the larvae were developing, can differ substantially due to factors such as bark thickness, tree diameter, and snow depth (Safranyik and Wilson, 2006).

3.2. Global larval proteome profile

In total 1139 proteins were detected in autumn-collected larvae, and 1051 proteins were detected in the spring-collected larvae. These were manually annotated and grouped into fourteen broad functional classes (Fig. 2). Of these proteins, 456 were exclusively present in autumn larvae and 368 in spring larvae (Fig. 3), highlighting the distinct seasonal difference in overall proteome composition. The proportions of proteins in each functional class were similar between autumn and spring. The largest classes were those involved in protein metabolism and modification; carbohydrate metabolism; and muscle, cytoskeletal and cuticlar processes.

3.2.1. Protein metabolism/modification class

Proteins in these functional groups made up 20.5% of the autumn larval proteome and 23.6% of the spring larval proteome. Protein synthesis enzymes made up almost half of this class, with the other half comprised mainly of protein degradation and protein-folding enzymes. The protein biosynthetic category was represented largely by ribosomal proteins, some translation factors, and a few tRNA aminoacylation enzymes. Proteasome subunits,

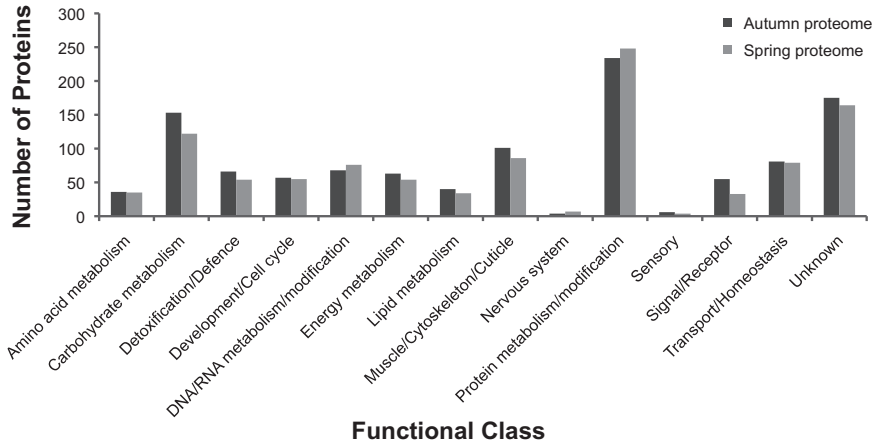

Fig. 2. Functional classification of all proteins detected in the autumn (black) and spring (grey) mountain pine beetle larval proteomes, including proteins that are common to both.

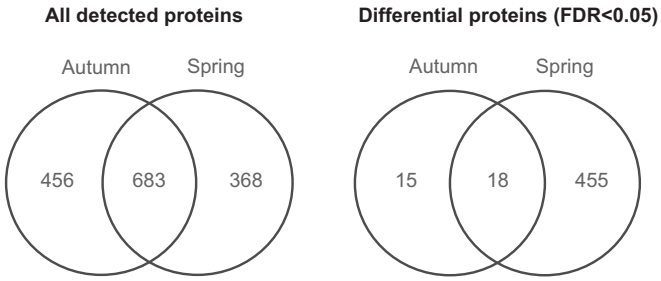

Fig. 3. Venn diagrams illustrating the proportion of all proteins from the mountain pine beetle larval proteome that are common between autumn and spring (left) and the proportion of differentially accumulated (FDR < 0.05) proteins that are common between autumn and spring (right).

ubiquitin-conjugating enzymes, and peptidases were grouped together under proteolysis. The protein-folding category was represented by heat shock proteins, T-complex subunits, protein disulfide isomerase, and peptidyl–prolyl *cis-trans* isomerase. We also observed some protein modification enzymes that are likely involved in protein sumoylation, GPI-anchor attachment, glycosylation, and acetylation.

3.2.2. Carbohydrate metabolism class

Enzymes involved in glycolysis; the citric acid cycle; metabolism of other sugars and their derivatives; and digestion made up 13.4% of the autumn larval proteome and 11.6% of the spring larval proteome. All ten enzymes involved in glycolysis were represented, as well as enzymes that regulate the supply of glucose to glycolysis, including glycogen phosphorylase, glycogen synthase, and phosphoglucomutase. All eight enzymes that function in the citric acid cycle were detected, as were subunits of pyruvate dehydrogenase and pyruvate carboxylase that feed carbon to the cycle. We also detected glycerol-3-phosphate dehydrogenase and sorbitol dehydrogenase, involved in the metabolism of polyols that are implicated in cryoprotection (Storey and Storey, 1981). Trehalose-phosphate synthase and UTP-glucose-1-phosphate uridyltransferase, which are important for protection against freezing and other stresses, were also observed. Phosphoenolpyruvate carboxykinase (PEPCK) was detected in both proteomes. PEPCK is important enzyme in maintaining glucose levels through gluconeogenesis and is important for glycerol biosynthesis in mountain pine beetle (Fraser, 2011). More than 20 digestive enzymes involved in cellulose and pectin degradation were found, some showing high similarity to those previously described in mountain pine beetle (Pauchet et al., 2010). These provide support to previous work indicating that beetles themselves, not just their gut flora, are able to breakdown plant cell walls (Pauchet et al., 2010; Watanabe and Tokuda, 2010), even at the larval stage.

3.2.3. Muscle/cytoskeleton/cuticle class

Structural and motor proteins comprised 8.9% of autumn larval proteins and 8.2% of spring larval proteins. These include multiple types of actin, myosin, tropomyosin, tropomodulin, troponin, zeelin, tubulin, and cuticle constituents. Changes to cellular and body structure are of significant importance in surviving low temperatures and dehydration (Thorne et al., 2011). In addition, developing larvae experience dramatic growth (particularly in the spring) and other changes prior to progression into the pupal stage.

