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Exposure of *Apis mellifera* to anti-*Varroa destructor* formic acid treatment induces significant proteomic alterations

Rachel Ward[a], Mary F. Coffey[b] and Kevin Kavanagh[a]*

[a]Department of Biology, Maynooth University, Maynooth, Co. Kildare, Ireland; [b]Plant Science Division (Bee Health), Department of Agriculture, Plant Health Laboratories, Celbridge, Co Kildare, Ireland

ABSTRACT

The parasitic mite *Varroa destructor* is a major threat to the health and productivity of *Apis mellifera* hives and induces its effect by feeding on the fat body of larvae/pupae and transmitting viruses. The effect of the anti-Varroa formic acid containing – Mite Away Quick (MAQ) strips on the proteome of *A. mellifera* was assessed. Samples of *A. mellifera* were isolated from hives one week prior to MAQ treatment, during the week of treatment and for two weeks after the end of treatment, proteins were extracted and analysed by label free quantitative proteomics. The results indicated that samples isolated during the week of treatment showed increased abundance of a range of cuticular proteins (+ 2.65 fold to + 6.64 fold) and decreased abundance of proteins that deal with xenobiotics (Cytochrome P450 subunits −11.38 fold to −2.16 fold). Interestingly some proteins associated with the oxidative phosphorylation pathway were increased in abundance (e.g., Cox5a and Cox5b) but others (e.g., Coxfa4) were decreased. The results presented here reveal that the application of MAQ strips caused a dramatic disruption to the proteome of *A. mellifera,* but the effect is transient and two weeks after the end of treatment the proteome has returned to resemble that of the untreated control. While MAQ strips are effective in reducing Varroa populations, the results presented here indicate they can adversely affect the proteome of *A. mellifera* and may contribute to the elevated stress in hives previously affected by Varroa parasitisation.

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Apis mellifera; formic acid; Varroa; parasite; proteomics

Introduction

The ectoparasitic mite of the honey bee *Varroa destructor* (Varroa) (Anderson & Trueman, 2000) is the single greatest threat to honey bee colonies worldwide, with "Varroa free" hives being difficult to find (Ramsey et al., 2019; Rosenkranz et al., 2010). The lifecycle of Varroa mites consists of two phases: phoretic (spreading throughout the hive on adult bees) and reproductive (reproducing and mating in larval cells) (Rosenkranz et al., 2010). Varroa mites were originally confined to *Apis cerana* colonies but have since expanded to parasitise *Apis mellifera* colonies, most likely when *A. mellifera* colonies were transported to Eastern Russia/Far East (Boecking & Genersch, 2008; Buchler et al., 1992; Oldroyd, 1999). There is a lack of a long term co-evolutionary relationship between Varroa mites and *A. mellifera* and this has resulted in insufficient levels of hygienic behaviour in *A. mellifera*. The absence of Varroa reproductive preference for drone larval stages of *A. mellifera* is also another factor contributing to the greater effect of Varroa parasitisation on *A. mellifera* colonies compared to that on *A. cerana* colonies (Ramsey et al., 2019; Traynor et al., 2020). Parasitisation by Varroa is termed varroosis and the effects are characterised by a reduction in the honey bee's body weight, immune responses, number of healthy larvae and adult workers in a hive and inhibition of protein and lipid production as a direct result of the Varroa feeding on the fat body (Ramsey et al., 2019). Varroa are also successful viral vectors and are known to harbour 18 honey bee viruses and facilitate viral reproduction (Chen & Siede, 2007; Rosenkranz et al., 2010).

As Varroa infestations are so widespread, treatment protocols are beneficial and aid in controlling the spread of Varroa in apiaries and reducing the damaging impact of Varroa mites on individual hives. Failure to treat hives can result in the rapid deterioration of hive health, due to the loss of healthy bees and an increase in viral diseases such as Deformed Wing Virus, many hives will fail after two years in the absence of treatments (Gisder et al., 2009; Rosenkranz et al., 2010; Traynor et al., 2020). There are several treatments

CONTACT Kevin Kavanagh Kevin.Kavanagh@mu.ie
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available to beekeepers which can be divided into three groups: hard acaricides – synthetic compounds, soft acaricides – organic acid treatments, and biotechnical methods – beekeeper intervention. This study focused upon characterising the effect of an organic acid treatment called Mite Away Quick strips (MAQs) on the proteome of *A. mellifera*.

