Mapping, Characterising and Targeting Acaricide Resistance in the Poultry Red Mite, *Dermanyssus gallinae*.

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Abstract

Dermanyssus gallinae represents a significant threat to poultry production in the UK and control is typically achieved through the use of acaricides, but farmers are facing increasing incidence of pesticide resistance. This must be managed through considered application of acaricides and to facilitate this, a map of *D. gallinae* perceived resistance has been created, to which future resistance can be compared. Toxicity testing has shown no geographical correlations in resistance, suggesting farms need testing on a per farm basis to provide a best practice recommendation. Additionally, a greater understanding of acaricide detoxification mechanisms is required. Research indicates that functional modification of P450s is responsible for the occurrence of pesticide resistance in many arthropods. In order to characterise resistance mechanisms, transcriptome data has been analysed and a database of putative pesticide metabolising sequences has been created. An attempt to develop a method for microsome preparation from *D. gallinae* was made for use with Activity Based Probes to identify pesticide metabolising protein sequences in the future. As well as identifying P450s, cytochrome b_5 and P450 oxidoreductase required identification. P450 oxidoreductase has been putatively identified using sequence data analysis but not yet successfully isolated. However, cytochrome b_5 from *D. gallinae* along with *Rhipicephalus microplus* and *Ixodes scapularis* has been successfully amplified, expressed and purified. A baculovirus expression vector has been created to express *D. gallinae* cytochrome b_5, which showed no effect on pesticide metabolism when amplified alone. A Glutathione S-transferase gene has also been cloned into an expression vector to compare the different mechanisms of metabolism. This will allow future links between *D. gallinae* genetics and resistance to be established, facilitating real-time tailoring of treatment programmes to ensure maximum efficacy.

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Abbreviations

% – Per cent

µl – Micro litre

AChE – Acetylcholinesterase

ALA – 5-Aminolevulinic acid hydrochloride

ART – Artemisinin

BCA – Bicinchoninic acid assay

BLAST – Basic Local Alignment Search Tool

BLASTn – Nucleotide BLAST search

BLASTp – Protein BLAST search

tBLASTx – Translated nucleotide BLAST search

bp – Base pair

$CaCl_2$ – Calcium Chloride

cDNA – Complimentary Deoxyribonucleic Acid

CDNB – 1-Chloro-2,4-dinitrobenzene

CDS – Coding Sequences

CHAPS – 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate

CO – Carbon Monoxide

CYP – Cytochrome P450

Cytb5 – Cytochrome b5

DDT – Dichdichlorodiphenyltrichloroethane

DEM – Diethyl maleate

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic Acid

dNTPs – Deoxynucleotide solution mix

EDTA – Ethylenediaminetetraacetic acid-

EST – Esterase

FAD – Flavin Adenine Dinucleotide

FMN – Flavin Mononucleotide

GABA – Gamma-Aminobutyric Acid

GSP – Gene Specific Primer(s)

GST – Glutothione-S-Transferase

HPRA – Health Products Regulatory Authority

IPTG – Isopropyl β-D-1-thiogalactopyranoside

IS – *Ixodes scapularis*

k – 1000 (one thousand)

kb – Kilo base

kDa – Kilo Dalton

L – Litre

LB – Luria Bertani

$MgCl_2$ – Magnesium Chloride

ml – Millilitre

MTT– 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

NCBI – National Center for Biotechnology Information

nM – Nanomole

OD – Optical density

P450 – Cytochrome P450

PBO – Piperonyl butoxide

PCR – Polymerase Chain Reaction

PDPs – Pland Dervived Products

POR – Cytochrome P450 Oxidoreductase

PRM – Poultry red mite

RACE – Rapid Amplification of cDNA Ends

RM – *Rhipicephalus microplus*

RNA – Ribonucleic Acid

rpm – Revolutions per minute

SDS-PAGE – Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis

Sf9 – *Spodoptera frugiperda* cell line

SNP – Single Nucleotide Polymorphism

TB – Terrific broth

TRIS – Trisaminomethane

TPP – Triphenyl phosphate

tRNAs –Ttransfer Ribonucleic Acids

VGSC – Voltage-gated sodium channel

xg – x gravity

Acknowledgements

Firstly I would like to thank my supervisors Robert Finn, Olivier Sparagano and David George, for giving me the incredible opportunity to do this PhD and for their endless help and support along the way.

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Declaration

I declare that the work contained in this thesis has not been submitted for any other award and that it is all my own work. I also confirm that this work fully acknowledges opinions, ideas and contributions from the work of others.

Any ethical clearance for the research presented in this thesis has been approved. Approval has been sought and granted by the Faculty Ethics Committee on 26/11/2014.

I declare that the Word Count of this Thesis is 44,390.

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Date: 02/01/2019

Signed:

Chapter 1: Introduction

1.1 Economic and Medical Importance of Acari

The acari are a subclass of small arachnids which comprises mites and ticks (class Arachnida, taxon: Acari) and there are more than 45,000 species of mites and ticks in the world. Acari are a form of arthropod but have not shared a common ancestor with insects for around 500 million years (Ullmann et al., 2005). Acari are incredibly diverse in both form and life strategies and live in almost every inhabitable place on earth, including fresh water and sea water (Krantz and Walter, 2009; Walter and Proctor, 1999). They have a vast amount of plant and animal hosts that include many agricultural, domestic and human pests, hence they have a large influence on human life (Dabert et al., 2010; O'Reilly et al., 2014).

Acari have jointed legs and a chitinous exoskeleton with an open circulatory system, ventral nerve cord, alimentary canal and striated muscles. Unlike insects and other arthropods, they do not have antennae or mandibles. The body of acari is divided into the gnathosoma and idiosoma, which contain various structures in different acari species (Dhooria, 2016). Acari are adapted to feed via haematophagy, using specially adapted mouth parts to feed on hosts such as birds and mammals (Bellgard et al., 2012).