3.3. Autumn larval proteome overview

We detected 1081 proteins in the autumn larvae with quantitative iTRAQ ratios, 203 of which showed significant shifts in accumulation ($p < 0.05$; Fig. 4). After application of the Benjamini–

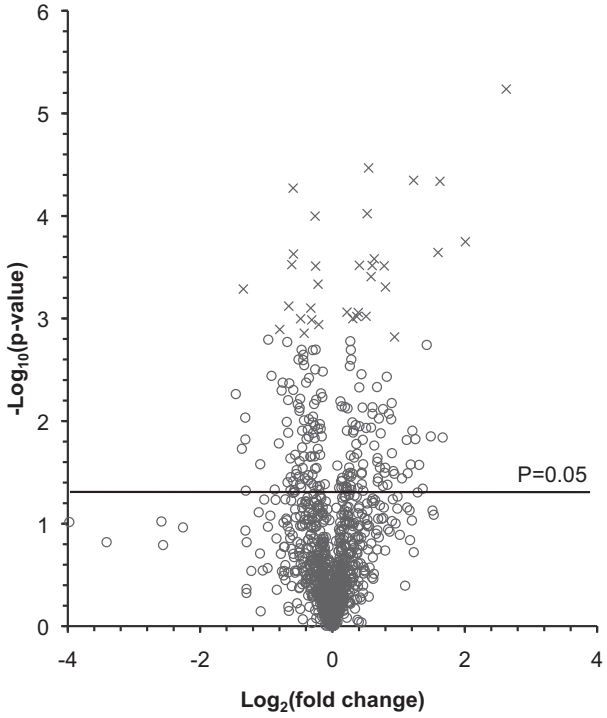

Fig. 4. Volcano plot representing all proteins with quantifiable iTRAQ ratio differences between the two autumn sampling dates. Proteins located further upward had more significant shifts and proteins located further outward from the origin shifted with higher absolute fold change. 203 proteins had *p*-values less than 0.05 when their iTRAQ ratios were compared between the two autumn sampling dates, shown here by points lying above the $p = 0.05$ line. After controlling for 5% FDR, 33 proteins still showed significant shifts in abundance, represented by 'x'.

Hochberg correction to control for false discovery, 33 proteins showed significant differential expression (FDR < 0.05) between the two autumn sampling dates (Fig. 4). Fold changes for those 33 proteins ranged from −2.5 to 6.2 for the 7 November samples relative to 26 September. These 33 proteins were functionally annotated and grouped into 13 functional classes (Fig. 5).

3.4. Spring larval proteome overview

We detected 991 proteins in the spring larvae with quantitative iTRAQ ratios, 554 of which showed significant shifts in accumulation ($p < 0.05$ Fig. 6). After controlling for 5% false discovery, 473 proteins showed significant differential expression (FDR < 0.05) between the two spring sampling dates (Fig. 6). Fold changes for these 473 significantly differentially accumulating proteins ranged from −69.2 to 22.1 for 27 May samples relative to 25 March. These 473 proteins were grouped into 14 broad functional classes (Fig. 7).

3.5. Proteins showing significant differential expression

3.5.1. Autumn

Of the 33 proteins (Supplemental Table 1) that showed statistically significant differential accumulation (FDR < 0.05) between the two autumn sampling dates, six were grouped under energy metabolism, with three of those involved in adenylate metabolism and the other three in the electron transport chain. Three proteins that are involved in protection against membrane peroxidation and toxic radicals show increasing expression during the autumn. This indicates that mountain pine beetles experience oxidative stress during overwintering, likely related to changes in basal

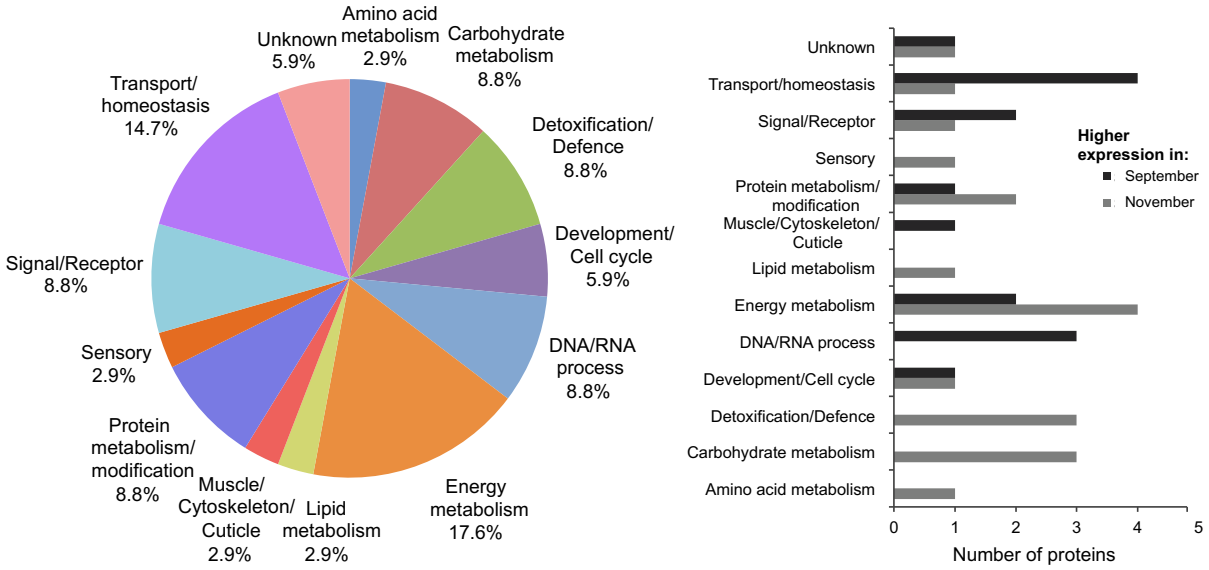

Fig. 5. Functional annotation (left) of the 33 proteins that significantly changed (FDR < 0.05) in autumn larvae and their expression breakdown (right).

