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Article *in* Journal of Apicultural Research · June 2016

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To cite this article: Carla Surlis, James C. Carolan, Mary F. Coffey & Kevin Kavanagh (2016): Proteomic analysis of Bayvarol® resistance mechanisms in the honey bee parasite Varroa destructor, Journal of Apicultural Research, DOI: 10.1080/00218839.2016.1196015

To link to this article: http://dx.doi.org/10.1080/00218839.2016.1196015

Published online: 28 Jun 2016.

Journal of Apicultural Research, 2016
http://dx.doi.org/10.1080/00218839.2016.1196015

ORIGINAL RESEARCH ARTICLE

Proteomic analysis of Bayvarol® resistance mechanisms in the honey bee parasite *Varroa destructor*

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(*Received 20 October 2015; accepted 20 May 2016*)

The haemophagous mite, *Varroa destuctor* is one of the most dangerous threats to the Western honey bee, *Apis mellifera.* Varroa mites parasitize the larval and adult stages of the honey bee and can have devastating effects on the health of the individual bee and colony. In recent years, varroa have shown resistance to the pyrethroid group of insecticides, including Bayvarol® which has flumethrin as the active ingredient. In the work presented here, changes in the expressed proteomes of mites, either sensitive or resistant to Bayvarol® were observed using 2D-SDS-PAGE and shotgun label-free proteomics. A number of detoxification proteins (e.g., glutathione-s-transferase, flavin-containing monooxygenase) were present at higher levels in the resistant mites, as were some proton pumping proteins (e.g., Na+/K+ ATPase alpha and beta subunit, E1–E2 ATPase protein). A decrease in the abundance of 12 cuticle proteins in the resistant mites was observed indicating that alteration to cuticle structure could be a potential resistance mechanism. A number of structural proteins such as myosin and alpha tubulin were expressed at higher levels in the resistant mites, which could indicate a change to the intracellular structure of the cuticle barrier or a change in the cell shape/surface, rather than the addition of extra cuticle proteins. The results presented here indicate higher levels of protein associated with cellular detoxification in Bayvarol®-resistant varroa mites.

Análisis proteómico de los mecanismos de resistencia a Bayvarol® en el parásito de la abeja de la miel *Varroa destructor*

El ácaro hemófago, *Varroa destuctor* es una de las amenazas más peligrosas para la abeja occidental de la miel, *Apis mellifera*. Los ácaros varroa parasitan los estadios larvarios y adultos de la abeja de la miel y pueden tener efectos devastadores sobre la salud de la abeja y la colonia individual. En los últimos años, Varroa han mostrado resistencia al grupo de insecticidas piretroides, incluyendo Bayvarol® que tiene la flumetrina como ingrediente activo. En el trabajo que aquí se presenta, se observaron cambios en los proteomas expresados por los ácaros, ya sea sensibles o resistentes a Bayvarol® utilizando 2D-SDS-PAGE y proteómica con marcaje libre *shotgun*. Ciertas proteínas de desintoxicación (por ejemplo, transferasa-s-glutatión, monooxigenasa incluyendo flavina) estaban presentes en niveles más altos en los ácaros resistentes, así como algunas proteínas de bombeo de protones (por ejemplo, las subunidades alfa y beta de la ATPasa Na + / K +, y la proteína ATPasa E1-E2). Se observó una disminución en la abundancia de 12 proteínas de la cutícula de los ácaros resistentes lo que indica que la alteración de la estructura de la cutícula podría ser un mecanismo de resistencia potencial. Ciertas proteínas estructurales, tales como la miosina y la alfa tubulina se expresaron en niveles más altos en los ácaros resistentes, lo que podrían indicar un cambio en la estructura intracelular de la barrera de la cutícula o un cambio en la forma o la superficie de la célula, en lugar de la adición de proteínas extra de la cutícula. Los resultados presentados aquí indican niveles más altos de proteínas asociadas con la desintoxicación celular en los ácaros varroa resistentes a Bayvarol®.

Keywords: *Apis mellifera*; Bayvarol®; proteomics; pyrethroid; resistance; *Varroa destructor*

Introduction

The parasitic mite varroa *Varroa destructor* (Anderson & Trueman, 2000) is one of the most serious threats to the Western honey bee, *Apis mellifera,* a pollinator of great economic importance. Varroa are not a natural parasite of *A. mellifera* but shifted host from *Apis cerana* in the early years of the twentieth century (Gómez-Moracho et al., 2015). Due to this recent host shift, the host–parasite relationship is imbalanced, with the result that *V. destructor* has negatively impacted on *A. mellifera* populations. Varroa are obligate parasites, feeding on the haemolymph of both larvae and adults leading to a weakening of the health and vitality of the individual bee and colony due to the transmission of viruses (Ball, 1985; Di Prisco et al., 2011; Navajas et al., 2008; Shen, Yang, Cox-Foster, & Cui, 2005; Yang & Cox-Foster, 2005). The elevated levels of viruses in bees have been correlated with high populations of varroa in colonies and are thought to be a major contributing factor to honey bee colony losses (Francis, Nielsen, & Kryger, 2013).

The most widely used chemical acaricides against *V. destructor* were the pyrethroids tau-fluvalinate and flumethrin, licensed under the trade names Apistan® (Vita UK) and Bayvarol® (Bayer), respectively (Rosenkranz,

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Aumeier, & Ziegelmann, 2010). First introduced in the 1980s, they were very effective in controlling varroa infestation with relatively few deleterious effects on the bee colony (Thompson, Brown, Ball, & Bew, 2002). The pyrethroid mode of action is via binding to sodium ion channels, the exact location of which is thought to be along the hydrophilic T929 on the IIS5 helix of the *para* sodium channels (O'Reilly et al., 2014). This binding prevents closure of the channel resulting in an influx of ions, affecting both the central and peripheral nervous system in arthropods by producing a surge in repetitive discharges, leading to eventual paralysis (Davies, Field, Usherwood, & Williamson, 2007; Yang & Cox-Foster, 2005). Only a small proportion of the overall number of sodium channels need to be modified by the chemical for the generation of these repetitive discharges (Davies et al., 2007).

Resistance to pyrethroids has become a major problem for the control of many pests in recent years due to its specific target site and mode of action. Numerous insecticide resistant mechanisms have been identified in organisms that exhibit pyrethroid resistance including target site alteration, to prevent binding of the chemical initially (e.g., alteration to the shape of sodium channel), increased tolerance to the effects of the chemical at a cellular level (such as the ability to contend with increased oxidative stress), increased detoxification due to cytochrome P450s and structural alterations that inhibit the path of the chemical through changes in cuticle or the epithelial lining of the digestive tract. These physical restrictive barriers are the least well understood, but are thought to often act synergistically with the other two mechanisms (Kasai et al., 2014).

Resistance to pyrethroids has also emerged in *V. destructor*. The haplodiploid nature of *V. destructor's* reproduction promotes the spread of resistance through its sibling mating habits, as resistance mechanisms from female mites are passed to the haplodiploid male firstborn, the father of all subsequent offspring (Sammataro, Untalan, Guerrero, & Finley, 2005). Resistance in *V. destructor* to pyrethroids is mainly due to a point mutation in the sodium channel (González-Cabrera, Davies, Field, Kennedy, & Williamson, 2013), the target site for the chemical, but also due to detoxifying elements in the cell which can help to clear the chemical and deal with the influx of ions into the cell following sodium channel inhibition (Maggi, Ruffinengo, Damiani, Sardella, & Eguaras, 2009; Mozes-Koch et al., 2000; Tan et al., 2007). These detoxification enzymes (e.g., microsomal p450 monooxygenases, glutathione-s-transerases and various esterases) may play an important role in the development of resistance in various other species such as *Drosophila* spp., *Heliothis virescens*, *Heliocoverpa armigera*, *Anopholes* spp. and *Aedes aegypti* (David et al., 2014; Scott, 1999). Pesticides can also lead to oxidative stress in the organism, and up-regulation of certain detoxification or stress-related proteins could provide a potential mechanism for protection (Abdollahi, Ranjbar, Shadnia, Nikfar, & Rezaie, 2004).