Both tick and mite species attack humans and animals and cause damage through direct feeding on the host and through the transmitting of disease. Many tick species are of medical importance and Table 1.1 summarises some of the infections transmitted to humans by tick species. *Ixodes* ticks are shown to cause human ehrlichiosis, tick borne encephalitis, Q fever, tularaemia, Lyme disease and tick paralysis. Lyme disease is caused by *Borrelia burgdorferi* and occurs in

27 European countries as well the USA, Canada, Africa and Australia – in eastern USA its vector is *Ixodes scapularis.* Lyme disease is the most common reportable vector borne zoonosis in the USA, with nearly 30,000 cases of Lyme disease confirmed in 2007, up from 10,000 in 1991, and being principally transmitted by birds (Service, 2012; Dumic and Severnini, 2018). Lyme disease is a multisystem infectious disease which affects the skin, joints, nervous system and heart, and it can lead to long term sequelae if left untreated (Smit and Postma, 2015; Stanek et al., 2011). One recent study found the cost of Lyme disease to be between $712 million and $1.3 billion each year, and this is expected to rise as incidence of the disease increases, presenting high economic and medical burdens of *I. scapularis* as a vector of disease (Adrion et al., 2015).

Table 1.1: Summary of vector-borne diseases transmitted to humans. *Summary of diseases transmitted to humans and the infective agent as well as principle tick vectors and main reservoir hosts. Adapted from Service (2012).*

Disease	**Infective agent**	**Principle tick vectors**	**Main reservoir hosts excluding ticks**
Tick-borne encephalitis	*Flavivirus*	*Ixodes ricinus, I. persulcatus*	Rodents, insectivores
Omsk haemorrhagic fever	*Flavivirus*	*Dermacentor reticulatus*	Muskrats, water voles
Kyasanur Forest disease	*Flavivirus*	*Haemaphysalis spinigera, H. turturis*	Monkeys, shrews, rodents
Crimean-Congo haemorrhagic fever	*Nairovirus*	*Hyalomma marginatum*	Hares, cattle, goats
Colorado tick fever	*Coltivirus*	*Dermacentor andersoni*	Rodents, rabbits
Rocky mountain spotted fever	*Rickettsia rickettsii*	*Dermacentor, Amblyomma and Rhipicephalus* species	Rodents
Mediterranean spotted fever	*Rickettsia conorii*	*Rhipicephalus sanguineus*	Rodents, dogs
African tick-bite fever	*Rickettsia africae*	*Amblyomma* species	Rodents, cattle
Q fever	*Coxiella burnetii*	Ixodid species	Sheep, goats, cattle, rodents
Human ehrlichiosis	*Ehrlichia chaffeensis*	*Amblyomma* and *Ixodes* species	Deer, rodents
Lyme disease	*Borrelia burgdorferi*	*Ixodes ricinus, I. scapularis, I. pacificus*	Birds, rodents
Tularaemia	*Francisella tularensis*	Ixodid species	Rabbits, hares, deer, beavers
Tick paralysis	*Tick toxins*	*Ixodes* and *Dermacentor* species	Not caused by pathogen

Another genus of interest is *Rhipicephalus,* specifically *Rhipicephalus microplus,* also known as the southern cattle tick. The southern cattle tick is a transmitter of babesiosis (caused by protozoal parasites *Babesia bigemina* and *Babesia bovis*) and anaplasmosis (caused by *Anaplasma marginale*). Tick borne diseases, such as babesiosis and anaplasmosis, cost African countries an estimated $5-$6 million per annum, a figure which is expected to be higher when calculated in more up to date studies (McLeod and Kristjanson, 1999; Leger et al., 2013; Nyangiwe et al., 2017). This increase is due to several reasons, key ones being that of climate change and development of pesticide resistance within the species (Stone et al., 2014; Miller et al., 2007).

1.2 Acari of Interest: *Dermanyssus gallinae*

The main acari of interest to this project is *Dermanyssus gallinae* (De Geer, 1778), known as the poultry red mite (PRM). PRM are an ectoparasitic mite which are ubiquitous as a poultry pest throughout the world (George et al., 2015; Kilpinen, 2001). They pose a significant threat to egg-laying hens in Europe, China, Japan and the USA, although northern fowl mite, *Ornithonyssus sylviarum* is more prevalent in the latter country (Sparagano et al., 2009; Wang et al., 2010).