MAQ strips are a single use saccharide gel strip applications that contain 46.7% formic acid which is placed along the top of the brood frames for a 7-day period (as per manufacturer's instructions). Several factors may affect the efficacy of MAQs including the size of the hive, positioning of the strips above or below the brood chamber, and the ambient temperature (Ostermann & Currie, 2004; Underwood & Currie, 2003). Optimum efficacy of MAQs application is achieved when external temperatures range between 10 °C and 30 °C (Satta et al., 2005). Variations in temperatures can affect the efficacy of the organic acid treatments such as formic acid use as it influences the evaporation in the hive (Rosenkranz et al., 2010). Organic acids such as formic, oxalic and lactic acid, are widely used to control Varroa populations as there is a low risk of the acids accumulating in the bee products and a low probability of Varroa developing resistance after repeated applications (Rosenkranz et al., 2010; Underwood & Currie, 2003). Formic acid is the only organic acid treatment that can be applied when brood is present as it effectively kills mites both in brood cells and on adult honey bees (Boecking & Genersch, 2008; Fries, 1991; Rosenkranz et al., 2010).

Formic acid is the simplest carboxylic acid, that can trigger metabolic acidosis by inhibiting cytochrome c oxidase at the sixth coordination position (Liesivuori, 2014). This causes disruption to the mitochondrial electron transport and energy production in the terminal electron acceptor of the electron transport chain, triggering histotoxic hypoxia (Liesivuori & Savolainen, 1991). Cell death from cytochrome oxidase inhibition by formic acid is believed to result in a partial depletion of ATP reducing energy concentrations so essential cellular functions cannot be maintained (Du et al., 2008; Liesivuori & Savolainen, 1991). Cytochrome oxidase inhibition by formic acid can also cause cell death by increased production of cytotoxic reactive oxygen species (ROS) that cause damage to essential components of cells. Formic acid is deemed miticidal when vaporised and is considered safe as it is hydrophilic so does not accumulate in wax and trace amounts are naturally found in honey (Rosenkranz et al., 2010).

Label free quantitative proteomics is a powerful technique that offers the possibility of characterising alterations in the abundance of proteins following exposure of cells or organisms to a treatment and whole cell (organism) response. The aim of the work presented here was to assess the treatment effect of MAQ strips administered as an autumn treatment on the proteome and subsequent overall physiology of the winter bee.

Materials and methods

Sample collection

Honey bee workers (*A. mellifera*) were collected from three colonies at an apiary maintained at Teagasc Research Centre, Oak Park, Co. Carlow, Ireland (52°51'53.3"N 6°54'09.7"W). All colonies were hived in commercial type boxes and headed with 2019 queens and standardised using brood area. Prior to the commencement of the trial, honey was harvested, and colonies were fed 4.5 litres of a 2:1 sugar: water syrup and based on the manufacturer's instructions, an empty "super" was placed on each of the test colonies. Natural mite fall (a measure of mite load in the colony) was estimated in the test colonies using a gridded insert underneath the brood box over a five-day period. Treatment was administered according to the manufacturer's instructions. The MAQ strips (registered for use in Ireland with the Health Product Regulatory Authority) were removed immediately after week one samples were collected. Four sets of samples were taken from the door of each colony: control – no treatment, week 1 – seven days after treatment was initiated, week 2–14 days post treatment initiation, and week 3–21 days post treatment initiation.

Protein extraction from A. mellifera

A single honey bee worker from each hive was decapitated using a sterile disposable scalpel and the heads collected in a microcentrifuge tube. Proteins were homogenised using a hand-held motorised pestle and extracted using 6 M urea, 2 M thiourea and a protease inhibitor tablet (PIC: Complete Series Roche) solution. Cellular debris was pelleted through centrifugation at 10000×*g* for 5 min. All supernatant was precipitated overnight at −20 °C at a ratio of 1:5 with 80% acetone. The acetone was removed, and proteins were re-suspended in 120 µl resuspension buffer (6 M urea, 2 M thiourea, 0.1 M tris-HCL, (pH 8.0) dissolved in deionised water). The Qubit™ protein quantification system (Invitrogen) was used to quantify 1 µl aliquots of protein sample. Ammonium bicarbonate (50 mM) was added to 20 µl of protein sample and proteins were reduced with 0.5 M dithiothreitol (DTT) (Sigma-Aldrich) at 56 °C for 20 min and alkylated with 0.5 M iodoacetamide (IAA) (Sigma-Aldrich) in the dark at room temperature for 15 min. Proteins were digested with Sequence Grade Trypsin (0.5 µg/ml) (Promega)