A proteomic analysis of Bayvarol® resistant and susceptible *V. destructor* is presented here in an attempt to elucidate the determinants and mechanisms of resistance at a protein level. Recent advances in mass spectrometry-based proteomics and the increasing availability of genomic and transcriptomic resources are now making it possible to investigate global systems level changes in an organism's proteome (Ozsolak & Milos, 2011). Quantitative expression proteomics permits the comparison of the proteomes of phenotypically different or experimentally treated organisms to identify either proteins, molecular processes, or biological pathways that may regulate or even contribute directly to the phenotype or characteristic itself. The most commonly used methods for expression proteomics are two-dimensional gel electrophoresis (2DE) and quantitative mass spectrometry, both of which were adopted for this study. 2DE involves separating proteins in two dimensions, the first based on differing protein isoelectric points and the second by protein mass. The resulting protein spots are then measured densitometrically and compared for differential abundance and numerous 2DE studies exist that have been conducted to investigate pyrethroid resistance in arthropods (Abdollahi et al., 2004; Brandt, Kerscher, Dröse, Zwicker, & Zickermann, 2003; Fragoso, Guedes, & Rezende, 2003; Hemingway, Hawkes, McCarroll, & Ranson, 2004; Kasai et al., 2014). Quantitative mass spectrometry can involve the labeling of proteins with a signal chemical, which can be measured and used as a basis to determine quantities (Bantscheff, Lemeer, Savitski, & Kuster, 2012). More recently "label-free" methods have been developed to measure the relative abundances of thousands of proteins across multiple sample groups in single mass spectrometry runs. Label-free quantitative (LFQ) proteomics was used here to obtain a deeper insight into the proteomic profile of Bayvarol® resistant mites in an attempt to better understand the mechanisms underlying pyrethroid resistance in this devastating pest of the honey bee.

Materials and methods

V. destructor *sampling*

V. destructor samples were obtained from mesh floor collections or from sticky floor inserts from various regions in Ireland by natural mite fall from colonies with known levels of resistance to Bayvarol® in the autumn following treatment. The samples were determined as being either sensitive or resistant to Bayvarol® using the Beltsville Method and the mite fall was collected for subsequent analysis.

Beltsville method for measuring chemical resistance in Varroa

The USDA Beltsville Method originally used for measuring susceptibility of bees to Apistan® was employed

here. *A. mellifera* adults were sampled from various locations and placed in a plastic beaker with a mesh lid. A Bayvarol® strip was then added to the beaker, and the container upturned. Bayvarol sensitive mites were collected as they fell and frozen at −70 °C. After 12 h, any mites that had not fallen were washed off and frozen at −70 °C. The percentage efficacy of Bayvarol® against *V. destructor* was determined. An overall efficacy of less than 50% indicated a resistant population of *V. destructor*. The mite fall from these colonies was collected and used for subsequent analysis and comparative proteomics against known sensitive samples.

Protein extraction for gel electrophoresis

Mites were crushed using liquid nitrogen to a fine powder in a pestle and mortar and resuspended in 500 µl of ice cold lysis buffer (PIPES (20 mM), NaCl (5 mM), Triton × 100 (.2% v/v)). Each protein extraction was left to lyse completely for one hour at 4 °C under constant agitation before centrifugation at 8000 *g* for 5 min to remove cellular debris. Supernatant was removed, and protein was quantified using the Bradford method and 400 µg of protein was acetone precipitated prior to focusing on 13 cm Immobiline DryStrip pH 4–7 for 2D SDS PAGE analysis.

Image analysis

Gels were stained using colloidal coomassie staining and three independent and reproducible 2-DE gel images were scanned using an Epson ImageScanner III. Gel images were subjected to analysis by Progenesis Samespot® software (version 4, nonlinear Dynamics; UK) for image quality control, spot alignment, filtration, normalization, and quantitation of spot volume. Obvious matching spots were firstly aligned manually, followed by matching in automatic alignment mode. The expression level of each protein spot was calculated in terms of its volume. Only spots with $p < .05$ were considered as statistically significant differences.

LC/MS identification of peptides

In-gel digestion was performed on two-dimensional gel spots of interest from a reference gel with proteins migrated to the same point between gels. The gel pieces were trypsin digested as described by Shevchenko et al. (2006). Tryptic peptide mixtures were spin-filtered (Agilent Technologies, .22 µm cellulose acetate), separated on extended liquid chromatography gradients on a nanoflow Agilent 1200 LC system and subjected to tandem mass spectrometry using an Agilent 6340 Ion Trap LC-MS System (Agilent Technologies, Santa Clara, CA). Database searches for identification of proteins were carried out using Spectrum Mill MS Proteomics Workbench (Revision B.04.00.127). Validation criteria were set to (i) maximum of two missed cleavages by trypsin, (ii) fixed modification: carbamidomethylation of cysteines, (iii) variable modifications: oxidation of methionine, and (iv) mass tolerance of precursor ions ±2.5 Da and product ions ±.7 Da were employed. Protein identities were confirmed by conducting a BLASTp search of the protein sequence against the Uniprot (www.uniprot.org) and NCBI (www.ncbi.nlm.nih.gov) databases.

Label-free proteomic analysis

Ten adult female *V. destructor* were chosen for each replicate for label-free proteomics, and were homogenized using a motorized pellet pestle in a sterile microfuge tube in 7 M urea, and 2 M thiourea buffer supplemented with protease inhibitor cocktail (Roche Complete mini inhibitors). After centrifugation at 9000 g for 5 min the supernatant was transferred to a fresh microfuge tube for Bradford analysis. Protein (300 µg) was removed and acetone precipitated overnight at −20 °C. Samples were centrifuged at 10,000 g for 10 min and the pellet was resuspended in 300 µl of 8 M urea. Samples were re-quantified using the Qubit® quantification kit, following the manufacturer's instructions (Life Technologies). Protein (75 µg) was reduced with 2 mM dithiotreitol (Sigma–Aldrich), and alkylated with 2.7 mM iodoacetamide (Sigma-Aldrich) and digested with sequence grade trypsin (Promega; Ireland) at a trypsin: protein ratio of 1:40, overnight at 37 °C. Tryptic peptides were purified for mass spectrometry using C18 spin columns (Medical Supply Company; Ireland) and 1 µg of peptide mix was eluted onto a QExactive (ThermoFisher Scientific; USA) high-resolution accurate mass spectrometer connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. Peptides were separated by an increasing acetonitrile gradient from 2 to 40% on a Biobasic C18 Picofrit™ 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 full MS scan at 140,000 resolution and a range of 300–1700 m/z was followed by an MS/MS scan, resolution 17,500 and a range of 200–2000, selecting the 15 most intense ions prior to MS/MS.

Quantitative mass spectrometry data 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 an annotated database derived from the *V. destructor* genome survey downloaded from Biomedical Central Genomics (Cornman et al., 2010). 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 two missed cleavage sites allowed. 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. LFQ intensities were calculated using the MaxLFQ algorithm (Cox et al., 2014) from razor and unique peptides with a minimum ratio count of two peptides across samples. Peptides with 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.

Result processing

Results processing, statistical analyses, and graphics generation were conducted using Perseus *V.* 1.5.0.31. LFQ intensities were log2-transformed and ANOVA of significance and *t*-tests between the proteomes of Bayvarol® sensitive and Bayvarol® resistant mites were performed using a *p*-value cut-off of .05. 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 three 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 .25 width in the downshift for standard deviation. The Blast2GO suite (www.Blast2GO.com) of software tools was utilized to assign gene ontology terms (GO terms) relating to biological processes, molecular function, and cellular component. A BLAST search was carried out on the peptide sequences of interest, followed by GO mapping, Annotation, and Enzyme code, and KEGG analysis. Graphs were compiled at a level three ontology using the mapping software on the Blast2GO resource.

Results

Analysis of Bayvarol® sensitive and resistant V. destructor *proteomes*

The proteome of varroa mites deemed either sensitive or resistant to Bayvarol® was resolved by 2D SDS-PAGE. In total, nine identified protein spots were shown to be altered in abundance in the resistant mites (Figure 1). Differentially abundant proteins were excised, digested, and identified by LC/MS as described (Table 1). An increase in abundance of proteins involved in detoxification was observed in the Bayvarol® resistant mites including glutathione-s-transferase (GST) (Spot 3; 2.8-fold increase; $p = .020$), aldehyde dehydrogenase (Spot 2; 5.9-fold increase; $p = .002$), and retinal dehydrogenase (Spot 1; 6.1-fold; $p = .01$).