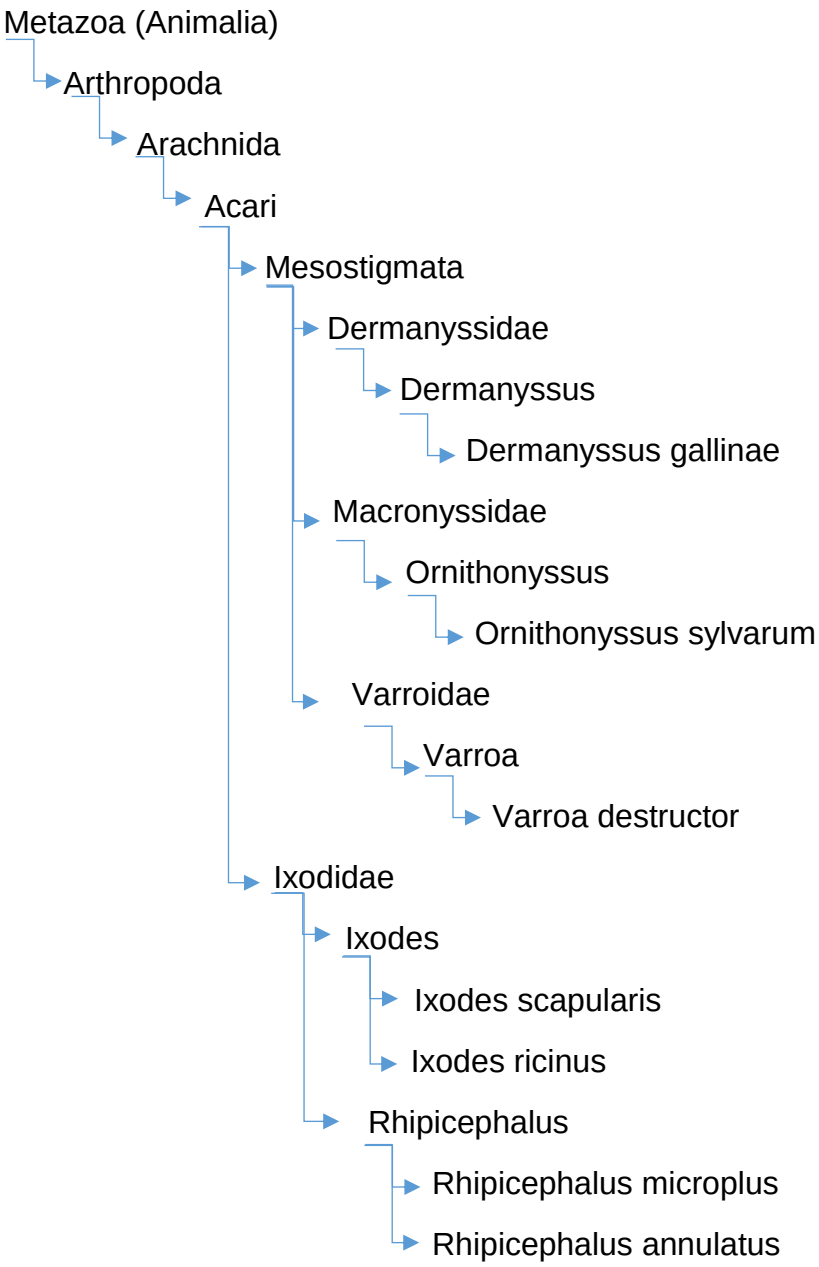


Figure 1.1: Phylogenetic tree showing the taxonomic classification of Dermanyssus gallinae as well as other species of interest. *Taxonomic classification of Dermanyssus gallinae, Ornithonyssus sylvarum, Varroa destructor, Ixodes scapularis, Ixodes ricinus, Rhipicephalus microplus and Rhipicephalus annulatus.*

Dermanyssus gallinae are members of the *Dermanyssidae* family, Figure 1.1, which are characterised by an idiosoma broadly rounded posterior and a second cheliceral article of female elongate which exceeds the basal segment in length.

Dermanyssus gallinae has a single dorsal shield that tapers posteriorly, the chelicerate are long and styliform and the sternal shield has two pairs of setae. A third pair is located posteriorly and is distinctly separate from the others. There is a genitoventral shield which is posteriorly rounded and bears one pair of seta and the anal shield has three setae (Di Palma et al., 2012). *D. gallinae* are a relatively small ectoparasitic mite being around 1.5 mm in length and change colour from a grey to red/brown after feeding on a blood meal (Figure 1.2).

A.

B.

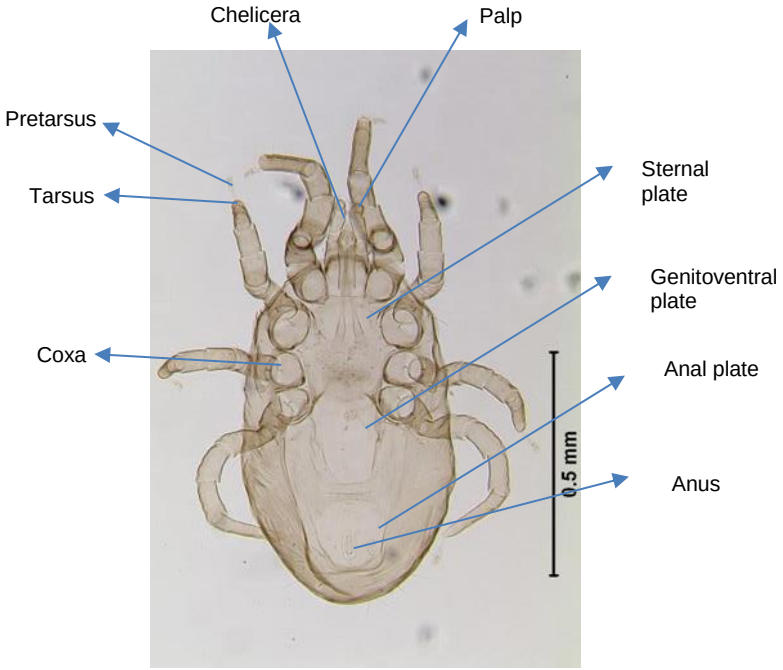


Figure 1.2: External and internal morphology of PRM. *A. Internal mite structures captured from the dorsal side at X 100 magnification on a fed mite (Pritchard et al., 2015). Gnth = gnathosoma (mouthparts), Os = oesophagus, Ca I-III = caeca I-III, Mp = Malpighian tubules, Mg = central midgut. B. External Mesostigmata mite morphology shown with labels detailing key external structures (Buss et al., 2014).*

The PRM digestive tract extends through the oesophagus, midgut and caeca and ends in the hindgut with the majority of blood digestion occurring in the expanded caecal pairings (Ca I-III) and central midgut. Malpighian tubules, involved in nitrogenous waste collection and osmoregulation, elongate along the idiosoma and are connected to the posterior hindgut. PRM body shape gets rounder and larger during blood feeding and the digestive tract completes most of the body cavity when full compared to an unfed mite.

1.3 Poultry Red Mite: Life Cycle and Ecology

The life cycle of PRM is very rapid which contributes to its status as a pest species (George et al., 2015). Development of PRM goes from egg to adult through one larval stage and two nymphal stages in approximately 2 weeks (Figure 1.3) (Sparagano et al., 2014).