metabolism, possible periods of hypoxia, and low-temperature stress (Storey and Storey, 2010). α,α,-Trehalose-phosphate synthase, involved in trehalose synthesis, increased significantly by 1.33-fold from September to November. Trehalose is a known cryoprotectant in insects (Baust and Lee, 1983), along with generally being the major sugar found in their hemolymph (Wyatt and Kalf, 1957). The levels of 2-deoxyglucose-6-phosphate phosphatase increased 6.18-fold in autumn, indicating that the larvae are concomitantly increasing production of 2-deoxyglucose which is known to be a suppressor of glycolysis in other animals (Wan et al., 2004). Ferritin, an iron storage protein linked to stress response in animals, plants, and cells (Carrasco et al., 2011; Kitteringham et al., 2010; Yamaguchi-Shinozaki and Shinozaki, 2006; Orino et al., 2001), increased 4.02-fold in November compared to September. One protein involved in isoprenoid biosynthesis showed higher levels in November with a fold change of 1.52.

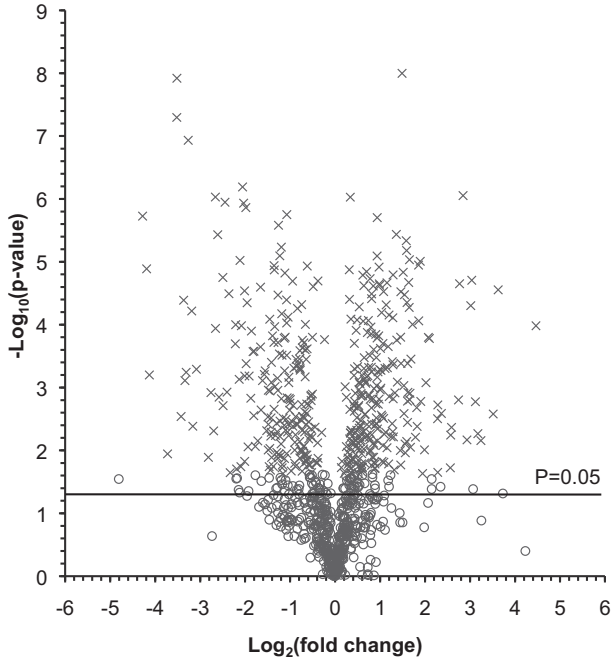

Fig. 6. Volcano plot representing all proteins with quantifiable iTRAQ ratio differences between the two spring sampling dates. Proteins located further upward had more significant shifts and proteins located further outward from the origin shifted with higher absolute fold change. 554 proteins had *p*-values less than 0.05 when their iTRAQ ratios were compared between the two spring sampling dates, shown here by points lying above the $p = 0.05$ line. After controlling for 5% FDR, 473 proteins still showed significant shifts in abundance, represented by 'x'.

3.5.2. Spring

The number of proteins with significantly changing levels in the spring was large, but not surprising considering the developmental changes that late-instar larvae go through in the two-month span leading up to pupation. Proteins involved in amino acid metabolism were generally found in reduced levels in May-collected larvae, with the exception of two enzymes involved in serine metabolism, and one enzyme involved in arginine biosynthesis (Supplemental Table 2). Two enzymes involved in proline metabolism ornithine transaminase and delta-1-pyrroline-5-carboxylate synthase, were found to decrease in May with −1.23- and −2.03-fold changes respectively. Increased levels of proline have been correlated with a number of environmental stresses in plants, including low temperature, water deprivation, and nutrient deficiency (Hare and Cress, 1997). Although not as well studied in insects, elevated proline levels have been correlated with overwintering and cold tolerance (Ramløv, 1999; Misener et al., 2001; Fields et al., 1998; Storey and Storey, 1981), as have levels of its associated metabolite alanine (Churchill and Storey, 1989). Proline oxidation in the mitochondria may also provide an important energy source for overwintering larvae (Ballantyne and Storey, 1984; Misener et al., 2001).

Almost all enzymes involved in carbohydrate metabolism (glycolysis, citric acid cycle, digestive enzymes) were found to have lower abundance in May with fold changes ranging from −1.4 to −69.2 compared to March larval samples. PEPCK exhibited the second largest negative fold change at −19.2. Two proteins showing similarity to glycogen phosphorylase were detected at lower levels in May compared to March (−6.3- and −4.1-fold changes), indicating the breakdown of glycogen for energy is reduced as the larvae approach the pupal stage. The only carbohydrate metabolism proteins that were found to increase in the spring were four that are