Comparative analysis of differential protein expression of Bayvarol® resistant and sensitive V. destructor *using label-free proteomics*

LFQ proteomics was conducted on Bayvarol® sensitive and resistant varroa mites. In total, 3757 peptides were identified representing 650 proteins with two or more peptides and 134 proteins were determined to be

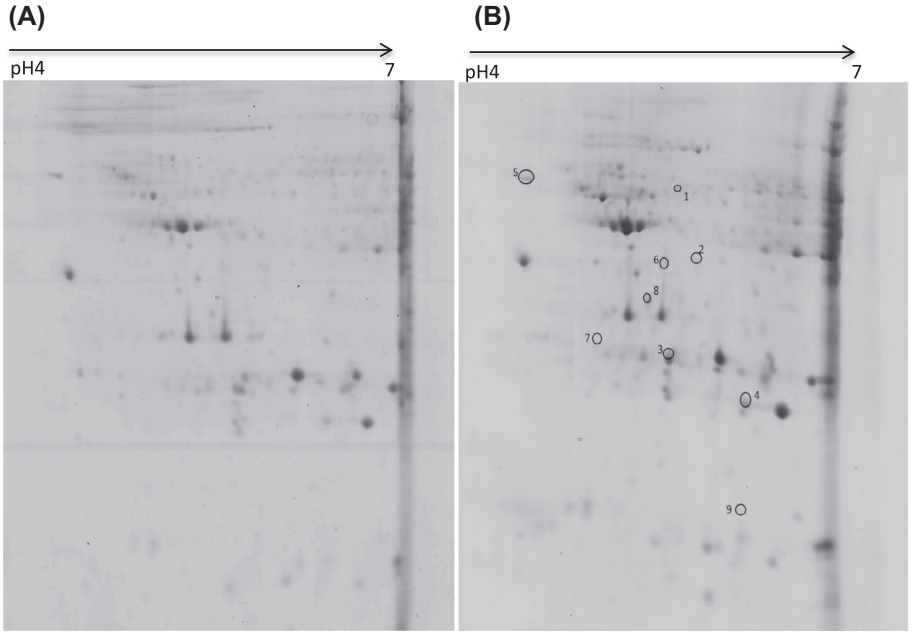
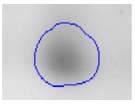
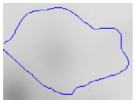
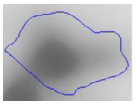
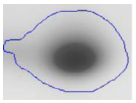
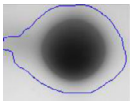
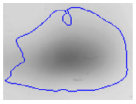
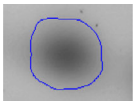
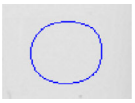
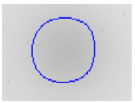
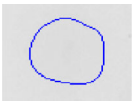
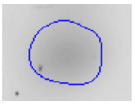


Figure 1. 2D SDS PAGE gel showing from varroa deemed sensitive and resistant to Bayvarol®. Representative 2D gel electrophoresis images of 400 μg of protein extracted from Bayvarol® sensitive (A) or resistant (B) mites. Statistically significant differentially abundant proteins were identified using Progenesis SameSpots® software and spots identified using LC-MS/MS are outlined and numbered.

Table 1. Table of differentially expressed proteins from the proteomic profiles resolved by two-dimensional SDS PAGE electrophoresis of sensitive and resistant varroa.

Protein spot and identity	Coverage (%)	Fold change increase in resistant mites	*P* value	Sensitive mites	Resistant mites
1. Retinal dehydrogenase 1-like	23.9	6.1	.0171		
2. aldehyde dehydrogenase	14.2	5.9	.00264		
3. Glutathione-s-transferase mu 1-like	10	2.8	.0253		
4. Secreted salivary gland peptide	35.4	5.9	.0348		
5. Beta tubulin	13.2	3	.0244		
6. Inorgnaic pyrophosphotase	20.1	1.8	.0371		
7. Proteosome subunit alpha type 5-like	11	3.9	.040		
8. Spermidine synthase like	52.6	2.3	.0288		
9. LOC100907454 secreted protein putative	16.4	3.9	.0129		

Notes: Each experiment was carried out in triplicate and statistically significant differentially expressed ($p < .05$) spots were identified. Spot profiles taken from Progenesis SameSpots®, the percentage coverage of identified peptides and relative fold change in resistant mites are shown.

differentially abundant (ANOVA, $p < .05$) with a fold change of >1.5, of which 89 were found in higher abundance in the resistant mites (Table 2) and 45 were found in higher abundance in the sensitive mites (Table 3).

A total of 41 proteins were present in all three resistant samples and absent in all three sensitive mite samples, and a further four were present in each replicate of the sensitive mites and absent in all three of the resistant samples (Table 4). These protein hits were also used in the statistical analysis of the total differentially expressed group following imputation of the zero values using a number close to the lowest value of the range of proteins plus or minus the standard deviation. After data imputation these proteins were included in subsequent statistical analysis (Tables 2 and 3). A number of related proteins were observed at similar abundance levels including a number of aldehyde dehydrogenases, myosin, heat shock proteins, and Na^+/K^+ subunit proteins which were at higher abundance in the resistant mites. Numerous proteins involved in cuticle structure and translation were found at higher abundance in sensitive mites (Figure 2).

The Blast2GO annotation software was used to group proteins based on conserved GO terms in order to identify processes and pathways potentially associated with Bayvarol resistance or sensitivity. GO terms were categorized by biological processes (BP; Figure 3) and molecular function (MF; Figure 4). No major changes

Table 2. Identities and expression values for proteins that were identified as being significantly higher in abundance in the Bayvarol resistant varroa.

Protein annotation	Peptides	Sequence coverage (%)	PEP	Mean LFQ Intensity	Expression in resistant *Varroa*
Myosin-9	3	46.2	7.71E − 110	6.39E + 09	18.9
Methylmalonate-semialdehyde dehydrogenase	5	47.6	7.23E − 99	2.73E + 09	12.5
Isocitrate dehydrogenase	4	65.2	1.48E − 50	1.62E + 09	7.2
Myosin-9	5	51.9	4.82E − 49	1.60E + 09	6.6
Alpha tubulin	6	42	1.62E − 254	1.52E + 09	6.5
Myosin-9	8	40.9	1.04E − 120	2.45E + 09	6.4
Ankyrin 2 3/unc44	8	36.3	2.49E − 106	9.77E + 08	6.0
Myosin heavy chain	9	46.7	2.36E − 167	1.91E + 09	5.8
Methylenetetrahydrofolate dehydrogenase	9	26.3	6.77E − 162	1.29E + 09	5.4
T-complex protein 1 subunit beta	6	38.9	2.62E − 70	9.77E + 08	5.1
Actin-5C	6	22.6	5.32E − 39	1.86E + 10	5.0
Aldehyde dehydrogenase	4	44.8	2.34E − 54	1.99E + 09	5.0
Myosin-9	10	24.8	2.03E − 42	8.28E + 08	4.4
Spermidine synthase	2	20.8	5.17E − 09	1.07E + 09	4.4
Quinone oxidoreductase	3	30.8	2.62E − 51	1.08E + 09	4.3
Chaperonin subunit	10	35	2.08E − 95	9.34E + 08	4.1
Translation elongation factor	4	38.4	1.63E − 91	2.02E + 09	4.1
Paxillin	9	43.2	3.60E − 114	1.32E + 09	3.8
Myosin heavy chain skeletal muscle	13	55	4.82E − 194	3.78E + 09	3.6
Cystathionine beta-lyase	2	67.7	1.03E − 58	5.42E + 08	3.6
Alcohol dehydrogenase	5	37.3	1.96E − 142	1.56E + 09	3.4
Succinate dehydrogenase	4	24.3	2.23E − 37	8.40E + 08	3.4
Spermatogenesis associated factor	4	49.7	9.82E − 49	8.28E + 08	3.3
Peptidyl-prolyl cis-trans isomerase	3	61.5	1.15E − 80	9.63E + 08	3.3
Paramyosin	16	36	6.58E − 274	9.38E + 09	3.3
Sumo-1-activating enzyme e1a	12	28	3.37E − 146	1.69E + 09	3.3
Aconitase	9	63.8	6.87E − 234	5.56E + 09	3.2
Vinculin	4	13.9	1.55E − 22	6.61E + 08	3.2
Myosin heavy chain skeletal muscle	14	64.2	1.81E − 138	2.89E + 09	3.1
Iron-containing alcohol dehydrogenase	5	40.5	2.27E − 65	2.60E + 10	3.0
Flavin-containing monooxygenase	4	36.3	3.64E − 62	9.60E + 08	2.8
Alpha-actinin-4	6	22.3	2.58E − 45	7.48E + 08	2.7
Myosin-9	6	51.6	5.00E − 172	8.62E + 09	2.7
Myosin heavy chain	16	50.7	6.15E − 164	4.06E + 09	2.7
Tubulin alpha-3 chain	4	55.4	5.66E − 77	1.95E + 09	2.7
60S ribosomal protein L19	2	17.9	9.75E − 06	1.11E + 09	2.6
Myosin-9	9	69.3	.00E + 00	8.33E + 09	2.6
GOT2 aspartate aminotransferase	2	25.2	4.70E − 07	6.74E + 08	2.5
Myosin-9	7	67.9	.00E + 00	5.13E + 08	2.5
Conserved protein (transmembrane)	2	23.6	3.70E − 12	4.58E + 08	2.5
6-phosphogluconolactonase	3	39.3	2.96E − 33	1.32E + 09	2.5
Processing peptidase beta subunit	2	30.1	2.01E − 15	6.20E + 09	2.4
Glycogenin-1	3	61.8	1.33E − 20	4.96E + 08	2.3
ADP/ATP translocase	9	46.8	1.72E − 57	2.86E + 09	2.2
Citrate synthase	6	26.9	2.56E − 35	2.60E + 09	2.2
Aminotransferase	2	14.5	3.76E − 45	5.81E + 08	2.2
LIM domain-containing protein	4	52.7	1.49E − 162	8.41E + 09	2.2
Aconitase	2	43.9	3.73E − 11	3.70E + 08	2.2
ADP/ATP translocase	7	64.1	1.33E − 25	3.79E + 09	2.2
Myosin-9	19	56.2	1.05E − 147	1.51E + 10	2.2
Ornithine aminotransferase	3	32.2	7.07E − 33	4.70E + 08	2.2
Glycerol-3-phosphate dehydrogenase	4	18.5	4.50E − 72	6.73E + 08	2.1
Phosphoglucomutase	4	40.6	1.34E − 40	7.89E + 08	2.1
Paramyosin	4	26.8	3.16E − 235	4.99E + 09	2.1
Formiminotransferase-cyclodeaminase	4	45.5	2.86E − 16	4.62E + 08	2.1
ADP/ATP translocase	8	48.5	1.23E − 35	3.11E + 09	2.1
Filamin	6	53.6	3.00E − 130	3.52E + 09	2.0
Acetyl-coa hydrolase	8	49	2.72E − 68	5.51E + 09	2.0
Hsp70	13	31.8	1.90E − 198	9.70E + 09	2.0
E1–E2 atpase	14	38.8	6.70E − 273	7.92E + 09	2.0
Hsp70	8	44.2	1.77E − 113	4.56E + 09	2.0