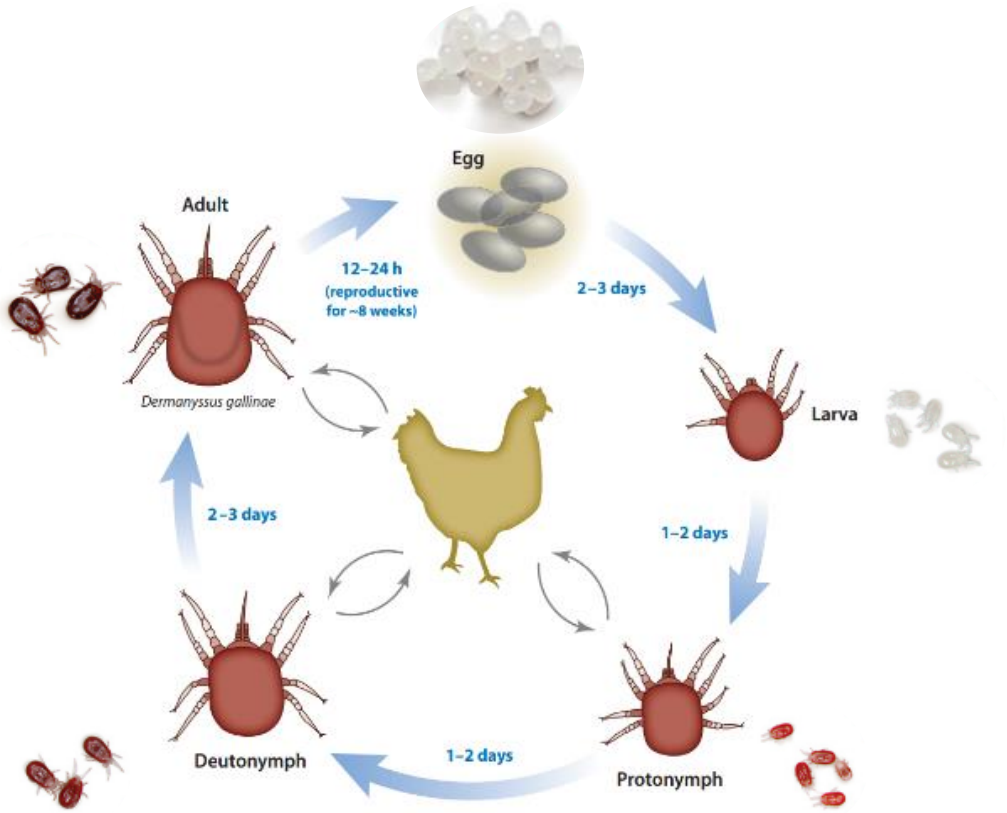


Figure 1.3: Life cycle of poultry red mite. *Life cycle of poultry red mite under favourable conditions. Diagram adapted from Sparagano et al. (2014).*

The process of development from egg to adult, as stated above, typically takes two weeks to complete, although it has been shown to take half this time at temperatures of between 25-37 °C, the most favourable temperature range for juvenile development (Maurer and Baumgartner, 1992). Females lay eggs in clutches of 4-8 and can lay up to eight clutches in between blood feeds. Typically, females will lay around 30 eggs in a lifetime in refugia where larva can remain without feeding before their first molt. High temperatures (10-35 °C), like those found in poultry housing, as well as high humidity (over 70 %) can facilitate the rapid reproduction of PRM which leads to the swift doubling of populations of infestations in egg laying facilities (Maurer and Baumgartner, 1992; Nordenfors et al., 1999). A more recent study has supported this, finding that the growth rate of the mite population increases 2 % for every 1 °C temperature increase above 20 °C (Mul et al., 2017). Densities of PRM often reach 50,000 mites per bird but have been reported as high as 500,000 mites per bird in more severe infestations (Kilpinen et al., 2005).

Host specificity is hard to define in mites due to them being opportunistic and potentially having multiple hosts during post-embryonic development (Roy et al., 2009). PRM have a variety of host species but are widely reported to be avian specific, with over 30 species of wild birds reported to being infested with PRM (Roy and Chauve, 2007). Poultry are the natural host of PRM, with egg laying facilities prone to large infestations due to the slow turnover of flocks (often exceeding 1 year) and ideal poultry house conditions. PRM are often present year round but infestations can peak in hot and humid seasons (Nordenfors et al., 1999; Mul et al., 2017). Even when flocks are changed and poultry houses are left empty, PRM can survive up to 8 months without blood feeding, meaning they can infect new flocks after long periods of time (Chauve, 1998).

PRM has been shown to change more readily between avian hosts than other species within the same genus. When removed from the hen host, PRM accepted canaries as a new avian host, something *Dermanyssus longipes* could not do (Roy et al., 2009). Interestingly, reports of PRM infestations on non-avian hosts are becoming more frequent, which is indicative of PRM's ability to adapt and its potential to be a human and mammalian pest (Abdigoudarzi et al., 2014; Sengul et al., 2017; Gavrilovic et al., 2015).

PRM spend most of their life away from their host and only parasitize birds in the darkness to feed. They can be found in all production types, ranging from small numbers of home reared birds to large barn systems. Mites generally find refuge in poultry housing and prefer to live in cracks or crevices (Figure 1.4), aggregating together in response to both thigmokinesis and pheromone cues (Entrekin and Oliver, 1982; Koenraadt and Dicke, 2010; George et al., 2015).

A. B.

Figure 1.4: Infestation with PRM. *A= red mite clustering on a cage in a laying farm. B= Red mites in straw litter from laying hen housing unit. Photos taken from Flochlay et al. (2017).*

In battery cage systems mites are often found under the conveyor belts of eggs and under cage supports but, in free range systems, more crevices are present

which often makes the infestation harder to control. Mites can be found under flooring systems, hidden in joints of nest boxes and perches, beneath troughs and in crevices in the walls of the housing (Chauve, 1998). Increasing mite infestation numbers can be, in part, attributed to the recent changes in legislation of housing systems in hen husbandry within EU countries (Directive 1999/74/EC). This directive has prohibited the use of traditional cages for poultry and has led to the increase in housing systems which consist of more complex environments, in the name of bird welfare. This increase in complexity of housing has allowed for increased areas of mite refuge and hence has intensified the issue of PRM infestations within EU countries (Flochlay et al., 2017).

Adult female PRM locate hosts using temperature stimuli, chemical signals, vibrations and carbon dioxide levels (Kilpinen, 2005; Kilpinen and Mullens, 2004; Zeman, 1988). Once the PRM has located a bird, it will only feed for 0.5-1.5 hours before returning to the refuge of the poultry housing. This will only happen every 2-4 days and is usually done under darkness, making PRM a difficult pest to treat (Nakamae et al., 1997). Adding to this, male PRM feed only intermittently and larvae do not feed (Chauve, 1998).

Kilpinen (2005) showed that in high light intensity PRM cease movement in response to CO_2 stimuli, which is an indication that they may have been seen by the host and face being eaten. When vibrations were detected by the mite it continued to move for the duration of the vibration, as the vibration is potentially an indication that the host has moved on and the risk of being eaten has diminished. In low light intensity, when mites would be hard to locate by the host, no freeze response was observed and PRM continued to search for the host to feed (Kilpinen, 2005).

1.4 Poultry Red Mite: Geographical Distribution and Prevalence

Incidence of infestations with PRM is on the rise, in part due to the vast poultry farming across the world with millions of birds being raised annually (Table 1.2). This prevalence data will need updating for the UK and other European countries, as traditional metal caging has been prohibited, and prevalence of PRM in enriched systems is expected to be higher (Figure 1.5). Infestations with PRM have been found in all countries where egg laying facilities have been sampled and in the UK between 60-87.5 % of egg laying facilities are expected to be infested (Fiddes et al., 2005; Guy et al., 2004).