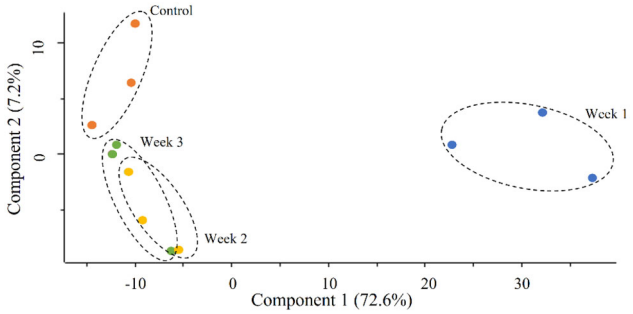

Figure 1. Principal Component Analysis (PCA) on label free proteomic analysis on control and treated samples. A clear distinction can be seen between week one and control/weeks 2 & 3. Total variation between samples is 79.8%. Protein intensity spectrum highlights the extent of down regulation (blue) and up regulated proteins (orange).

and incubated overnight at 37 °C. Trifluoroacetic acid (1 µl of 100%) (Sigma-Aldrich) was added to inhibit tryptic digestion during a 5 min incubation. Samples were centrifuged at 13,000×*g* for 10 min. Peptides were purified using C-18 spin columns (Pierce) and dried in a SpeedyVac concentrator (Thermo Scientific Savant DNA120) at 39 °C for 2 hours. Samples were resuspended in 2% acetonitrile and 0.05% trifluoroacetic acid followed by sonication in a water bath for 5 min and centrifugation for 5 min at 15,500x*g*. The supernatant was extracted and used for mass spectrometry.

Mass spectrometry

Digested *A. mellifera* protein samples (1 µg) were loaded onto a QExactive Mass Spectrometer (ThermoFisher Scientific) connected to a Dionex Ultimate™ 3000 (RSLCnano) chromatography system. Separation was determined by an acetonitrile gradient in a BioBasic™C18 PicoFrit™ COLUMN (100 mm in length, 75 mm inner diameter) using a 135 min reverse phase gradient at a flow rate of 250nL/min. The mass spectrometer was operating in an automatic dependent switching more to acquire all data. A high-resolution MS scan (300–200 Dalton) was performed using the Orbitrap to select the 15 most intense ions prior to MS/MS. Protein identification and LFQ normalisation of MS/MS data were carried out using the Andromeda search engine MaxQuant version 1.6.6.0 (https://maxquant.org/) to correlate the data against the predicted protein set derived from the *A. mellifera* genome (Amel 4.5 assembly, Honey bee Genome Sequence consortium, 2006).

The following search parameters were used: first search peptide tolerance of 20 ppm, second search peptide tolerance 4.5 ppm, carbamidomethylation of cysteines of N-terminals were set as variable modifications and a maximum of 2 missed cleavage sites allowed. False Discovery Rates (FDR) was set to 1% for both peptides and proteins and the FDR was estimated following searches against a target-decoy database. Peptides with a minimum length of seven amino acids were considered for identification and proteins were only considered identified when more than one unique peptide for each protein was observed. The MS proteomics data and MaxQuant search output files have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the dataset identifierPXD026058.

Data analysis

Results processing, statistical analysis, and graphics were generated using Perseus v. 1.6.6.0. LFQ intensities were $\log_2$ transformed and proteins that had a non-existent values (indicative of absence or very low abundance in a sample) were used in statistical analysis following the imputation of representative numbers based on the lowest value for each data set, which was calculated as a 1.8 downshift from the mean value allowing for 0.3 width in the downshift for the standard deviation. ANOVA significance was calculated between treated and control samples was performed using a p-value of 0.05, only ANOVA significant proteins were used for further analysis. Volcano plots were generated in Perseus to visualise differentially abundant proteins between control and treated groups and principal component analysis (PCA) was completed on all ANOVA significant proteins. Hierarchical clustering was performed on Z-score normalised intensity values for all statistically significant differentially abundant (SSDA) proteins (proteins with a real fold change > 2) by clustering all samples and proteins using Euclidean distance and complete linkage. The Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) v11.0 was used to map known and predicted protein-protein interactions and used to identify protein clusters associated with specific pathways and processes. Pathways were examined further using Kyoto Encyclopaedia of Genes and Genomes (KEGG) using the "KEGG Mapper – Search&Color Pathway" tool. Graphs of protein intensities were generated using Rstudio version 4.0.3 (2020-10-10). Boxplot