(*Continued*)

Table 2. (*Continued*).

Protein annotation	Peptides	Sequence coverage (%)	PEP	Mean LFQ Intensity	Expression in resistant *Varroa*
Gars/airs/gart	5	15.3	4.74E − 15	3.38E + 08	1.9
NADH:ubiquinone oxidoreductase NDUFS2/ 49 kda subunit	3	33.3	2.77E − 15	4.36E + 08	1.9
Filamin-C	8	65.1	7.52E − 63	5.93E + 09	1.9
ATP-binding cassette subfamily E member 1	3	40	1.53E − 120	4.08E + 09	1.9
Vacuolar H+ – atpase V1 sector subunit B	9	79.8	2.48E − 88	3.14E + 09	1.9
Paxillin	5	38.4	5.15E − 71	8.50E + 09	1.8
Spectrin alpha chain	4	73	6.07E − 39	4.02E + 08	1.8
Tubulin beta-1 chain	11	66	5.43E − 192	1.11E + 10	1.8
Aldo-keto reductase	3	34.5	4.50E − 68	8.63E + 09	1.8
THO complex subunit	3	28.4	4.27E − 29	5.55E + 08	1.8
THO complex subunit	33	71.6	.00E + 00	4.28E + 10	1.8
Arginine kinase	4	33.7	.00E + 00	1.04E + 10	1.7
Phosphoribosylamidoimidazole-succinocarboxamide synthase	5	48.4	7.59E − 117	4.93E + 09	1.7
4-aminobutyrate aminotransferase	2	38.5	1.53E − 15	3.79E + 08	1.7
Acetyl-coa acetyltransferase	4	35.6	4.38E − 67	4.15E + 09	1.7
Na+/K+ atpase beta subunit	3	22.2	9.35E − 08	1.26E + 09	1.7
Na+/K+ atpase alpha subunit	8	33.5	4.48E − 87	3.75E + 09	1.7
Hsp90 protein	14	34.2	1.36E − 203	1.41E + 10	1.7
Aldehyde dehydrogenase	7	46.4	1.81E − 59	1.13E + 09	1.6
Aminoimidazole-4-carboxamide ribonucleotidetransformylase	3	46.2	1.50E − 84	3.82E + 09	1.6
Chaperonin subunit	2	34.4	3.16E − 45	8.08E + 09	1.6
Translation elongation factor EF-1 alpha/Tu	6	41.7	3.78E − 63	1.35E + 10	1.6
Phosphoglycerate kinase	4	41.7	2.85E − 56	5.54E + 09	1.6
Heat shock protein	12	53.1	.00E + 00	3.83E+10	1.6
Glutamine synthetase 2 cytoplasmic	5	38.5	9.27E − 189	1.05E + 10	1.5
Phosphoglycerate mutase	2	18.6	4.93E − 27	8.57E + 08	1.5
Heat shock protein	10	71.5	1.42E − 283	1.12E + 10	1.5
Chaperonin subunit	14	63	5.73E − 207	1.47E + 10	1.5

Notes: Relative fold changes in expression of proteins in the profile of Bayvarol® resistant mites, and the number of matched peptides, sequence coverage, PEP and mean LFQ intensities. Proteins that had more than two matched peptides, with a *t*-test probability < .05 and that were found to be differentially expressed at a 1.5-fold change and greater in resistant compared to sensitive mites are given.

were evident between the profiles of resistant and sensitive mites, with the most noticeable change being in the proportion of proteins involved in single-organism metabolic process, which accounted for 3% of the overall proportion of GO terms for proteins involved in the sensitive mites but 12% of the protein composition of resistant mites. A comparison of the overall proportion of GO terms for proteins found in sensitive and resistant mites, grouped based on molecular function assignment is also presented (Figure 5). The largest increased groups in resistant mites compared to sensitive mites were carbohydrate derivative binding, small molecule binding, ion binding, and oxidoreductase activity. Proteins involved in cuticle and ribosome structure were present at lower levels in the resistant mites (Figure 5).

Using KEGG pathway analysis, pathways involving proteins of differential abundance between the sensitive and resistant mites were examined for possible association with the resistant phenotype. The P450 metabolic pathway involved in xenobiotic detoxification was one pathway which showed differences between the sensitive and the resistant mites (Figure 5) with a number of enzymes found at higher levels in the resistant mites (Figure 6).

Discussion

Flumethrin, the active ingredient in Bayvarol®, is a member of the pyrethroid group of insecticides which acts upon the sodium channel of the cell membrane resulting in the loss of the ability to close correctly, leading to over saturation of potassium and sodium within the cell and subsequent cell death (Martin, Ochou, Hala-N'Klo, Vassal, & Vaissayre, 2000; Vontas, 2001). Resistance to pyrethroids can be due to knock-down resistance of the target site of the chemical, but evidence suggests that this type of resistance does not exist independently, and that other resistance mechanisms such as increased detoxification can also operate (Martin, 2004; Vontas et al., 2005). The cross resistance of *V. destructor* mites to unrelated chemicals supports the theory that metabolic resistance could play a role in the development of resistant populations (Martin, 2004; Vontas et al., 2005). Resistance due to changes in the rate or efficacy of metabolism is thought to be mainly attributed to up-regulation of metabolic enzymes such as esterases, gluthathione-S transferases, and P450 mono-oxygenases (Puinean et al., 2010; Wang et al., 2015).

Table 3. Identities and expression values for proteins that were identified as being significantly lower in abundance in the Bayvarol® resistant varroa.