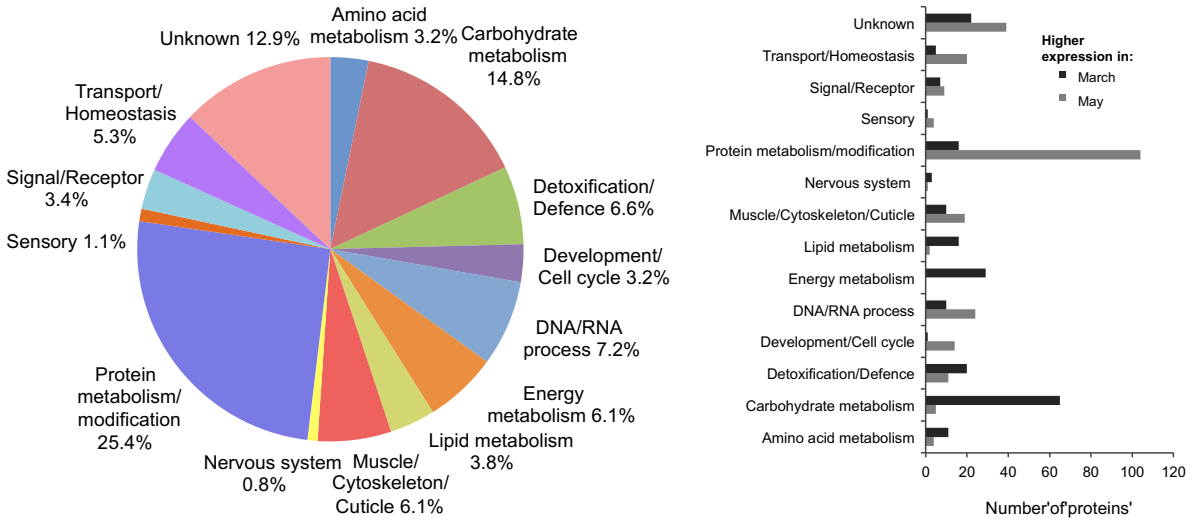

Fig. 7. Functional annotation (left) of 473 proteins that significantly shifted (FDR < 0.05) in abundance in spring larvae, and their expression breakdown (right).

involved in chitin metabolism with ~1- to 3-fold changes, along with one enzyme involved in fructose metabolism. Trehalose biosynthesis may also decline with the warmer temperatures, as shown by reductions in two proteins with similarity to α,α-trehalose-phosphate synthase (UDP-forming) with fold changes of −2.1 and −2.4 in larvae in May compared to March.

Proteomic evidence indicates that fatty acid metabolism was lower in May than in March. One enzyme involved in prostaglandin metabolism had a large change of −9.6-fold in May compared to March. All significantly shifting proteins involved in the regulation of DNA and chromatin (i.e., transcriptional regulation) increased in May, indicating that the transcriptome changes in response to the need for different proteins during late-stage development. Enzymes involved in nucleotide and nucleic acid metabolism were roughly split evenly between proteins exhibiting increasing and decreasing levels, indicating high levels of mRNA turnover in the spring. RNA processing enzymes also showed increases in accumulation between March and May larval samples.

Twelve proteins involved in development and the cell cycle showed higher expression in May than in March, with fold changes ranging from 1.8 to 4.9. Some of these have gene ontologies associated with cell growth and proliferation, neurogenesis, limb development, and circadian timing for adult eclosion. We found four nervous system proteins with increased levels in the spring (~2- to 5-fold changes), indicating nervous system activity and development, perhaps in preparation for adult activities such as dispersal flight and detection of semiochemicals. Levels of six cuticle proteins also increased in the spring (2.2- to 8.7-fold changes). Cytoskeleton proteins mainly increased in spring larvae between March and May, probably in preparation for the rapid developmental and anatomical changes that occur with pupation. Seven muscle and motor proteins were found and were roughly split between small but significant increases and decreases.

Levels of proteins involved in proteolysis generally increased during the spring, indicating substantial shifts in cell physiology and bodily restructuring. Similarly, all significantly shifting ribosomal proteins and translation factors increased from March to May. Proteins involved in guiding protein folding generally showed significant increases in May compared to March, with the exception of some heat shock proteins that showed significant decreases. In particular, proteins showing similarity to subunits of heat shock protein 70 significantly decreased 11.5-, 11.4-, 4.2-, and 2.4-fold in May larvae compared to levels in March larvae. Heat shock proteins are associated with stressors such as cold (Rinehart et al., 2007) or toxic chemicals (Feder and Hofmann, 1999), so decreases in the spring indicate a shift away from those influences. These same heat shock proteins were found to increase significantly in adult mountain pine beetles during the first 24 h of host colonization (unpublished), indicating their likely involvement in stress physiology relating to host secondary metabolites. Signaling and receptor proteins were roughly split between higher and lower expression in May, potentially involved in activating or deactivating certain genes during release from winter cold and ongoing spring larval development.

Levels of transport and homeostasis proteins mostly showed significant increases during the spring, likely in response to shifts in cell physiology during late-instar larval development. Ferritin, the iron storage protein that increased significantly during the autumn, exhibited a large significant decrease (−17.5-fold) in the spring. The same enzyme thought to be involved in isoprenoid biosynthesis that increased significantly across the autumn sampling dates, decreased significantly from March to May (−2.5-fold).

3.5.3. Autumn and spring

Eighteen proteins showed significant changes in both the autumn and spring (Fig. 3; Table 1). All but one of these proteins showed opposite shifts in accumulation in the two seasons. One noteworthy protein is ferritin, due to its large fold changes seen in both autumn and spring (4.02-fold in autumn and −17.49-fold in spring). Other proteins of interest related to overwintering and development physiology are α,α-trehalose-phosphate synthase (1.33-fold in the autumn and −2.36-fold in the spring); a lipid storage droplets surface-binding protein (−1.51-fold in the autumn and 6.81-fold in the spring); antennal-specific protein 3c (3.08-fold in the autumn and −3.50-fold in the spring); an isoprenoid biosynthesis protein (1.52-fold in the autumn and −2.52-fold in the spring); β-tubulin (−1.74-fold in the autumn and 8.09-fold in the spring); and 2-deoxyglucose-6-phosphate phosphatase (6.18-fold in the autumn and −69.24 in the spring).