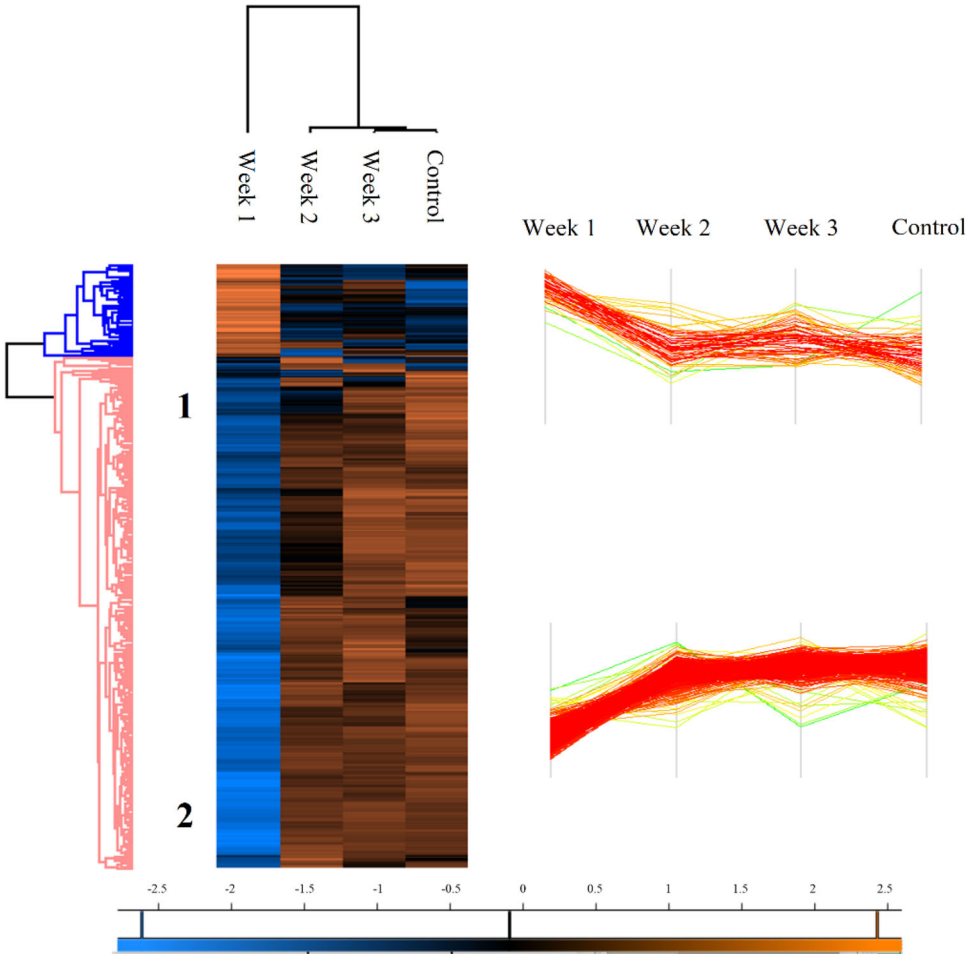

Figure 2. Hierarchical clustering and intensity heatmap on label free proteomic analysis on control and treated samples. Protein expression values of all statistically significant differentially abundant proteins. 2 distinct groups were identified.

significance was calculated using Tukey HSD (honestly significant difference) test and error bars in the line-graphs were by R-studio calculated using the standard deviation of the intensity values from the samples.

Results

Characterisation of proteomic response of A. mellifera to MAQ strip exposure

MAQ strips were applied to hives according to the manufacturer's instructions and weekly mite fall was monitored using adhesive inserts (Figure S1). An average of 70 mites was collected in the control during the week prior to application of the treatment, 400 mites fell during Week 1 (treatment week) and 240 during Week 2. Thereafter the mite fall was approximately 80 per week. In order to characterise the effect of MAQ strip treatment on *A. mellifera,* the proteome of control bees was compared to treated bees using label free quantitative (LFQ) mass spectrometry. Protein extractions were performed on three replicates per sample group. Initially, a total of 3,070 proteins were identified, after filtration and ANOVA test (p-value $>$ 0.05)712 proteins remained. Proteomic analysis revealed a strong response to treatment application in the proteome of workers exposed to MAQ strips in the first week of treatment compared to control or workers sampled on Week 2 or Week 3 (Figures 1 and 2). Principal Component Analysis (PCA) highlights a large separation between Week 1 samples versus control samples and Weeks 2 and 3 samples (Figure 1).