Protein annotation	Peptides	Sequence coverage (%)	PEP	Overall intensity	Expression in resistant varroa
Conserved hypothetical protein	2	48.5	1.22E − 28	1.64E + 09	−13.3
Ribosomal protein L37A	3	28.5	7.14E − 11	1.01E + 09	−5.0
Gastric triacylglycerol lipase	3	21.5	8.82E − 13	6.63E + 08	−4.0
Cold-inducible RNA-binding protein	2	32.7	9.66E − 41	1.12E + 09	−3.9
Ferritin	6	36.4	1.94E − 200	5.21E + 10	−3.2
Nucleolysin TIA-1	1	5	2.08E − 14	7.58E + 07	−2.9
Cytochrome B5	2	19.4	1.01E − 06	5.22E + 08	−2.9
Ubiquitin carboxyl-terminal hydrolase	2	22.1	3.82E − 07	1.82E + 08	−2.5
Cuticular protein	6	45	3.94E − 129	3.84E + 09	−2.4
Cuticular protein	8	65.1	5.60E − 220	2.99E + 10	−2.4
Cuticular protein	15	75.1	.00E + 00	1.62E + 10	−2.3
Transferrin receptor	4	57.9	2.86E − 127	5.34E + 09	−2.1
Cuticular protein	7	25.5	3.94E − 150	8.29E + 09	−2.1
60S ribosomal protein L23	4	38.8	1.93E − 82	5.25E + 09	−2.0
Structural constituent of cuticle	6	51.6	5.20E − 278	4.80E + 10	−2.0
Cuticular protein	7	46	.00E + 00	7.82E + 10	−2.0
Ribosomal protein L8	4	19.3	1.42E − 08	2.74E + 09	−2.0
Secreted protein	2	16.2	5.15E − 11	3.39E + 08	−1.9
Angiotensin-I converting enzyme	4	26.5	4.69E − 94	6.63E + 08	−1.9
Cuticular protein	4	52.1	6.18E − 259	3.19E + 10	−1.9
Ribosomal protein S18	5	54.5	1.12E − 172	8.42E + 09	−1.9
Cuticle protein	2	35.4	3.32E − 34	8.54E + 08	−1.9
60S ribosomal protein L9	5	38.6	4.95E − 94	3.96E + 09	−1.8
Secreted salivary gland peptide	4	32	2.82E − 54	6.78E + 10	−1.8
Cuticular protein	5	70.6	.00E + 00	7.12E + 10	−1.8
Transferrin receptor	4	47.2	2.10E − 100	5.34E + 09	−1.8
Rho guanine dissociation factor	2	37.3	1.25E − 83	2.62E + 09	−1.8
Alternative splicing factor srp20/9G8	2	11.9	8.10E − 58	7.50E + 09	−1.8
Ribosomal protein L15	4	27.9	7.39E − 32	2.54E + 09	−1.6
Cuticular protein	5	53.5	7.36E − 241	1.35E + 11	−1.6
Histone H4	12	55.3	2.62E − 92	3.38E + 10	−1.6
Transformer-2 sex-determining protein	3	46.2	4.55E − 65	3.80E + 09	−1.6
60S ribosomal protein L3	3	21.2	4.59E − 25	3.39E + 09	−1.6
60S ribosomal protein rpl7a	6	26.6	1.37E − 56	2.56E + 09	−1.6
Calmodulin-A	15	94.6	9.25E − 287	2.75E + 10	−1.6
40S ribosomal protein S13	5	33.3	1.29E − 12	3.91E + 09	−1.6
Beta-galactosidase precursor	6	59.6	7.44E − 31	1.16E + 09	−1.6
Glyceraldehyde 3-phosphate dehydrogenase	7	72	.00E + 00	1.77E + 11	−1.5
Ribosomal protein L13	9	47.2	8.31E − 70	8.07E + 09	−1.5
40S ribosomal protein S2	5	36	3.37E − 32	1.75E + 09	−1.5
Cuticular protein	5	45.5	1.76E − 89	1.21E + 10	−1.5
Conserved hypothetical protein	4	43.2	3.51E − 21	4.44E + 09	−1.5
Heat shock protein 20.6	7	44.4	3.43E − 304	3.57E + 10	−1.5
Ribosomal protein L3	4	48.5	4.35E − 45	5.07E + 09	−1.5
60S ribosomal protein L30	5	49.2	1.46E − 68	6.57E + 09	−1.5
60S ribosomal protein L10	3	27.6	1.04E − 44	7.92E + 09	−1.5

Notes: Relative fold changes in expression of proteins in Bayvarol resistant mites, the number of matched peptides, sequence coverage, PEP, and overall intensity. Proteins that had more than two matched peptides, with a *t*-test probability <.05 and that were found to be differentially expressed at a 1.5-fold change and greater in sensitive compared to resistant mites are given.

The comparison of the proteomic profiles of both sensitive and resistant varroa mites showed significantly different patterns of expression, with a total of 9 proteins identified as being differentially expressed by 2DE and 179 identified as being differentially abundant by LFQ analysis. This overview of the proteomic profile made possible by mass spectrometry, gives an excellent opportunity to observe differences between two phenotypes of the same organism on a large scale, highlighting differences that may be contributing towards the observed phenotype, such as the resistant mites. A number of proteins were observed to be differentially expressed between the profiles of sensitive and resistant mites that have previously been linked with resistance to pyrethroid, as well as some novel groups of proteins that could potentially be helping confer resistance

In the comparative 2DE, a significant increase in the abundance of GST was observed in Bayvarol® resistant mites compared to the level in sensitive mites. GST has previously been associated with detoxification against

Table 4. Exclusively expressed proteins in Bavarol® sensitive and resistant mites.

Protein annotation	LFQ intensity sensitive_1	LFQ intensity sensitive_2	LFQ intensity sensitive_3	LFQ intensity resistant_1	LFQ intensity resistant_2	LFQ intensity resistant_3
Conserved hypothetical protein	5.6E + 08	1.2E + 09	1.0E + 09	0	0	0
Translation initiation factor 3	1.5E + 08	2.6E + 08	1.4E + 08	0	0	0
Sulfotransferase	1.0E + 08	9.7E + 07	9.8E + 07	0	0	0
Trailer hitch, isoform D	4.5E + 07	4.8E + 07	5.2E + 07	0	0	0
Spermidine synthase	0	0	0	2.7E + 08	3.0E + 08	3.4E + 08
Coronin-6	0	0	0	8.5E + 07	1.3E + 08	7.6E + 07
Myosin-9	0	0	0	2.3E + 08	3.6E + 08	2.0E + 08
Alpha tubulin	0	0	0	4.6E + 08	4.0E + 08	4.1E + 08
Dipeptidyl peptidase III	0	0	0	1.2E + 08	1.9E + 08	1.2E + 08
Myosin-9	0	0	0	2.8E + 08	6.3E + 08	3.4E + 08
Spectrin alpha chain	0	0	0	1.4E + 08	1.5E + 08	1.3E + 08
Peptidyl-prolyl cis-trans isomerase	0	0	0	3.4E + 08	2.1E + 08	3.9E + 08
Glycosyl hydralase sucrase-isomaltase	0	0	0	4.2E + 08	8.6E + 08	5.9E + 08
Aconitase	0	0	0	9.2E + 07	1.7E + 08	1.3E + 08
26S proteasome subunit S9	0	0	0	1.0E + 08	1.6E + 08	1.1E + 08
Formiminotransferase-cyclodeaminase	0	0	0	1.6E + 08	2.0E + 08	1.3E + 08
DEAD box ATP-dependent RNA helicase	0	0	0	5.7E + 07	7.7E + 07	4.6E + 07
Glycogenin-1	0	0	0	1.2E + 08	1.9E + 08	1.7E + 08
Ornithine aminotransferase	0	0	0	1.5E + 08	1.6E + 08	1.6E + 08
Aminotransferase	0	0	0	1.6E + 08	1.6E + 08	2.2E + 08
Isocitrate dehydrogenase	0	0	0	4.8E + 08	4.3E + 08	4.9E + 08
Myosin-9	0	0	0	1.5E + 09	3.5E + 09	1.2E + 09
60S ribosomal protein L19	0	0	0	2.8E + 08	2.1E + 08	2.1E + 08
Methylmalonate-semialdehyde dehydrogenase	0	0	0	1.2E + 08	2.6E + 08	1.3E + 08
Methylmalonate-semialdehyde dehydrogenase	0	0	0	8.8E + 08	1.1E + 09	8.6E + 08
THO complex subunit	0	0	0	1.4E + 08	1.6E + 08	1.8E + 08
Vinculin	0	0	0	2.2E + 08	2.5E + 08	2.0E + 08
Chaperonin subunit	0	0	0	2.8E + 08	4.3E + 08	2.0E + 08
T-complex protein 1 subunit beta	0	0	0	2.6E + 08	3.9E + 08	2.9E + 08
Gars/airs/gart	0	0	0	9.6E + 07	1.4E + 08	1.3E + 08
Ankyrin 2 3/unc44	0	0	0	2.4E + 08	3.2E + 08	3.0E + 08
Alpha-actinin-4	0	0	0	1.6E + 08	3.4E + 08	1.9E + 08
Spectrin alpha chain	0	0	0	8.5E + 07	1.7E + 08	8.8E + 07
Flare, isoform A	0	0	0	1.4E + 08	1.7E + 08	2.3E + 08
Nucleolysin TIA-1	0	0	0	1.6E + 07	2.8E + 07	3.6E + 07
Succinate dehydrogenase	0	0	0	1.8E + 08	3.4E + 08	2.5E + 08
Enoyl-coa hydratase	0	0	0	1.0E + 08	1.6E + 08	8.3E + 07
Spermatogenesis associated factor	0	0	0	2.7E + 08	3.0E + 08	3.2E + 08
26S protease regulatory subunit 6A	0	0	0	1.5E + 08	1.2E + 08	1.3E + 08
Phosphofructokinase	0	0	0	1.3E + 08	2.2E + 08	1.7E + 08
GOT2 aspartate aminotransferase	0	0	0	1.8E + 08	2.4E + 08	1.5E + 08
Myosin-9	0	0	0	1.2E + 08	2.1E + 08	1.6E + 08
Polyadenylate-binding protein	0	0	0	1.4E + 08	1.5E + 08	1.5E + 08
Phosphoglucomutase	0	0	0	2.2E + 08	3.4E + 08	2.4E + 08
Prolylcarboxypeptidase	0	0	0	3.7E + 07	3.9E + 07	4.7E + 07

Notes: Proteins were considered exclusive to a sample when normalized LFQ intensities were obtained for all three replicates in a given sample. A zero value indicates a protein that was absent or undetected in a sample.