3.5.3.1. Ferritin. Ferritin has been associated with stress response in many organisms (Carrasco et al., 2011; Kitteringham et al., 2010; Yamaguchi-Shinozaki and Shinozaki, 2006; Larade and Storey, 2004). Specifically looking at genes and their protein products

Table 1
Eighteen proteins that show significant differential accumulation (FDR < 0.05) in both the autumn and spring mountain pine beetle larval proteomes.

Translated EST contig ID[a]	Representative EST Accession No.	Annotation (BLASTx-derived)	Subject	E-value	Putative function (UNIprot-derived)	Autumn[d]		Spring[d]	
						Fold change[b]	*p*-value	Fold change[c]	*p*-value
h_cluster_01391 − 1 + 3	GT428448	Similar to CG6950-PB, isoform B	tca:655252	0	Amino acid metabolism	1.24	0.00100	−1.50	0.00657
h_cluster_01449	GT330404	2-deoxyglucose-6-phosphate phosphatase	tca:663913	1.00E-76	Carbohydrate metabolism	6.18	0.00001	−69.24	0.00000
h_cluster_10352 − 1 − 2	GT328775	Similar to Phospholipid hydroperoxide glutathione peroxidase	tca:658307	2.00E-66	Detoxification/Defence	1.50	0.00039	−2.76	0.01809
h_cluster_06534 − 1 + 3	GT389502	Similar to CG18811-PA	tca:661585	9.00E-101	Development/cell cycle; Caprin homolog	−1.34	0.00139	2.74	0.00016
h_cluster_03321 + 2	GT404183	Similar to CG32626-PA, isoform A; K01490 AMP deaminase	ame:409444	0	Energy metabolism	1.74	0.00049	−2.11	0.01123
h_cluster_19266 − 3	GO486898	Similar to CG32626-PA, isoform A; K01490 AMP deaminase	ame:409444	1.00E-97	Energy metabolism	1.46	0.00003	−1.77	0.00041
h_cluster_08578 + 2	GT363201	Mitochondrial ATP synthase F chain	cqu:CpipJ_CPIJ004713	1.00E-40	Energy metabolism	1.31	0.00087	−3.06	0.00078
h_cluster_00739 + 3	GT321473	Similar to ferritin, heavy polypeptide 1	tca:656261	2.00E-65	Homeostasis	4.02	0.00018	−17.49	0.00063
h_cluster_04731 − 1 + 2	GT320100	Similar to CG10585-PA	tca:658669	0	Isoprenoid biosynthetic process	1.52	0.00031	−2.52	0.00097
h_cluster_01210 − 1 + 3	GT490368	Lipid storage droplets surface-binding protein 1	cqu:CpipJ_CPIJ003879	2.00E-18	Lipid transport/storage	−1.51	0.00005	6.81	0.00002
h_cluster_01720 − 1 + 2	GT349497	Similar to ubiquitin specific peptidase 14	tca:657507	0	Proteolysis	1.44	0.00010	−1.62	0.01183
h_cluster_04232 − 1 + 3	GT398758	Similar to CG7008-PA	tca:663751	2.00E-92	RNA interference; Tudor-SN	−1.25	0.00079	1.26	0.00526
h_cluster_01288 − 2 + 2	GT369651	Similar to CG5651-PA, isoform A; K06174 ATP-binding cassette, sub-family E, member 1	tca:656379	0	RNAse inhibitor; mRNA turnover	−1.15	0.00115	1.23	0.01650
h_cluster_04093 + 3	n/a	Asp3c; antennal-specific protein 3c	ame:406094	5.00E-27	Sensory	3.08	0.00005	−3.50	0.00281
h_cluster_02648 − 1 + 1	GT326361	Similar to beta1-tubulin	api:100168148	0	Structural; cytoskeleton	−1.74	0.00128	8.09	0.00005
f_cluster_c97 + 2	GT484670	Similar to CG4104-PA; K00697 α,α-trehalose-phosphate synthase (UDP-forming)	tca:659356	0	Trehalose biosynthesis	1.33	0.00030	−2.36	0.00029
h_cluster_01030 − 1 + 1	GT388432	Similar to ganglioside-induced differentiation-associated-protein 1	tca:659156	7.00E-93	Unknown	1.42	0.00095	−2.74	0.00008
h_cluster_00121 − 2 + 1	GT412511	Similar to CG1240-PA	ame:408520	7.00E-29	Unknown	1.17	0.00087	2.13	0.00548

[a] Suffix (+1, +2, +3, −1, −2, −3) indicates translation frame. Non-translated contig sequences can be found in supplementary text file.
[b] 7 November samples relative to 26 September.
[c] 27 May samples relative to 25 March.
[d] % coverage and ProtScore values for autumn and spring can be found in Supplemental Tables 1 and 2, respectively.

related to cold treatments, Carrasco et al. (2011) compiled bioinformatics data across 34 species, and found that ferritin was upregulated in response to cold in *Rana sylvatica* (frog), *Megaphorura arctica* (collembolan), *Arabidopsis thaliana* and *Thellungiella halophila* (plants), and *Chrysemys picta marginata* (turtle). This storage protein sequesters free iron, which can cause the production of toxic radicals if left freely soluble. The significant increase of both superoxide dismutase and phospholipid hydroperoxide glutathione peroxidase in the autumn proteome is evidence that oxidative stress is an issue for overwintering larvae, as in many insects (Storey and Storey, 2010). Simultaneous production of ferritin may further help to reduce the effects of oxidative stressors. Ferritin can be synthesized rapidly in response to iron stress because cells often maintain a constitutive supply of ferritin mRNA (Nichol et al., 2002; Shull and Theil, 1982, 1983), which accounts for the large fold change in the fall in overwintering mountain pine beetle larvae compared to other proteins.

