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Quantitative proteomics reveals divergent responses in *Apis mellifera* worker and drone pupae to parasitization by *Varroa destructor*

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ABSTRACT

Varroa destructor is a haemophagous ectoparasite of honeybees and is considered a major causal agent of colony losses in Europe and North America. Although originating in Eastern Asia where it parasitizes *Apis cerana,* it has shifted hosts to the western honeybee *Apis mellifera* on which it has a greater deleterious effect on the individual and colony level. To investigate this important host-parasite interaction and to determine whether *Varroa* causes different effects on different castes we conducted a label free quantitative proteomic analysis of *Varroa*-parasitized and non-parasitized drone and worker *Apis mellifera* pupae. 1195 proteins were identified in total, of which 202 and 250 were differentially abundant in parasitized drone and worker pupae, respectively. Both parasitized drone and worker pupae displayed reduced abundance in proteins associated with the cuticle, lipid transport and innate immunity. Proteins involved in metabolic processes were more abundant in both parasitized castes although the response in workers was more pronounced. A number of caste specific responses were observed including differential abundance of numerous cytoskeletal and muscle proteins, which were of higher abundance in parasitized drones in comparison to parasitized workers. Proteins involved in fatty acid and carbohydrate metabolism were more abundant in parasitized workers as were a large number of ribosomal proteins highlighting either potentially divergent responses to *Varroa* or a different strategy by the mite when parasitizing the different castes. This data improves our understanding of this interaction and may provide a basis for future studies into improvements to therapy and control of Varroasis.

1. Introduction

Varroa destructor is an ectoparasite of the western honeybee, *Apis mellifera*, and its deleterious effects have been responsible for large scale losses in both natural and commercial settings. *Varroa* is a completely host-dependent mite, lacking a free living stage with its life cycle completed within the bee brood cells. *Varroa* feed communally and repeatedly on developing larvae and pupae leading to the spread of pathogens within colonies (Parker et al., 2012). Parasitization by *Varroa* has a serious effect on the health of the bee through loss of haemolymph, transmission of viruses and other microbes, and by causing a reduction in body weight (Bowen-Walker and Gunn, 2001, Levin et al., 2016, Wilfert et al., 2016).

Although originating in Eastern Asia where it parasitizes *Apis cerana,* it has shifted hosts to the western honeybee *Apis mellifera* on which it has a greater deleterious effect on individual and colony level fitness (Chen et al., 2017, Beaurepaire et al., 2015). The lack of a definite preference by *Varroa* for drone brood during host finding in *A. mellifera* colonies and the poor ability of *A. mellifera* to dislodge feeding *Varroa* during grooming contribute to much higher infestation levels in *A. mellifera* colonies than in *Apis cerana* which was the original host of *V. destructor* (Büchler et al., 1992, Fries et al., 2006, Parker et al., 2012). Worker bees are the largest caste in the colony and are essential to the survival of the colony, and the lack of drone brood preference by *Varroa* in *A. mellifera* contributes to their negative effect on colonies (Parker et al., 2012). *Varroa* have adversely affected apiculture in every country where present, leading to the loss of hundreds of thousands of colonies (Zakar et al., 2014, Kuster et al., 2014). Given the importance of *Apis mellifera* in honey production and a provider of key ecosystem and agricultural services such as plant pollination, it is surprising how little is known about this host-parasite interaction at the molecular level. To address this a label free quantitative proteomic analysis of *Varroa*-parasitized and non- parasitized drone and worker pupae was conducted.

Abbreviations: BP, biological processes; FDR, False Discovery Rates; GO, gene ontology terms; KEGG, Kyoto Encyclopedia of Genes and Genomes; SSDA, statistically significant differentially abundant; MP, molecular function; SSDA, statistically significant differentially abundant

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Recent advances in proteomic and genomic technologies has resulted in significant insights into honeybee biology and the *Apis-Varroa* interaction at the molecular level (Qi et al., 2015). High-resolution quantitative mass spectrometry (MS) routinely quantify and identify thousands of proteins across multiple samples in a single run which gives an unprecedented opportunity to examine changes in proteomic profile of a particular biological fraction or organism. We previously utilised this technology to study the mechanisms conferring resistance to Bayvarol in *V. destructor* and the results indicated a decreased abundance of cuticle associated proteins and an increase in the abundance of proteins involved in cellular detoxification as possible factors in the resistant phenotype (Surlis et al., 2016). Similar proteome-wide analysis has been utilised to investigate the molecular determinants of hygienic behaviours and resistance/sensitivity to *V. destructor* (Parker et al., 2012; Hu et al., 2016). Proteomics has also been used to investigate developmental processes and differentiation of drone and worker bees (Fang et al., 2014, Fang et al., 2015) and the phosphoproteome of the hypopharyngeal gland in *A. mellifera* worker bees (Qi et al., 2015) has also been reported.

In the work presented here label free quantitative (LFQ) mass spectrometry was utilised to compare the proteomes of *Varroa*-parasitized and non-parasitized drone and worker *A. mellifera* pupae to gain insight into the interaction between the mite and honeybee host.