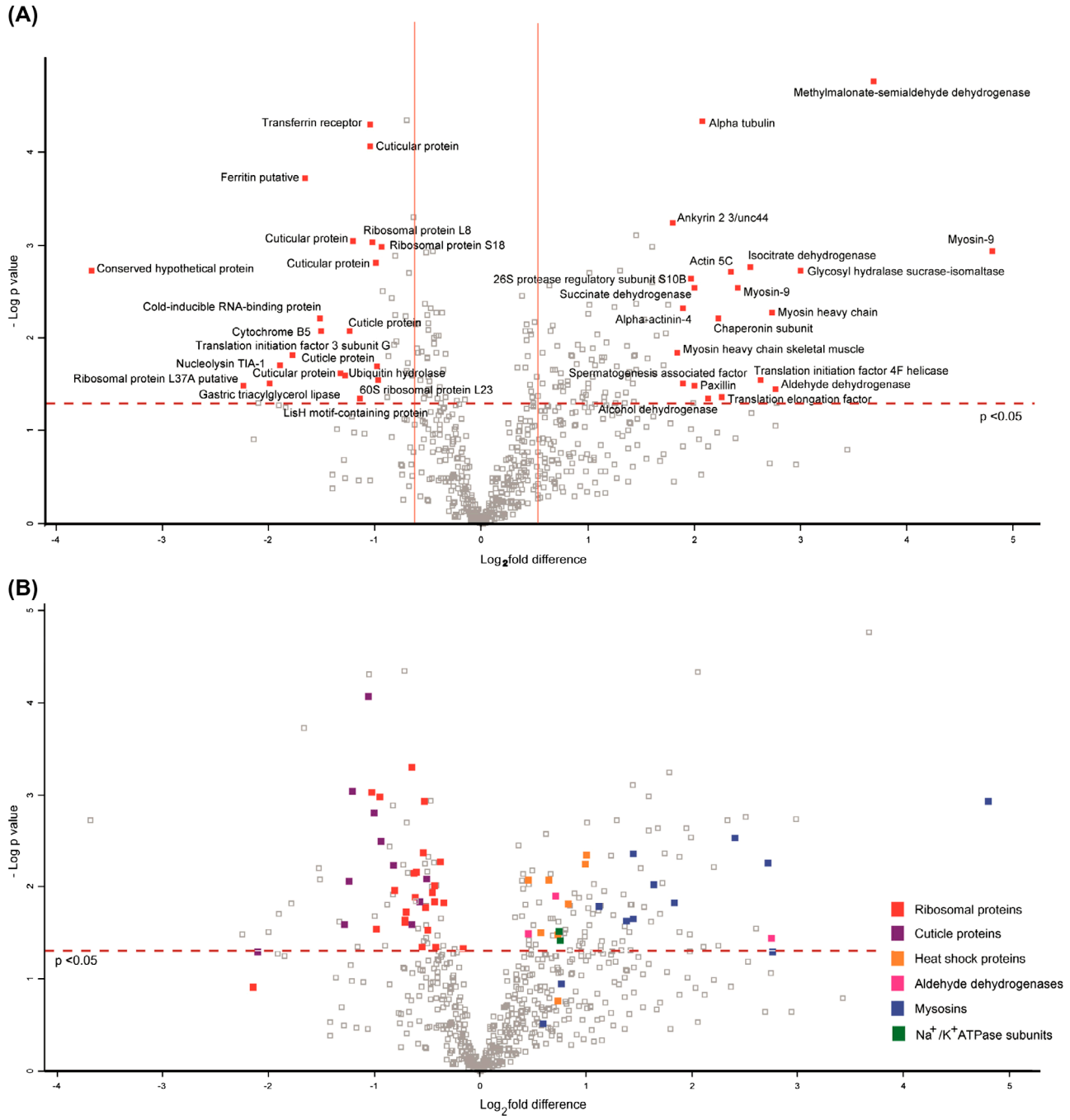


Figure 2. Volcano plot of all identified proteins based on relative abundance differences between sensitive and resistant varroa. (A) Volcano plot of protein intensity difference (−log2 mean intensity difference) and significance in differences (−log *P*-value) based a two-sided *t*-test. Proteins above the line are considered statistically significant (*p* value < .05) and those to the right and left of the vertical lines indicate fold changes ≥1.5 in resistant and sensitive mites, respectively. The top 20 differentially abundant proteins are annotated. (B) Same volcano plot indicating proteins of similar expression profile potentially associated with Bayvarol® resistance, annotated according to biological process ontology term.

insecticides (Fragoso et al., 2003; Kostaropoulos, Papadopoulos, Metaxakis, Boukouvala, & Papadopoulou-Mourkidou, 2001) and has been linked to the metabolic resistance to pyrethroids in the spotted mite *Tetranychus urticae* (Stumpf & Nauen, 2002). Pyrethroid exposure leads to oxidative stress by inducing lipid peroxidation and by depletion of reduced glutathione, and superoxide dismutases, catalases and GSTs provide defense against this stress (Nardini, Christian, Coetzer, & Koekemoer, 2013).

A substantial number of proteins from the dehydrogenase superfamily were present at much higher levels in the Bayvarol® resistant mites with some also detected only in the resistant mites, with their detection

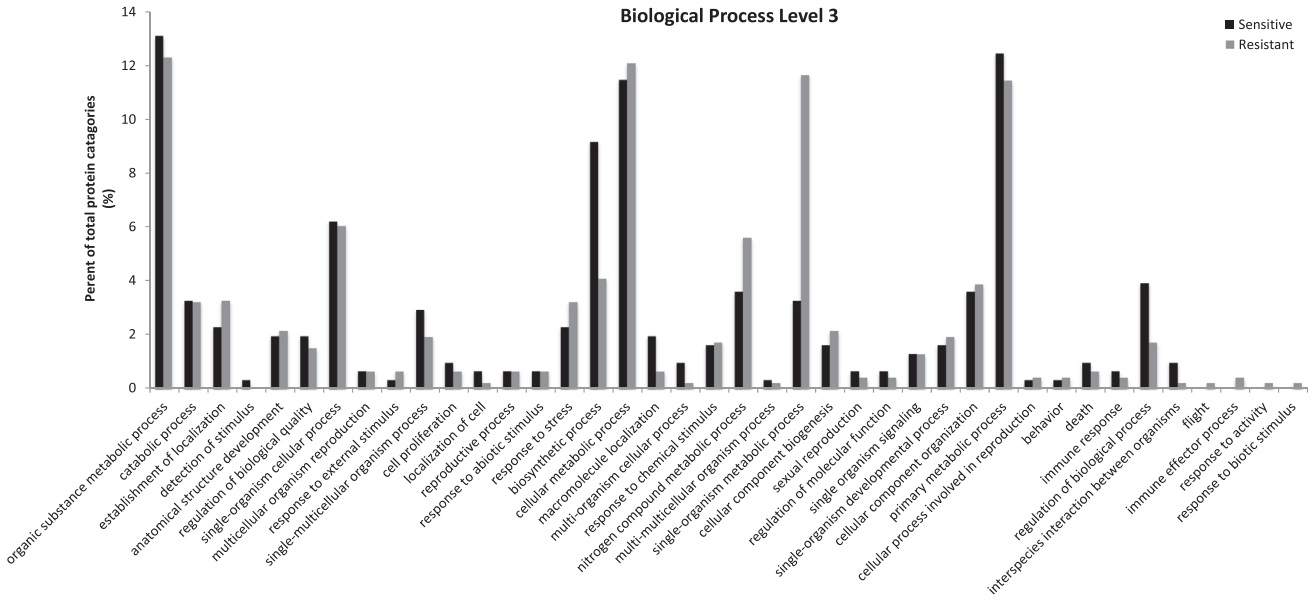


Figure 3. Bar chart highlighting the difference in protein differences involved in selected biological processes. Comparative bar chart of level three biological process annotation for resistant and sensitive mite proteins based on percentage proportion of the total proteins found in the proteomic profile.