3.5.3.2. Trehalose-6-phosphate synthase. Trehalose is the main insect hemolymph sugar, comprising over 90% of total hemolymph sugars in some insects (Wyatt and Kalf, 1957). It is synthesized by the fat body and is used for functions as diverse as energy for flight muscle (Becker et al., 1996) and as a cryoprotectant that is accumulated in some insects at low temperature (Baust and Lee, 1983; Khani et al., 2007; Overgaard et al., 2007). In arthropods such as Arctic springtails, that undergo cryoprotective dehydration (Thorne et al., 2011), trehalose acts to replace the water molecules surrounding proteins and cell membranes, stabilizing their functional state (Crowe and Crowe, 1986), and preventing oxidative damage (Oku et al., 2005). As such, trehalose is highly important in maintaining a number of physiological processes in insects and other organisms. In the insect fat body, trehalose-6-phosphate synthase combines glucose 6-phosphate, from the breakdown of glycogen, and UDP-glucose to form trehalose-6-phosphate (Becker et al., 1996). Trehalose-6-phosphate phosphatase then completes the second step in the formation of trehalose. The significant increase in α,α-trehalose-6-phosphate synthase levels in the autumn proteome, and the opposite and significant change in the spring proteome (Table 1) supports the hypothesis that trehalose is being produced by overwintering mountain pine beetle larvae for survival during cold periods. Further supporting evidence is the significant increase of UTP-glucose-1-phosphate uridylyltransferase 2 in the autumn, indicating that UDP-glucose is being supplied for trehalose synthesis.

3.5.3.3. Lipid storage droplets surface-binding protein. Insects (and other eukaryotes) have been shown to store neutral lipids in the form of lipid droplets in the cytosol of the fat body and other cells (Brasaemle, 2007; Arrese et al., 2008; Teixeira et al., 2003). Lipid droplets surfaces are associated with a variety of proteins that regulate the transport of the droplets throughout the cell and also act to influence the rate of lipolysis (Brasaemle, 2007). Triglyceride energy requirements are, in part, met by the action of cytosolic lipases. Cytosolic lipase activity is regulated by several types of surface proteins present on lipid droplet membranes. Two proteins involved in lipid storage droplet homeostasis, Lsd1 and Lsd2, have been found in insects (Arrese et al., 2008). Lsd1 is specifically involved in the activation of triglyceride lipolysis (Patel et al., 2005), while Lsd2 aids in the accumulation of triglycerides in the lipid droplet (Grönke et al., 2003). One protein, which shows a significant decrease in the autumn (−1.51-fold) and a large significant increase in the spring (6.81-fold), shows similarity to Lsd1. This expression pattern indicates that triglycerides are being stored through the winter and then being converted to usable energy later in the spring when larvae undergo rapid development. These proteomics data also support recent work indicating that triglycerides are not the source of glycerol cryoprotectant production by mountain pine beetle larvae in the autumn (Fraser, 2011)

3.5.3.4. Antennal-specific protein 3c. We found a chemosensory protein that shows similarity to the antennal-specific protein 3c (ASP3c) found in honeybees. In *Apis mellifera* L., ASP3c was suggested to be a carrier for the brood pheromone, based on its affinity for binding fatty acids and their derivatives that make up the pheromone (Briand et al., 2002). In mountain pine beetle, this protein could be involved during digestion in general fatty acid transport (Lartigue et al., 2002) to fat body cells or in the transport of a similar lipid-based pheromone or hormone (Gilbert and Chino, 1974). The expression pattern (increasing in autumn, decreasing in spring) is consistent with either of these hypotheses. Alternatively, this protein may be involved in development of the antennal imaginal disc. However, we would not expect its expression to decrease in the spring. Its expression pattern is more consistent with a function that is specifically required in autumn larvae; such as transport of nutrients or as a general sensory protein involved in navigating around pockets of resin toxins.

3.5.3.5. Isoprenoid biosynthesis protein. This protein shows high similarity to an uncharacterized "candidate tumor suppressor protein" in *Tribolium castaneum*, which has regions similar to some prenyltransferase enzymes involved in isoprenoid biosynthesis, namely decaprenyl diphosphate synthase subunit 2 and geranylgeranyl diphosphate synthase. Prenyltransferases catalyze reactions leading to the synthesis of prenylated proteins, sterols, ubiquinones, pheromones, and juvenile hormone in insects (Sen et al., 2007). Based on these descriptions, and its significant fold changes of 1.52 in autumn and −2.52 in spring, it is possible that this protein is involved in the synthesis of a growth-suppressing hormone. Alternatively, this enzyme could be acting in the synthesis of ubiquinone (Quinzii et al., 2008), an essential component of the mitochondrial electron transport chain. More recently, ubiquinone has been recognized as an effective antioxidant in preventing damage to lipid membranes, proteins, and DNA (Bentinger et al., 2007). Although the downstream product of this enzyme is unknown, its significance and fold change directions in the autumn and spring proteomes point to its role in synthesizing an isoprenoid molecule important for developmental regulation or stress tolerance.