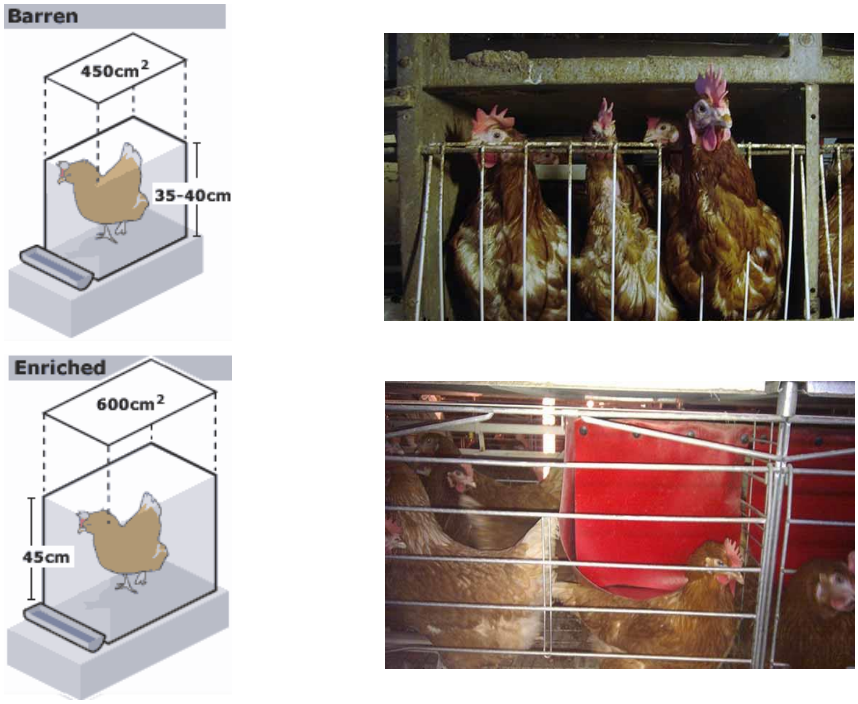


Figure 1.5: Comparison between traditional barren cages and enriched cages. *All cages must include access to fresh water and adequate food. Cages have a sloping floor to allow eggs to roll out and several hens may be kept in one cage. Cage size indicated space per hen when four or more hens are kept in one cage. Enriched cages also include 150cm^2 nest box space as well as litter, perches and claw trimming devices. Image adapted from BBC (2002); Daniels (2015) and Barlow (2018).*

In a more recent report, it is suggested that 83 % of European farms are infested, with PRM prevalence reaching as high as 94 % in The Netherlands, Germany and Belgium (Mul, 2013). Limited studies have been completed on the prevalence of PRM worldwide and more surveys are required to provide a complete picture of geographical distribution of the pest species. Prevalence in industrial poultry farms in Tunisia was measured and 28 egg laying facilities were tested with a 34 % prevalence of PRM, however this study was only completed in one region of Tunisia (North East) and a systematic survey was recommended (Gharbi et al., 2013). Prevalence data of PRM in other European countries is sparse or dated, for example data for Sweden was last recorded in 1995, finding 33 % of deep litter flocks to be infected with PRM upon inspection (Hoglund et al., 1995). A more recent study in Egypt found 40% of samples obtained were confirmed as *Dermanyssus gallinae*, however samples were only taken from three farms and hence true prevalence is unlikely to be represented (Eladl et al., 2018). Larger surveys have been completed in China, with 833 respondents sending PRM samples to the lab in which 64.1 % contained *Dermanyssus gallinae* (Wang et al., 2010).

Table 1.2: Key data for poultry production and PRM prevalence. *Table shows countries from which information is known for PRM prevalence, the egg production (Tonnes) per country and the number of birds in each system (1,000 hens) (FAOSTAT, 2016). PRM prevalence is listed per system where information is available. References for PRM prevalence have been provided.*

Country	Egg production (Tonnes), 2017	Enriched cages (1,000 hens)	Barn (1,000 hens)	Free range (1,000 hens)	PRM prevalence (%)	Reference
China	31,338,856	1,260,000	126,000	14,000	64.1 (caged)	(Wang et al., 2010)
Portugal	141,211	6,764	340	131	95.8	(Waap et al., 2019)
Italy	740,320	40,952	17,008	2,352	74.1 (caged)	(Cafiero et al., 2008)
The Netherlands	719,564	5,103	20,940	6,881	82 (caged) 83 (barn) 78 (organic)	(Mul et al., 2010)
UK	752,000	16,225	2,088	18,313	87.5 caged	(Guy et al., 2004)
Sweden	137,762	1,721	4,450	882	4 (caged) 67 (backyard) 33 (deep litter)	(Hoglund et al., 1995)
Poland	594,560	32,905	3,915	829	100	(Cencek, 2003)
Romania	314,893	4,987	1,522	80	100 (battery-cages) 57.14 (deep litter)	(Gruianu et al., 2018)
Tunisia	107,100	-	-	-	34	(Gharbi et al., 2013)
Iran	782,000	75,000	0	0	39.3	(Yakhchali et al., 2013)
Kenya	79,389	-	-	-	60 (free range)	(Mungube et al., 2008)
Turkey	1,205,075	65,000	0	0	72.39 (backyards)	(Konyali et al., 2018)
Kosovo	33,402	-	-	-	50	(Hamidi et al., 2011)

Only two surveys of mite prevalence have been completed in the United Kingdom. One survey had a response rate of 60 %, with 29 total farms being

surveyed which had a prevalence of 87.5 % and had a significantly higher level of both nymph and adult mites in free range systems compared to barn or caged systems. However, the survey was limited due to the farms being surveyed only from the North of England and being outdated as it was completed in 2004 (Guy et al., 2004). A second survey was completed in the UK which had a total of 43 questionnaire responses with over 60 % reported infestations with PRM which also had a higher level of infestation with birds from free range systems (Fiddes et al., 2005). This is of interest as the survey was completed in 2002 and the proportion of birds kept in free range systems has more than doubled in recent years, indicating the need for a more up to date reflection of PRM prevalence in the UK (Figure 1.6).