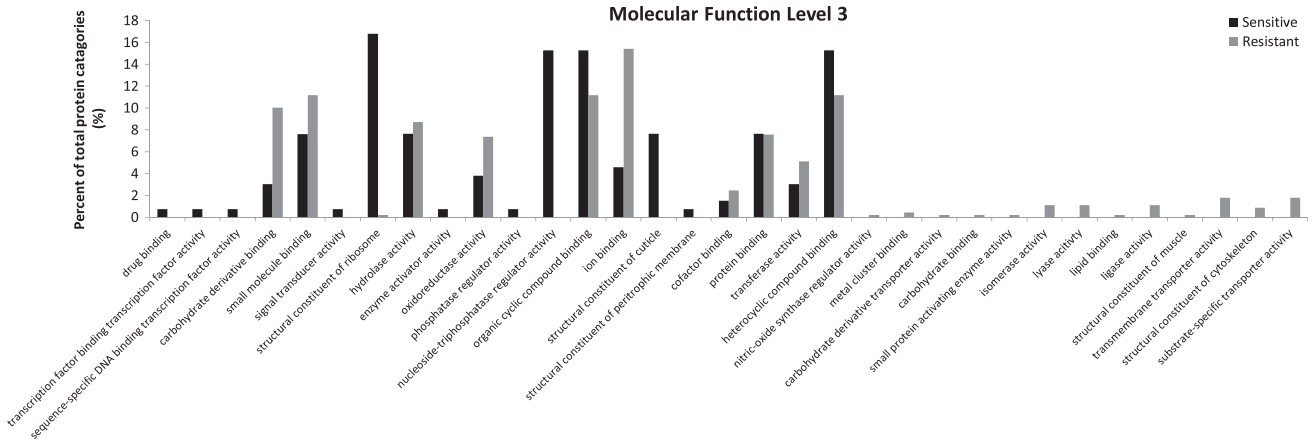


Figure 4. Bar chart highlighting the difference in protein differences involved in selected molecular function. Comparative bar chart of level three molecular function annotation for resistant and sensitive mite proteins based on percentage proportion of the total proteins found in the proteomic profile.

completely absent in the sensitive mites. This group of dehydrogenases included two alcohol dehydrogenases (5-fold and 1.6-fold increase, Table 2), retinal dehydrogenase (6.1-fold increase, Table 1), methylmalonate semi-aldehyde dehydrogenase (12.5-fold increase, Tables 2 and 3), isocitrate dehydrogenase (12.5-fold increase, Tables 2 and 3) and methylenetetrahydrofolate dehydrogenase (5.4-fold increase, Table 2). Dehydrogenases have been previously linked to insecticide resistance due to their ability to exhibit esterase-like activity (Kedishvili, Goodwin, Popov, & Harris, 2000). Variations in dehydrogenase expression has been observed in pyrethroid resistant insects, such as the increase of aldehyde dehydrogenase in *Anopheles gambiae* after exposure to permethrin (Vontas, 2001) which is similar to the increase observed here in Bayvarol® resistant mites. An additional study on pyrethroid/DTT resistant *A. gambiae* reported similar changes in the levels of dehydrogenases with two members of the family showing higher levels in resistant mosquitoes at both the genetic and proteomic level (Lumjuan, Wicheer, Leelapat, Choochote, & Somboon, 2014). Methylmalonate-semialdehyde dehydrogenase was expressed exclusively in the proteomic profile of the Bayvarol® resistant mites (Table 3). It exhibits esterase activity, typical of all dehydrogenases, but is unique among the members of this family because coenzyme A is required for the reaction and a CoA ester is produced (Kedishvili et al., 2000). The presence

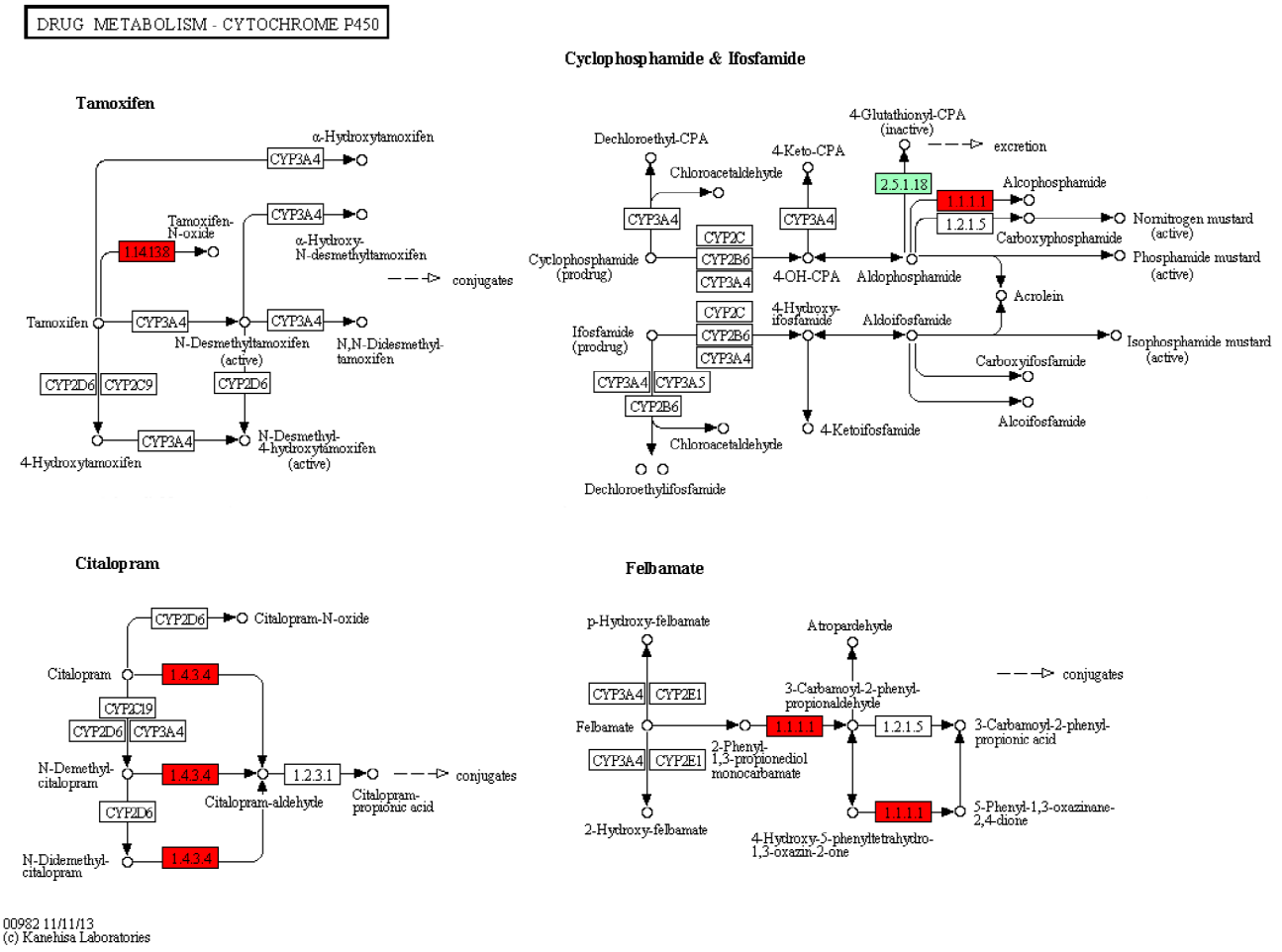


Figure 5. KEGG analysis of a drug detoxification pathway showing a number of proteins increased in abundance in Bayvarol® resistant varroa. Various parts of the cytochrome P450 drug metabolism pathway that were found to be present at significantly higher levels in mites that showed resistance to Bayvarol® (Red indicates up regulation) are given.

of these dehydrogenases in the resistant mites at such increased levels of abundance indicates that they may be playing a role in conferring the resistant phenotype. The combined elevated levels of these proteins could potentially allow for a more efficient means of xenobiotic degradation.