2. Materials and methods

2.1. Mellifera pupae collection

A. mellifera purple-eye stage pupae were collected from three separate colonies within an apiary in Kilmessan, County Meath, Ireland (coordinates 53.558980, -6.660039). For the drone collection, brood boards were removed from hives, stored in insulated boxes and transported to the laboratory where they were stored at −80 °C prior to protein extraction from drone pupae. Time from removal of brood boards from hive to freezing in laboratory in < 1 h. Following removal of the drone boards, a queen trapping method was used to concentrate *Varroa* within worker brood cells by placing a queen excluder on a single brood frame, and moving to a new board every week following removal of the worker brood. Boards were stored at −80 °C for subsequent worker brood protein extraction and analysis. To avoid variability between samples pupae were chosen at the purple eye stage with no darkening of the cuticle. Capped drone and worker cells were opened and pupae were inspected for *Varroa* and where present, the mature adult female and immature mites were enumerated. Pupae from cells containing 4 adult female mites and 8 immature offspring were considered heavily parasitized and were chosen for subsequent analysis. Pupae from three different colonies were collected, mixed and randomly chosen. Four independent biological replicates were used for the analysis. Pupae were not screened for the presence of viruses or bacteria.

2.2. Sample preparation and mass spectrometry

Individual parasitized or unparasitized pupae (number of replicates = 4) were homogenized using a pellet pestle in 7 M urea; 2 M thiourea buffer supplemented with a protease inhibitor cocktail tablet (Complete™ Protease Inhibitor Cocktail (Roche)). Samples were centrifuged at 9000*g* for 5 min, the lipid layer was removed using a spatula, and the supernatant transferred to a fresh tube. Proteins were quantified by Bradford assay and 300 µg of protein was removed and acetone precipitated overnight at −20 °C. Proteins were centrifuged at 10,000*g* for 10 min and the pellet resuspended in 300 µl of 8 M urea. Samples were re-quantified using Qubit Fluorometer (Invitrogen), 75 µg was removed, reduced with dithiotreitol (DTT; 200 mM), alkylated with iodoacetamide (IAA; 1 M) and digested with sequence grade trypsin (Promega) at a trypsin:protein ratio of 1:40, overnight at 37 °C. Tryptic peptides were purified for mass spectrometry using C18 spin filters (Medical Supply Company) and 0.70 µg of the peptide mix was eluted onto a QExactive (ThermoFisher Scientific, U.S.A) high resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient on a Biobasic C18 PicofritTM column (100 mm length, 75 mm ID), using a 120 min reverse phase gradient at a flow rate of 250 nL /min. All data were acquired with the mass spectrometer operating in automatic data dependent switching mode. A high-resolution MS scan (300-2000 Dalton) was performed using the Orbitrap to select the 15 most intense ions prior to MS/MS.

2.3. Data and bioinformatic analysis

Protein identification from the MS/MS data was performed using the Andromeda search engine in MaxQuant (version 1.2.2.5; http://maxquant.org/) to correlate the data against the predicted protein set derived from the *A. mellifera* genome (Amel_4.5 assembly, Honeybee Genome sequence consortium, 2006). The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm with cysteine carbamidomethylation as a fixed modification and N-acetylation of protein and oxidation of methionine as variable modifications and a maximum of 2 missed cleavage sites allowed, all standard (Michalski et al., 2011). False Discovery Rates (FDR) were set to 1% for both peptides and proteins and the FDR was estimated following searches against a target-decoy database. Peptides with minimum length of seven amino acid length were considered for identification and proteins were only considered identified when more than one unique peptide for each protein was observed.

Results processing, statistical analyses and graphics generation were conducted using Perseus v.1.5.0.31 (www.maxquant.org). LFQ intensities were log2-transformed and ANOVA of significance and t-tests between the parasitized and non-parasitized pupae proteomes were performed using a p-value cut-off of 0.05 to identify statistically significant differentially abundant (SSDA) proteins. Proteins that had intensity values of zero (indicative of absence or very low abundance in a sample) were included in the study only when they were completely absent from one group and present in at least three of the replicates in the second group. These proteins were also included in the statistical analysis after imputation of representative numbers based on the lowest value for each data set, which was calculated as a 1.75 downshift from the mean value, allowing for 0.25 width in the downshift for standard deviation. Volcano plots were generated in Perseus to visualize differentially abundant proteins between control and parasitized groups and a principal component analysis (PCA) was performed using the normalized intensity values. Hierarchical clustering was performed on Z-score normalized intensity values for all differentially abundant proteins by clustering both samples and proteins using Euclidean distance and complete linkage.

The Uniprot identifier for each protein was obtained by conducting a BLAST search of all proteins identified against the Uniprot sequence set for *Apis mellifera* (WWW.Uniprot.org downloaded April 2017). The top Uniprot match was obtained for each identified protein and used to query the Perseus annotation file (downloaded April 2017) and extract terms for biological process, molecular function, Kyoto Encyclopaedia of Genes and Genomes (KEGG) name, KEGG pathway, protein family (pfam) and InterPro. The 'categories' function in Perseus was utilised to highlight and visualize the distribution of various pathways and processes on volcano plots. GO and KEGG term enrichment analysis was performed on the major protein clusters identified by hierarchical clustering using a Fisher's exact test (a Benjamini-Hochberg corrected FDR of 2%) for enrichment in Uniprot Keywords, gene ontology biological process (GOBP), gene ontology cellular component (GOCC) and KEGG (FDR < 2%).

The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) v10 (http://string-db.org/) was used to map known and predicted protein: protein interactions and to identify groups of proteins associated with specific pathways and processes. FASTA files were generated