Hierarchical clustering analysis (Figure 2) was performed on statistically significant differentially abundant (SSDA) proteins. Week one samples are least like the control samples and are not clustered with Weeks 2 and 3 samples. Cluster analysis reveals an enrichment of cuticular protein in cluster 1 but only 2 cytochrome c oxidase proteins – Cox6b1 and Cox5a, which are associated with an anaerobic environment. Cluster 2 however, shows an abundance of xenobiotic enzymes and 3 cytochrome c oxidase proteins – Cox6a1Coxfa4 and Cox4l1.

Comparative proteomic analysis between control and MAQ-treated samples

Control vs week 1:

Volcano plot analysis between control and Week 1 samples revealed the highest number of SSDA

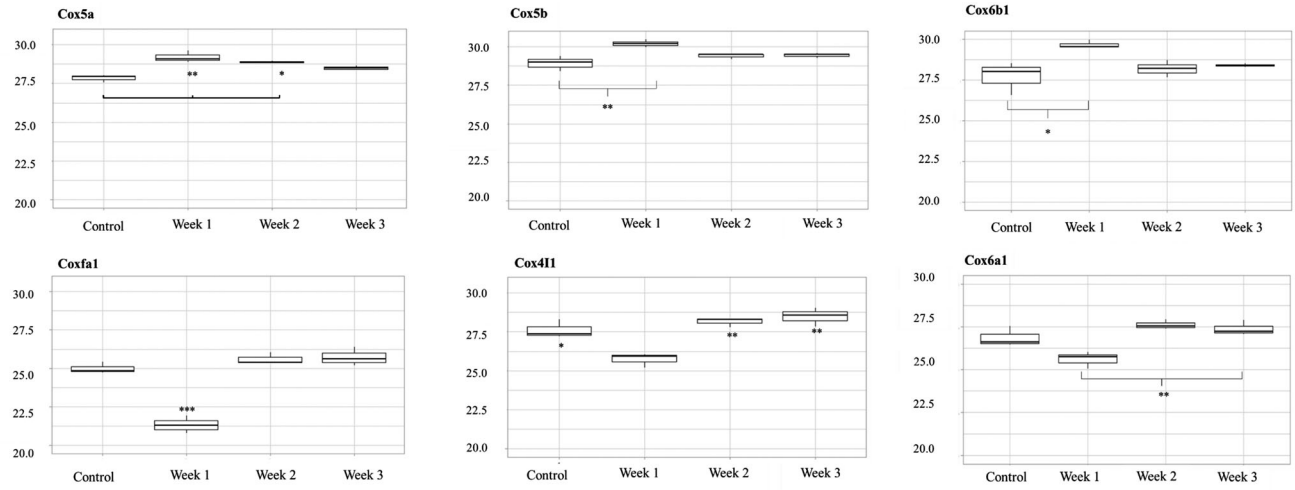

Figure 3. Protein intensity values on significantly increased/decreased cytochrome c oxidase subunits 5a, 5b, Coxfa1 and Cox4l1. Significance is represented by 0 "***", 0.001 "**", 0.1 "*".

proteins with a real-fold change ranging between (−28.06 to 13.49 fold) (Figure S2). The most prominent SSDA proteins increased in abundance in Week 1 samples were cuticular proteins: pupal cuticle protein (+ 6.64 fold), Cpap3-e (+5.99 fold), CPF2 (+5.46 fold), Capap3-d (+5.07 fold), endocuticle structural glycoprotein SgAbd-1 (+4.83 fold), CPR1 (+3.50 fold), and CpR5 (+2.65 fold). Interestingly some cytochrome c oxidase subunits were increased in abundance in Week 1 samples (Cox6b1 (+3.90 fold), Cox5a (+2.57 fold), Cox5b (+2.42fold)) while others were decreased in relative abundance (Coxfa4 (−12.59 fold), Cox4l1 (−3.73 fold), Cox6a1 (−2.43fold). Several xenobiotic proteins were decreased in relative abundance in Week 1 samples including Cytochrome P450 – CYP6AS8 (−11.38 fold), CYP6AS5 (−2.16 fold) Glutathione S transferases – GST-mic1 (−2.92 fold), UDP-glucuronosyltransferase – LOC408788 (−10.57 fold), LOC413043 (−7.80fold), LOC409203 (−4.28 fold), ATP-binding cassette (ABC) proteins – LOC409666 (−6.73 fold), LOC413252 (−3.91 fold), ATP-binding cassette sub-family F member 1 (−3.43 fold), ATP-binding cassette sub-family E member 1 (−2.3 fold) and several other antioxidant proteins (Figure S2).