3.5.3.6. β-tubulin. The expression of some forms of tubulin is known to be developmentally regulated (Kawasaki et al., 2003). In *Drosophila* cells, one β-tubulin subunit was found to be regulated by 20-hydroxyecdysone, a steroid hormone that regulates the process of ecdysis (Sobrier et al., 1986). The late-stage significant accumulation of this protein in the spring proteome is consistent with its role as a developmentally-regulated cytoskeletal protein, potentially involved with adult eclosion. Its significant decrease from September to November shows that it may be necessary at some point in development from embryo to first-instar larvae, as in *Drosophila melanogaster* (Rudolph et al., 1987), but that it is not required during the bulk of larval development. This protein may be useful as a marker for developmental stage progression.

3.5.3.7. 2-Deoxyglucose-6-phosphate phosphatase. With the largest fold change of any protein in the autumn and spring proteomes respectively, and its high significance, we predict that 2-deoxyglucose, the product of this enzyme, is important for overwintering survival in the mountain pine beetle. 2-Deoxyglucose has been found to inhibit glycolysis and cell growth in cultured cells and mammals (Barban and Shulze, 1961; Wan et al., 2004),

resulting in its continual development as a target for cancer cell treatment (Sahra et al., 2010). In *Saccharomyces cerevisiae*, the *DOG2* gene responsible for producing this enzyme was found to be induced under conditions of oxidative and osmotic stress or under glucose starvation (Tsujimoto et al., 2000). In rats, dietary supplements of 2-deoxyglucose resulted in improved stress adaptation, suggesting that dietary restriction is used as a pre-conditioning for other stressful conditions (Wan et al., 2004). In overwintering mountain pine beetle, it is possible that 2-deoxyglucose is acting to divert energy from glycolysis toward cryoprotectant production; restrict development and growth during autumn; as a stress hormone to signal the expression of other stress physiology responses; and/or as a cryoprotectant metabolite.

3.6. Glycerol metabolism in overwintering larvae

Our proteomics data support recent RT-qPCR findings that examined genes involved in glycerol metabolism in larval *D. ponderosae* that were sampled at the same trees and have the same corresponding temperature data (Fraser, 2011). Glycerol is a major cryoprotectant accumulated by overwintering insects employing a freeze avoidance strategy (Storey and Storey, 1996), and specifically by mountain pine beetle (Bentz and Mullins, 1999). Glycerol metabolism has been examined in a number of overwintering insects, and the pathways for its synthesis and breakdown can vary (Joanisse and Storey, 1994a,b; Storey and Storey, 1986), even among bark beetles (Lombardero et al., 2000).

Our proteomic results mirror quite closely the transcriptomic shifts noted by Fraser (2011), supporting the finding of glycerol biosynthesis in this insect being mainly via glycogenolysis and gluconeogenesis. Proteins that show similar shifts in accumulation include glycogen phosphorylase (two glycogen phosphorylase proteins shifted by 2.3- and 2.4-fold, respectively in autumn and −4.09- and −6.32-fold, respectively, in spring) and PEPCK (3.17-fold change in autumn and −19.4-fold change in spring).

Fraser (2011) found no evidence that glycerol production in mountain pine beetle larvae occurs through a lipolytic source. Transcript abundance for triacylglycerol lipase was found to remain constant through the autumn and spring study periods. The LSD1 protein – responsible for activating triglyceride breakdown – decreases in the autumn proteome and increases in the spring, consistent with this finding. Additionally, trends in some enzymes involved in glycogenesis and the citric acid cycle support the observation (Fraser, 2011) that glycerol does not seem to be reconverted to glycogen, or metabolized via the citric acid cycle in this insect when temperatures warm.

3.7. Developmental and energy metabolism

As mountain pine beetle larvae overwinter in a non-feeding state (Régnière and Bentz, 2007), their finite quantity of energetic stores must be allocated between development, maintaining basal metabolism, and producing cryoprotectants (Fraser, 2011). The larvae alternate rapidly between states of quiescence and development, on a daily basis (Powell and Logan, 2005), allowing more energy to be allocated toward cold hardening mechanisms when most needed. Recent evidence indicates that *adult* overwintering mountain pine beetle may have the ability to go through a stage of completely arrested development (facultative diapause), and that this could possibly occur during earlier life stages too (Lester and Irwin, 2012).

In the autumn proteome, we detected 43 proteins related to developmental and cell cycle processes. Only one showed a significant shift in accumulation (−1.3-fold in autumn) – a caprin homolog associated with embryo development in *D. melanogaster* (Papoulas et al., 2010). In the spring, we found 51 proteins related to developmental and cell cycle processes, twelve of which significantly increased, and two that significantly decreased. The two proteins that decreased show similarity to juvenile hormone epoxide hydrolase, and have −3.0- and −8.5-fold changes in May relative to March, indicating that juvenile hormone may be degraded earlier than March, as a signal for major development to begin. It seems that mountain pine beetle larvae do not make large investments into development until spring, in order to focus resources on winter survival.

Enzymes involved in maintaining basal metabolism through glycolysis and the citric acid cycle remained at consistent levels when compared between September and November larvae, but most of them significantly decreased in May compared to March. These central metabolic pathways are intertwined with cryoprotectant production, and the expression trends of their enzymes are consistent with the distinct shifts between overwintering survival and spring development. Enzymes involved in the electron transport chain also remain relatively constant in the autumn and then show significant decreases in the spring, with a few exceptions – two subunits of NADH dehydrogenase showed small significant decreases (−1.16- and −1.24-fold) in the autumn, and a succinate dehydrogenase subunit showed a small significant increase (1.29-fold) in the autumn. Given that the NADH dehydrogenase complex is the primary producer of oxygen free radicals (Storey and Storey, 2010), and that this production is exacerbated during respiratory stress, some reorganization of these enzymes may be necessary during overwintering. Overall, the expression trends we found for mitochondrial enzymes involved in energy metabolism fit with the observation that freeze-avoiding insects selectively regulate these enzymes during the winter, rather than degrading mitochondria to reduce energy metabolism (Storey and Storey, 2010).