Certain components of the cytochrome P450 detoxification system were also altered in abundance (Figure 6). P450 components have previously been linked with metabolic resistance of various insecticides including pyrethroids (Puinean et al., 2010; Scott, 1999; Zhang & Scott, 1996). A large increase in NADH-Cytochrome b5 reductase was observed and this has previously been associated with resistance in cypermethrin resistant house flies (Zhang & Scott, 1996). Cytochrome C oxidase subunit and cytochrome C reductase subunit were also increased in abundance in the resistant mites, however it was not deemed significant (Figure 6). Cytochrome b5 was significantly reduced in abundance in the resistant mite population, and a small non-significant reduction was also observed in cytochrome C oxidase. This varied expression in the cytochrome subunits could implicate altered oxidative detoxification in the resistant phenotype, as pyrethroids are known to cause oxidative stress (Scott, 1999).

An additional protein, flavin-associated mono-oxygenase was expressed 2.8-fold higher in resistant mites compared to those sensitive to Bayvarol® (Table 2). Flavin-associated mono-oxygenase is strongly linked to the detoxification of xenobiotic substances in a wide range of organisms (Berenbaum & Johnson, 2015; Cashman, Perotti, Berkman, & Lin, 1996; Krueger & Williams, 2005; Naumann, Hartmann, & Ober, 2002). Its detoxification characteristics have been linked to the development of resistance to certain chemical pesticides including pyrethroids (Dawkar et al., 2013; Lumjuan et al., 2014; Tian, Sun, & Su, 2014). Thioredoxin reductase-2 has a role in the oxidoreductase response of a cell to stress and was increased by 5.9-fold in resistant mites compared to the levels in susceptible mites (Table 1). Thioredoxin reductase was present at higher levels of abundance in resistant *A. gambiae* as part of an overall up-regulation of a number of detoxification proteins (David et al., 2005).

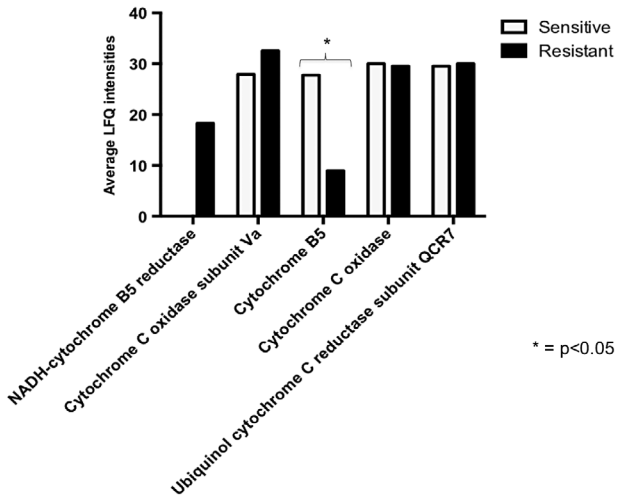


Figure 6. Average LFQ intensities of a number of cytochrome p450 components differentially expressed in the proteomic profile of Bayvarol® sensitive and resistant mites. A number of cytochrome P450 components that were found to be differentially expressed between the sensitive and resistant *V. destructor* mites.
Note: *Indicates a statistically significant change in expression levels at $p < .05$.

The elevated levels of these detoxification enzymes in resistant *V. destructor* could confer a greater ability to withstand the oxidative stresses caused by the chemical and lead to the organism becoming more tolerant to the effects of the chemical.

A number of cuticle proteins were at lower levels of abundance in the proteomic profile of the resistant mites (Figure 2(b)). One possible means by which organisms are thought to become resistant is by alteration of the route of entry of the chemical such as a thickening or hardening of the cuticle or other epithelial barriers (Hemingway et al., 2004). A number of cuticular proteins were present in lower amounts in resistant mites (Figure 2(b)), which partly contradicts the trend observed in previous reports (Puinean et al., 2010; Silva, Jander, Samaniego, Ramsey, & Figueroa, 2012; Vontas et al., 2005; Wang et al., 2015). The protein that showed the lowest abundance in the resistant mites, exclusively expressed in the profile of sensitive mites, was a conserved hypothetical protein (Table 3). This protein is possibly a cuticular protein as it shows homology to *gasp* from *Drosophila melanogaster*, a chitin binding cuticle constituent. Previous research has demonstrated that a down-regulation of some cuticle proteins alongside an up-regulation of others does occur, suggesting that an alteration in cuticle composition may occur, leading to decreased rates of entry of the chemical (Vontas et al., 2005; Wang et al., 2015). However, no cuticle proteins were observed here to be of higher abundance in resistant mites which may indicate that the cuticular changes in resistant mites involve the removal of certain proteins without their replacement.

A number of proteins with structural roles were found to be significantly increased in mites that showed resistance to Bayvarol® (Figure 2(b)). Some of these were also present exclusively, indicating presence and absence in all three replicates of the resistant and sensitive mites, respectively (e.g., coronin, alpha tubulin) (Table 4). Proteins involved in cytoskeleton structure have previously been shown to be changed in abundance in deltamethrin resistant mosquitoes (Bonizzoni et al., 2012). A large group of myosins were also found at much higher levels in resistant mites and some were exclusively present in the resistant mite population. Similar results have previously been reported in deltamethrin resistant *Culex pipiens pallens*, where it has been shown that the over-expression of a light chain regulatory myosin confers resistance in a cell line derived *Aedes albopictus* (Yang et al., 2008). It is not unreasonable to postulate that proteins associated with altering the structure of cells and cell junctions may contribute to resistance to a chemical, in particular chemicals in which the mode of entry is through the exoskeleton or the digestive tract such as the pyrethroid insecticides.

A number of other groups of proteins were also differentially expressed in the Bayvarol® sensitive and the resistant *V. destructor* including a group of ribosomal proteins the majority of which were present at a lower levels in the resistant mites (Table 3). Deltamethrin resistant mosquitoes demonstrated a 23-fold increase in the expression of the L39 gene (Tan et al., 2007), but this was not the case in the Bayvarol® resistant mites studied here. Yu et al. (2014) demonstrated that another ribosomal protein s29 regulates metabolic resistance by binding to CYP6N3, so perhaps the unbound versions of certain ribosomal proteins were more abundant during proteomic analysis of the sensitive mites.

A number of proteins involved in ion transport were also greater in abundance in resistant mites. NADH ubiquinone oxidoreductase NDUFS2/49 kDa subunit is thought to act as a proton pump for sodium and potassium and was present at higher levels (1.9-fold) in resistant mites (Brandt et al., 2003) (Table 2). Mosquitoes that were resistant to pyrethroids had similar elevated levels of this protein post-treatment with permethrin (Vontas et al., 2005). E1-E2 ATPase putative, vacuolar H^+ – ATPase V1 sector subunit B, Na^+/K^+ ATPase beta subunit and alpha subunit were all expressed at higher levels in the proteome of resistant mites (2, 1.9, 1.7, and 1.7-fold, respectively) (Table 2). These proteins act as proton pumps for detoxification of the cell, and higher levels of these proteins in the cell could help combat the extra ions due to malfunctioning sodium transport channel (Emery, Billingsley, Ready, & Djamgoz, 1998). As pyrethroid treatment results in the influx of ions into the cell due to the inhibition of the sodium channels in the membrane, perhaps the extra abundance of proteins involved in alternative proton pumping mechanisms observed in resistant mites presented here counteract this influx.

Pyrethroids are metabolized by hydrolysis of ester bonds, oxidation at the acid and alcohol moieties, and several conjugation reactions such as hydrophilic and lipophilic conjugates (Mikata, Osobe, & Kaneko, 2011; Strachecka, Borsuk, Olszewski, Paleolog, & Lipiński, 2013). Here, a number of proteins involved in hydrolysis (processing peptidase beta subunit putative, acetyl-CoA hydrolase putative, aminoimidazole-4-carboxamide ribonucleotidetransformylase/IMP were present in higher levels in pyrethroid resistant mites (Table 2) and these could be potential effectors of the more efficient breakdown of the chemical in resistant mites. These could potentially be leading to the phenotype observed in the resistant mites by helping to overcome the oxidative stress induced by the synthetic pyrethroid. In addition, the ion transport and transferase proteins were also expressed at higher levels in the resistant mites and this could also be contributing toward the resistant phenotype by helping to combat the influx of ions induced by the pyrethroid.

The work presented here offers novel insights into the metabolic resistance mechanisms that could play a role in the resistance of *V. destructor* to Bayvarol® and may open the possibility of identifying targets to circumvent this phenotype.

Disclosure statement

No potential conflict of interest was reported by the authors.

Funding

Carla Surlis is the recipient of an Irish Research Council scholarship. This work was part funded by a grant from the Federation of Irish Beekeepers Associations. The Qexactive mass spectrometer was funded under the SFI Research Infrastructure Call 2012 [grant number 12/RI/2346 (3)].

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