Week 1 vs weeks 2

Comparative analysis between Week 1 and Week 2 samples yielded 150:276 SSDAs respectively with a real fold range between −21.06 to 27.42 (Figure S3). Xenobiotic detoxification proteins were decreased in relative abundance in Week 2 samples compared to Week 1 samples: Cytochrome P450s – CYP6AS8 (−10.09 fold), CYP6AS5 (−3.11 fold), and CYP6a14 (−3.05 fold), CYP9e2 (−2.39 fold). Detoxifying enzymes such as ABCs – LOC413252 (−3.22 fold), Hmt-1 (−2.86 fold), Glutathione S transferases – GST-mic1 (−2.56 fold), UDP-glucuronosyltransferase – LOC408788 (−7.97 fold), UDP-glucuronosyltransferase 2B15 isoform X1 (−4.86 fold), LOC409203 (−4.73 fold), ABCs – LOC413252 (−3.22 fold), Hmt-1 (−2.86 fold) and multidrug resistance protein – LOC551167 (−11.61 fold) were also decreased in abundance in Week 2 samples. Three cytochrome c oxidase proteins were decreased in relative abundance in Week 2 samples – Coxfa4 (−19.36 fold), Cox4l1 (−5.38 fold) and Cox6a1 (−4.06 fold), while Cox6b1 (+2.78 fold) was increased in relative abundance in Week 1 samples.

There was an increased number of cuticular proteins associated with Week 1 samples relative to Week 2 samples: Cpap3-e (+14.09 fold), Cpap3-d (+11.35), extension (+7.76), pupal cuticle protein (+5.84), CPF2 (+4.82), LOC725804 (+4.56), CPR5 (+3.25), CPR27 (+3.04) and CPR24 (+2.95). Interestingly there is also an increased abundance of three odorant binding proteins associated with Week 1 samples: OBP19 (+9.56 fold), OBP21 (+5.44 fold) , OBP4 (+4.97 fold).

Week 1 vs Weeks 3

Results from the Week 1 versus Week 3 comparative analysis resulted in 77:298 SSDAs with a fold change range of −22.03 to 30.44 respectively (Figure S4). Xenobiotic detoxification proteins were also decreased in relative abundance in Week 3 samples compared to Week 1 samples: Cytochrome P450s – CYP6a14 (−11.2 fold), CYP6AS5 (−3.52 fold), Glutathione S transferases – GST-mic1 (−2.35fold), LOC408788 (−10.05 fold), UDP-glucuronosyltransferases– UDP glucuronosyltransferase 2B15 isoform X1 (−7.9 fold), LOC413043 (−5.45 fold), LOC409203 (−5.35 fold), ABCs – LOC409666 (−7.79 fold), LOC413252 (−4.05 fold), pix (−2.04 fold) and multidrug resistance protein – LOC551167 (−18.86 fold). Cytochrome c oxidase proteins were decreased relative abundance in Week 3 samples: Coxfa4 (−20.96 fold), Cox4l1 (−6.78 fold), Cox6a1 (−3.48 fold). Cox6b1 (+2.40) was the only cytochrome c oxidase subunit found in higher abundance in Week 1

samples. Cuticular proteins were upregulated in Week 1 samples:Cpap3-d (+8.39 fold), pupal cuticle protein (+3.7 fold), CPF2 (+2.85 fold), extension (+2.33 fold), CPR27 (+2.01 fold).