We also found significant shifts in enzymes related directly to energy balance – those involved in adenylate metabolism. Two AMP deaminases, which convert AMP to IMP, significantly increased in the autumn (1.74- and 1.46-fold) and significantly decreased in the spring (−2.11- and −1.77-fold). A mitochondrial ATP synthase subunit increased in the autumn (1.31-fold) and decreased in the spring (−3.06). These three detections of enzymes which act on the adenylate pool, and their significant changes in both the autumn and spring, indicate a mechanism for larvae to maintain a high energy charge (Atkinson, 1968) even while resources are being exhausted, such that energy-consuming biosynthetic pathways may continue (Churchill and Storey, 1989). Because glycerol biosynthesis is an ATP-consuming process (Storey and Storey, 1990), maintaining a high cellular energy charge is probably necessary for glycerol biosynthesis to proceed (Churchill and Storey, 1989). We additionally found four enzymes – adenylosuccinate synthase, IMP cyclohydrolase, phosphoribosylaminoimidazole carboxylase, and IMP dehydrogenase – that significantly changed in the spring proteome and could be involved in clearance of excess IMP and restoration of energy balance.

3.8. Detoxification of host tree defense compounds

While larvae prepare for winter through the production of glycerol and other cryoprotective agents, they must simultaneously defend themselves from an onslaught of toxic secondary metabolites released by their host tree (Boone et al., 2011). Lodgepole pines that have been attacked by mountain pine beetles have been shown to maintain high levels of terpenoids, even weeks after the initial attack when eggs hatch and larvae begin to feed (Clark et al., 2012). Cytochromes P450, carboxylesterases, and glutathione-S-transferases (GST), are three classes of enzymes produced by

insects for detoxification and clearance of these compounds (Li et al., 2007). There are also the sensory, signaling, and transporting proteins that help transmit information from the sensory appendages to induce production of these detoxification enzymes.

In the autumn proteome we detected approximately ten each of Cytochromes P450, GSTs, and esterases, but none of them significantly changed between the September and November samples. The only defense-related proteins that significantly changed were the three previously mentioned (Section 3.5.3.1) that are involved in prevention of oxidative stress and toxic radical damage, and the ASP3c-similar protein (discussed in Section 3.5.3.4) that could be involved in sensory perception of host secondary metabolites or transport of nutrients.

In the spring proteome, we again detected a number of these detoxification proteins, but saw several significant changes. Three Cytochromes P450 enzymes decreased significantly in May compared to March: CYP6DE1 (−2.4-fold); and two detections of CYP349B1 (−18.2- and −3.2-fold). CYP6DE1 was also found to significantly increase in female mountain pine beetles during host colonization (unpublished), further support for its role in secondary metabolite detoxification. Two GSTs significantly decreased (−5.3- and −1.9-fold), and one significantly increased (1.9-fold). Three esterases significantly decreased (−5.0-, −2.7-, −2.2-fold changes), while one significantly increased 1.5-fold.

These results show that detoxification proteins are expressed very early in the early larval stages and constitutively throughout the autumn. Colonized trees release and retain very high levels of several terpenoids for weeks after mountain pine beetle attack and larval hatching (Clark et al. 2012), so constitutive expression of such enzymes upon hatching is expected for larval survival. Conversely, the trend of their decreasing levels as the spring progresses indicates that they are no longer needed as the dead hosts cannot synthesize or even retain secondary metabolite stores.

4. Conclusion

In total, we have discovered 1507 unique larval mountain pine beetle proteins. Of these, 456 were unique to the autumn larvae, 368 were unique to the spring larvae, and 683 were common to larvae during both seasons. Thirty-three proteins showed significant differential accumulation between the two autumn sampling dates, and 473 proteins between the two spring sampling dates. Eighteen proteins showed differential accumulation in both the autumn and spring, most of them showing opposing fold changes between the two seasons.

After a cold snap in the autumn, larvae show significantly increased levels of some enzymes commonly implicated in stress response: UTP-glucose-1-phosphate uridyltransferase and α,α-trehalose-phosphate synthase, involved in trehalose biosynthesis; ferritin, involved in iron ion homeostasis; phospholipid hydroperoxide glutathione peroxidase and superoxide dismutase, functioning in oxidative stress response; and 2-deoxyglucose-6-phosphate phosphatase, potentially important for multiple strategies in overwintering survival. As they warm up in the spring, larvae significantly lower their levels of most of these proteins. Spring larvae significantly increase expression of developmental proteins; transcription and translation proteins; and cuticle proteins, in preparation for the many changes that occur as they approach pupation.

In addition to stress response caused by low temperatures and oxidative stress, larvae show evidence of an early response to host toxins. The presence of several P450s, GSTs, and esterases in autumn larvae, a number of which show large significant decreases in the spring, indicates that these enzymes may be expressed constitutively from a very early time point in larval development and that expression drops when they are no longer needed.

The large number of significantly changing proteins, and the magnitude of the change in many cases, highlights the extremely active physiology of mountain pine beetle larvae during winter cold hardening and, in particular, during the shift toward further development in the spring. While not completely neglected, the larval and pupal stages of this insect have not been as heavily researched as have aspects of adult physiology and behavior related to host finding, colonization, and eventual reproduction. These substantial proteomics data should allow, and spur on, new, detailed research into these life stages that are so vital to understanding mountain pine beetle outbreaks and infestations.

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Appendix A. Supplementary material

Supplementary material related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2012.08.003.

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