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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