The Volcano plots reveal an absence of oxidative phosphorylation proteins in Week 1 in comparison to Weeks 2 and 3 in comparison to the control samples. There is also a significant loss of xenobiotic metabolism proteins in Week 1 samples. The reduction of oxidative phosphorylation proteins and the absence of xenobiotic proteins in Week 1 samples could indicate an extensive effect on bees exposed to MAQs in the seven days of treatment. Interestingly in Week 2 samples (7 days after treatment was removed), there is a significant increase in both oxidative phosphorylation and xenobiotic metabolism proteins. Cytochrome c oxidase (the target of formic acid) the fourth complex in oxidative phosphorylation pathway shows a dual response to MAQ; subunits Cox5a, Cox5b and Cox6b1 are in significantly higher abundance in Week 1 samples, whereas subunits Coxfa1, Cox4l1 and Cox6a1 are significantly decreased in abundance in Week 1 (Figure 3). Cytochrome c oxidase was the only complex to have subunits both increased and decreased in abundance in the comparisons, none of the subunits from the other oxidative phosphorylation complexes were identified in Week 1 samples. This suggests disruption to the oxidative phosphorylation pathway as a whole and not just the cytochrome c oxidase subunit in response to MAQ strip exposure.

Discussion

Varroa mites are a constant threat to the health and success of beehives across the world. Understanding the impacts of commonly used anti-Varroa treatments on bee physiology is essential as these treatments may induce stress responses in bees, further adding to the strain they are undergoing due to Varroa parasitisation. This study focused on the use of Mite Away Quick strips and the potential proteomic alterations that may occur in the bees due to treatment exposure. MAQ strips are a quick and easy to use treatment to reduce high Varroa populations in hives and are a popular choice among beekeepers but may have unanticipated effects on the health of honey bees. MAQ strips contain formic acid (47.6%) as the active ingredient, which targets the mite's ability to deal with oxidative stress and causes disruption to the oxidative phosphorylation system in the mitochondria leading to cell death (Liesivuori & Savolainen, 1991; Nicholls, 1976).

Proteomic analysis identified that MAQ strip application induced a significant change in the proteomes of bees sampled on Week 1 (treatment week). Overall, there is an absence of proteins associated with xenobiotic metabolism and a reduced presence of oxidative phosphorylation proteins in Week 1 samples in comparison to control and Weeks 2 and 3 samples. Formic acid targets cytochrome c oxidase which has been suggested to be the main regulation site for the oxidative phosphorylation pathway (Li et al., 2006; Liesivuori & Savolainen, 1991). Interestingly, there was a significant decrease in the abundance of three cytochrome c oxidase subunits; Cox4l1, Cox6a1, and Coxa41, but a significant increase in the abundance of Cox5a, Cox5b and Cox6b1 subunits in Week 1 samples compared to the control. Cytochrome c oxidase subunit 5 is the largest of the cytochrome c oxidase subunit, COX5a is expressed under normoxic conditions and COX5b is expressed in low oxygen conditions (Dodia et al., 2014). These data suggest that the formic acid within MAQ strips inhibit/disrupts cytochrome c oxidase activity and subsequentially the oxidative phosphorylation pathway in *A. mellifera* samples. This response is limited to the time the treatment is within the hive as by Week 2 the abundance of Cox5b decreases and the abundance of COX5a rises. Cytochrome c oxidase 4 isoform 1 is essential for the assembly and respiratory function of the enzymatic complex (Li et al., 2006), which was found at significantly lower intensities in Week 1 samples. The subunit that was identified to be least abundant in Week 1samples was cytochrome c oxidase subunit NDUFA4 which has recently been renamed as an additional cytochrome c subunit CoxFA4 (Pitceathly et al., 2013; Pitceathly & Taanman, 2018). The deficiency of CoxFA4 in cells has been observed to cause a reduction in cytochrome c oxidase function and could potentially alter the protein complex synthesis (Pitceathly et al., 2013; Pitceathly & Taanman, 2018). CoxFA4 has been associated with Cox5 subunit and may be essential for the structure of cytochrome c complex IV (Balsa et al., 2012). A reduction in CoxFA4 and an abundance of Cox5a and Cox5b in Week 1 samples of MAQ treatment suggests that MAQ strips cause an adverse effect in honey bees by disrupting the function of cytochrome c oxidase and therefore altering the oxidative balance in cells. This effect seems to be temporary as samples from weeks 2 and 3 more closely resembled control samples, so disruption to cytochrome c oxidases may be limited to the presence of the MAQ strips within the hive.