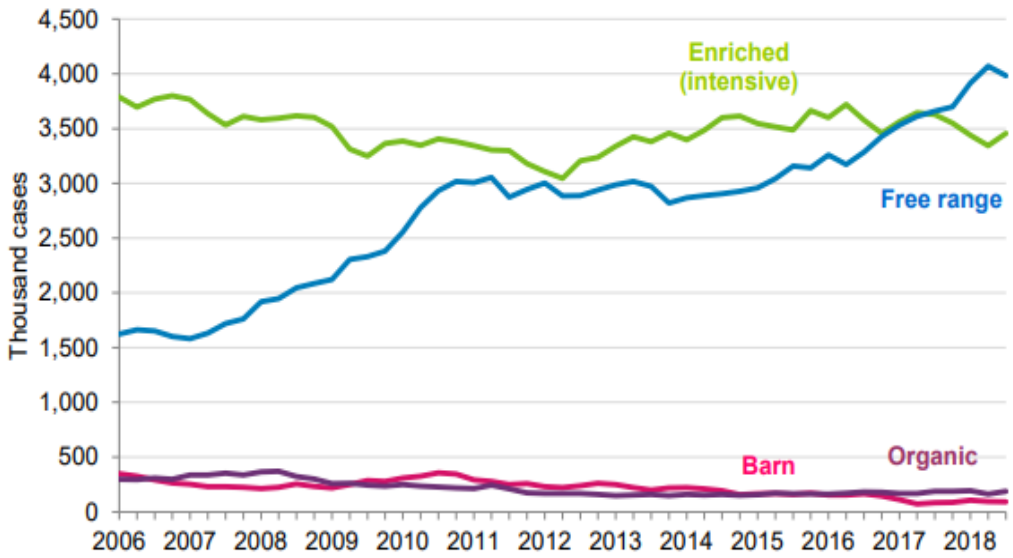


Figure 1.6: UK egg throughput by production method. *Egg throughput is shown per thousand cases and four methods of production are included. Figure taken from Rumsey (2018).*

1.5 Pesticide Classes

The control of PRM is typically achieved using synthetic acaricides. The different pesticides classes which are commonly used to treat infestations with PRM have different mechanisms of action. Both carbamates and organophosphates are acetylcholinesterase (AChE) inhibitors whilst pyrethroids are sodium channel modulators. AChE is an enzyme which hydrolyses acetylcholine, a neurotransmitter. Carbamates and organophosphates inhibit AChE by the modification of the active site of the enzyme, leading to the inhibition of the termination of nerve impulses, causing death in PRM (Figure 1.7) (Aldridge, 1950; David et al., 2013; Antonio-Nkondjio et al., 2016).

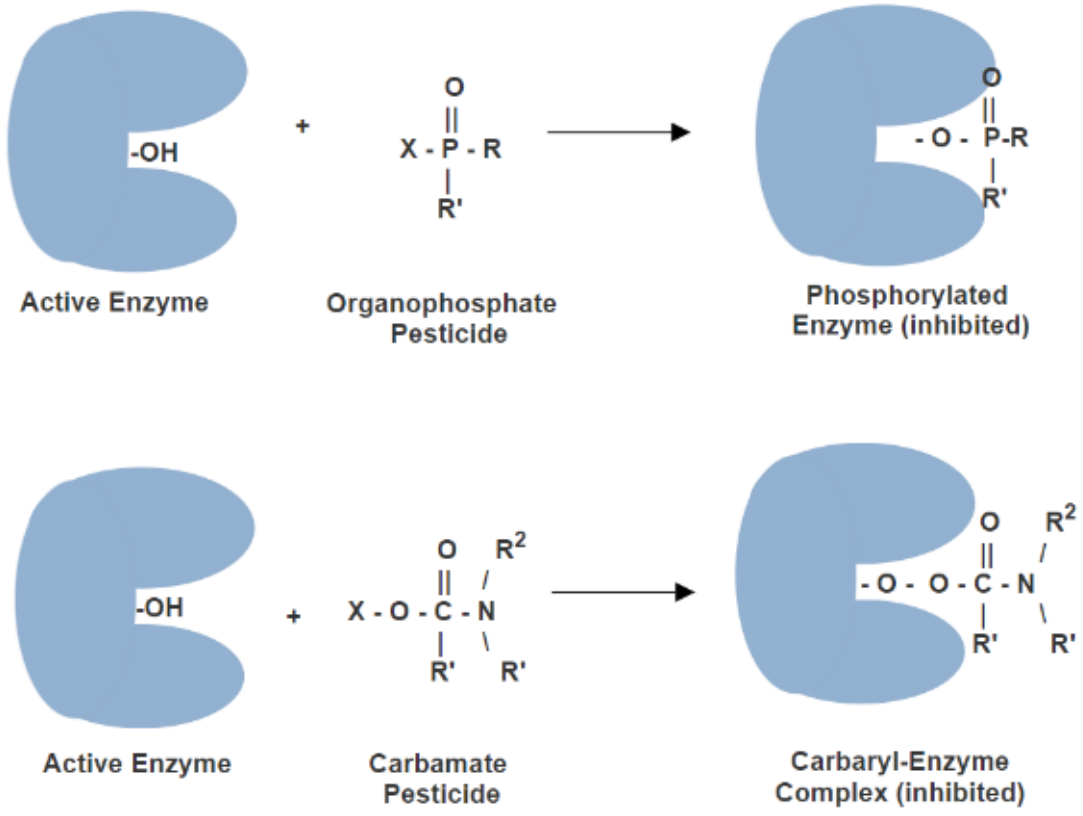
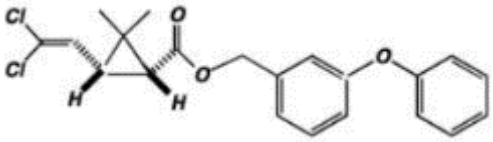


Figure 1.7: Inhibition of AChE. *Mechanism of inhibition of AChE with organophosphate and carbamate pesticides. Figure recreated from Pogacnik and Franko (1999).*

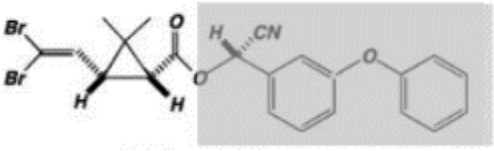
Pyrethroids are synthetic analogues of pyrethrins, which are botanical chemicals derived from chrysanthemum flowers (Ismail et al., 2013). Pyrethroids are structurally diverse and are traditionally split into two classes, type 1 (absence of α-cyano group) and type 2 (presence of α-cyano group) (Figure 1.8).