Metabolism of xenobiotics in insects is associated with an increase in metabolic processes and drug metabolism enzymes (Li et al., 2007). The results presented here identify an absence of metabolic proteins and xenobiotic enzymes in Week 1 samples compared to control samples and Weeks 2 and 3 samples. There are three phases of xenobiotic

metabolism: Phase I – toxic structure is altered preventing it from interacting with target sites this is primarily completed with cytochrome P450 monooxygenases (P450) and carboxylesterases (CCE) (Berenbaum & Johnson, 2015). Phase II – conjugation of products from Phase I for solubilisation and transport carried out by glutathione-S-transferases (GST) (Berenbaum & Johnson, 2015; Ketterman et al., 2011). Phase III – is transporting the products from Phase II for excretion this is mainly done by multi drug resistant proteins and ATP-binding cassette transporters (Berenbaum & Johnson, 2015; Dermauw & Leeuwen, 2014; Poquet et al., 2016). Two cytochrome P450 6 A subunits, one NADH reductase cytochrome P450 subunit and one 932 cytochrome P450 subunit were identified as SSDAs in Week 2 and 3 samples in comparison to Week 1 samples. Three ATP-binding subunits were also identified as SSDAs in Week 2 and 3 in comparison to Week 1. The role of cytochrome P450s in the metabolism of an acaricide (pyrethroid, another Varroa treatment) has been studied and was determined that pyrethroid is safe to use to reduce Varroa populations in hives as honey bees utilise cytochrome P450 activity for the metabolism of this treatment (Johnson et al., 2006; 2012; Rand et al., 2015). CYP9 and CYP6 subfamily members have been identified as those responsible for tau-fluvalinate and pyrethroid metabolism (Mao et al., 2011; Yang et al., 2006). This study has also identified proteins belonging to those cytochrome P450 gene clans that decreased in abundance in Week 1 samples. Although no significant changes in protein intensities were observed, there was an overall decrease in cytochrome P450 abundance in Week 1samples which recovered to levels mirroring the control in Week 2 and 3 samples (Figure S5).

Increased metabolic processes have been observed in several studies investigating its association with insecticide and pollutant resistance (Kliot & Ghanim, 2012; Poquet et al., 2016; Rand et al., 2015). Results presented here highlight the abundance of metabolic and oxidative phosphorylation proteins in Weeks 2 and 3 samples in comparison to those in Week 1 samples, indicating an increased detoxification response in these samples. This indicates a decrease in protein abundance Week 1 proteomes due to the presence of MAQ strips, which recovers by Week 2 and 3 (7 and 14 days post treatment removal respectively) as these proteomes more closely resemble that of the control (pre-treatment) samples. The increased abundance of cytochrome P450 and NADH dehydrogenase (the driving complex of the oxidative phosphorylation pathway) in Weeks 2 and 3 samples further indicates the delayed reaction to insecticide resistance as workers are working to achieve homeostasis through increased metabolic and detoxification processes.

Several cuticular proteins were observed in higher abundance in Week 1 samples compared to control and Weeks 2 and 3 samples. A previous study has identified the negative impacts formic acid has upon the proteolytic barrier, however, the impacts of formic acid on the workers' proteome is dependent upon the stage of development (Strachecka et al., 2012). The increase in these proteins in Week 1 samples may indicate a response to formic acid penetration of the cuticle, possibly as a means of slowing insecticide penetration into the internal organs thereby allowing metabolic detoxification to begin (Wood et al., 2010; Yahouédo et al., 2017). A study on the response of *Anopheles funestus* to short exposures of insecticides (pyrethroid) concluded that tolerant/resistant *A. funestus* are likely to have a thicker cuticle layer than susceptible *A. funestus* (Wood et al., 2010; Yahouédo et al., 2017).

Conclusions

This study has identified a temporary adverse effect of MAQ strip treatments on the proteome of *A. mellifera*, which is most pronounced in the first seven days of treatment. Almost all proteins associated with the oxidative phosphorylation system were downregulated in samples taken at Week 1 (treatment week) with the exception of Cox5a, Cox5b and Cox6b. This decrease in cytochrome c oxidase proteins may result in a rise in cellular stress as it correlates with an overall decrease in oxidative phosphorylation and xenobiotic proteins in workers and could lead to further unknown damage to workers, such as apoptosis due to oxidative stress (Song & Scharf, 2009). Disturbance to the hive's natural structure is not uncommon with MAQ treatment and has been observed by several beekeepers and is stated in the instruction sheet for the treatment application process (NOD Apirary Products USA). Unintended side effects are not uncommon in the use of treatments to reduce or eliminate infections or pests (Johnson et al., 2012). MAQ strips are effective at reducing Varroa infestation levels over a short period of time, however, care and caution should be taken when treating honey bee colonies as MAQ strips may induce a short term stress response in bees which may add to the pressure of already Varroa-infected hives.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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