Type I

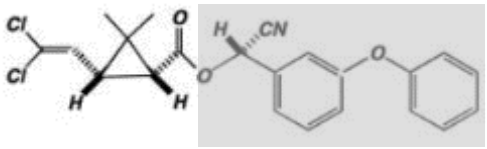


Permethrin

Type II



Deltamethrin



Cypermethrin

Figure 1.8: Structures of pyrethroids. *Structures of Type I and II pyrethroids used in pesticides to treat PRM. The α-cyano moiety, which differentiates between the type I and II structural classes, has been shaded grey in the structure of deltamethrin and cypermethrin. Figure adapted from Soderlund (2012).*

Pyrethroids function by blocking the voltage-gated sodium channels. Voltage gated sodium channels are essential for the initiation of the action potential in neurons and excitable cells (Dong et al., 2014). Pyrethroids bind to receptor sites on the sodium channels which enhances their activation and inhibits deactivation, resulting in prolonged channel opening and leads to death. Insect sodium channels are much more sensitive to pyrethroids than mammalian sodium

channels, which make them a popular active ingredient in pesticides due to their specificity (Du et al., 2013; Soderlund, 2012; Silver et al., 2014; Dong et al., 2014).

1.6 Aims of Study

The poultry red mite is a haematophagous, ectoparasitic pest which presents a significant threat to British poultry production and often leads to heavy production losses through the downgrading of eggs and reduced laying. Despite the importance of the use of acaricides in controlling populations, poultry farmers are faced with several problems such as the growing incidence of pesticide resistance and tighter legislation and restrictions on the chemicals that can be used. This project aims to target the issue of resistance by carrying out a survey of the occurrence of perceived resistance and acaricide usage across the UK, with laboratory toxicity testing independently verifying these observations. Secondly, it aims to isolate the cytochrome P450 complex, the components of which have been shown to be overexpressed in resistant insect strains, and to investigate novel ways of targeting this complex in resistant strains using a baculovirus expression model.

To this end, a survey will be designed to assess the perceived effectiveness of the acaricidal products on the market and this effectiveness will be plotted on a map of the UK, which will be the first of its kind. Poultry red mite will then be collected from farms which show signs of resistance in order to use them in toxicity assays with commercial acaricides, active ingredients of acaricides and with synergists.

Transcriptomic data has been provided by collaborators and will be used for extensive bioinformatics analysis. This aim of this analysis is to identify PRM

accessory protein sequences, using homology to known accessory protein sequences, which can then be purified from PRM for the first time. As well as this, a bank of PRM putative xenobiotic metabolising sequences, also based on homology to known metabolising sequences in other species, can be created which can be compared to future peptide data.

In addition to using the PRM for toxicity testing, they will be used to make a microsome preparation for use in Activity Based Probe (ABP) testing. A protocol will need to be adapted from a protocol used for mosquito preparations, as this technique is novel in PRM. APBs are a chemically modified form of the acaricides of interest which will bind to the specific enzyme involved in the metabolism of the acaricide, without being metabolised. PRM will be homogenised and used to make a microsomal preparation which will first be tested for P450 activity using a resorufin florescence assay.

As well as the isolation of cytochrome P450s, accessory proteins are essential components of the P450 complex. It is therefore important to isolate and express PRM b_5 in order to assess its role in the detoxification of pesticides. As well as expressing PRM b_5 in both soluble and membrane bound form, it will also be expressed from similar pest species *Rhipicephalus microplus* and *Ixodes scapularis.* POR is also a vital part of the P450 complex and attempts will be made to isolate and express this protein from the PRM. To better understand the mechanism of resistance in PRM, any accessory proteins which are isolated and expressed will be cloned into a baculovirus expression system to allow the generation of an *in vitro* cell based assay in which their role in acaricide resistance can be tested. A collaborator based in the Moredun institute has identified PRM GSTs and these will also be used *in vitro* in a cell based assay to compare the role of GST and P450 accessory proteins in the detoxification process.

Chapter 2: A preliminary assessment of the current state of resistance/susceptibility to the active compounds used in Acaricides for Poultry Red Mite, *Dermanyssus gallinae*, in the United Kingdom.

2.1 Abstract

Poultry red mite (PRM) presents a significant threat to British poultry production and control of PRM is typically achieved through the use of synthetic acaricides. However, poultry farmers are faced with the growing incidence of pesticide resistance. To begin combatting the issue of resistance in PRM, a survey of the occurrence of resistance and acaricide usage across the UK was carried out which produced 51 responses from 17 counties across England, Scotland and Wales. The survey results showed a 98-100% PRM prevalence rate and the products used in the survey had low perceived effectiveness in certain regions. Ficam (active ingredient of bendiocarb) scored 5/10 in the East Riding of Yorkshire but scored 10/10 in Dumfries and Galloway (scores could not be statistically analysed due to only receiving one response per county. The variability of responses shows the lack of an overall effective product on the market. The highest average perceived effectiveness score for a product was grease (9/10) which was notable due to its mechanical action and the inability for PRM to form resistance to it. A selection of individual farms were chosen for toxicity testing which indicated widespread resistance to specific commercial products and their actives, such as Fendona which scored and average of 19.76% mortality across the 6 farms tested and Milben Ex which scored 21.56% (both products contain cypermethrin as the active ingredient) compared to Perbio choc (active ingredient permethrin) which scored and average of 99.7% mortality. As well as toxicity testing, synergists were tested on PRM from two farms, along

with the active ingredients in order to improve efficacy of the actives. Permethrin and piperonyl butoxide significantly increased mortality compared to permethrin and diethyl maleate ($P = 0.0461$) and permethrin and triphenyl phosphate ($P = 0.0253$). Individual farm testing is required to show a full picture of resistance and to allow for best practice recommendations.

2.2 Introduction

The poultry red mite (PRM), *Dermanyssus gallinae*, represents a significant threat to poultry production throughout the UK and worldwide. Infestation rates in Europe average over 80 % in egg laying facilities (Figure 2.1).

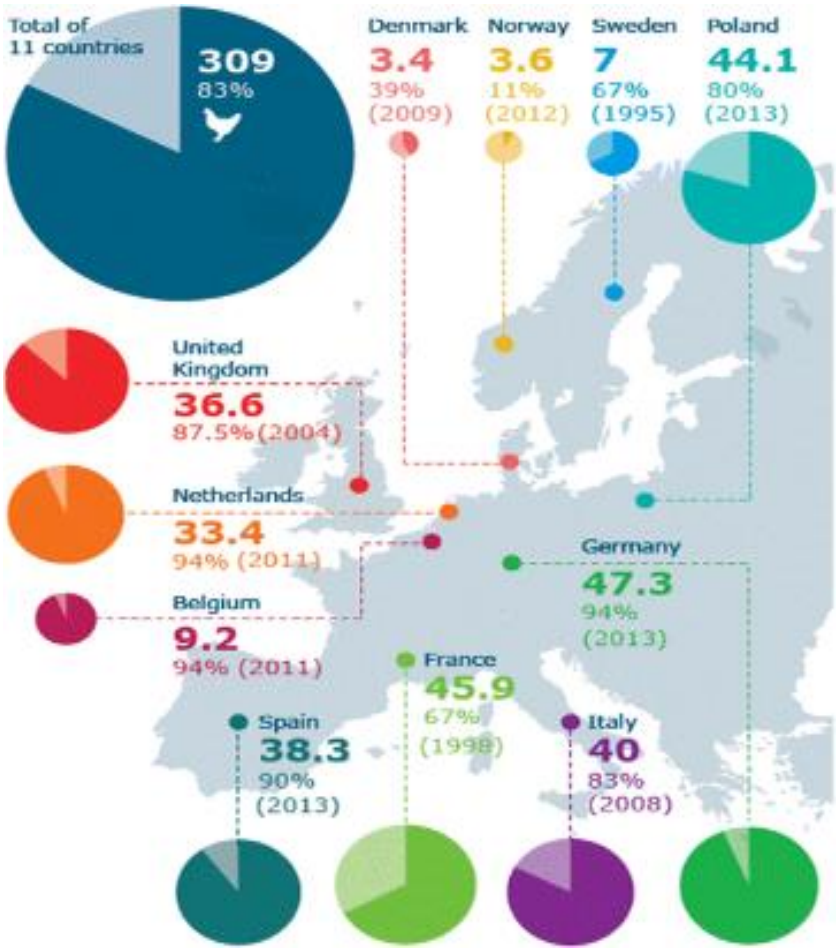


Figure 2.1: Infographic of laying hens data. *Number of laying hens per country in millions and the percentages of farms infested by Dermanyssus gallinae. Data shown from 2012. Image reproduced from Mul (2013).*

One of the main consequences of infestation with PRM is the negative impact on the overall health of the hens. In high levels of infestation, hens may become anaemic from repeated blood feeding by the mites, and in extreme cases, death by exsanguination has been observed (Cosoroaba, 2001; Wojcik et al., 2000). A

laying hen can lose 3 % of its blood volume every night, despite red blood cell production being increased, and reports have shown a 10 fold increase in hen mortality from heavy infestations with PRM as well as a 10 % decrease in egg laying (Cosoroaba, 2001; Wojcik et al., 2000; Van Emous, 2005). This intense feeding from the host results in high levels of stress within the hens and this is represented by a series of stress behaviours such as increased aggression, increased noise levels, excess grooming and feather pecking. (Van Emous, 2005; Chauve, 1998; Mul et al., 2009; Kilpinen, 2005; Kilpinen et al., 2005). Impacts from such behaviours are currently being limited by beak trimming, but legislation on this practice is changing. Beak trimming is highly controversial. Austria, Finland and Sweden have already banned the practice, Germany has agreed to put in place a scheme to encourage the ending of the practice of beak trimming and the Netherlands have pledged to stop beak trimming from September 2018 following a review of the practice in 2017 (Beak Trimming Action Group, 2015).

A study by Kowalski and Sokol (2009) has shown a 1.5 fold increase in corticosterone, a 22 % decrease in β globulin levels and double the adrenaline in the blood samples of birds infested with PRM compared to blood samples from control groups, which supports the theory of PRM inducing high stress levels and immunosuppression in hosts (Kowalski and Sokol, 2009).

As well as being detrimental to the health of the laying hens, PRM can cause large economic losses for poultry farmers from a range of different issues caused by infestations. In large infestations, there is a negative impact on the feed conversion ratio as feed and water intake is increased during infestation accompanied by a decrease in bird growth rates (Chauve, 1998; Mul et al., 2009; Mul, 2013). As well as this increased cost of feed, egg quality declines during

infestation. Egg shells are thinned and blood spots are present on the shells, due to rolling over recently fed mites, which makes them unfit for sale and reduces productivity (Chauve, 1998; Cosoroaba, 2001). The proportion of second grade eggs increased 2-14 % after infestation with PRM (Zenner et al., 2009).

The economic cost of PRM is difficult to quantify and is presumed to be underestimated due to the difficulty in confirming infestations and the stigma of infestations often leads farmers to not acknowledge the issue on surveys and reports. Production losses stemming from infestations with PRM have been estimated by Van Emous at €130 million per year in Europe, with equally large losses from other areas (Van Emous, 2005; Sparagano et al., 2009). This figure is likely to be underestimated due to the increase in numbers of laying hens since it was calculated. Number of laying hens in Europe in 2005 was 350 million, which had risen to 382.8 million in 2015 (Windhorst, 2017).

In the 2005 study, Van Emous calculated the cost per hen of PRM infestation to be €0.43 (€0.14 direct cost of treatment and €0.29 indirect cost of productivity losses) (Van Emous, 2005). In 2017, Van Emous calculated the cost of PRM infestation in The Netherlands had increased to €0.60 per hen (€0.15 direct cost of treatment and €0.45 indirect cost of productivity losses) and overall costs attributed to PRM infestations is now estimated to be €231 million in Europe (Flochlay et al., 2017; van Emous, 2017) This rising economic cost of PRM highlights the necessity to successfully treat infestations.

Additional to the detriment of the health of the birds and the rising economic cost of PRM is the role PRM play as a vector of disease. Absolute vector competence has not yet been confirmed in PRM, however the importance of PRM as a disease vector cannot be disregarded (Sparagano et al., 2014). The significance of PRM

as a pest is demonstrated by the proven ability to carry both bacterial and viral pathogens, a compilation of which are detailed in Table 2.2.

Table 2.2: Bacterial and viral pathogens which are associated with Dermanyssus gallinae. *Table recreated from Sparagano et al. (2014).*

	Pathogen	**Details**	**Associated References**
Bacteria	*Salmonella gallinarum*	Isolated from mites	Zeman et al. (1982)
	Salmonella enteritidis	Transmission demonstrated	Valiente Moro et al. (2009)
	Pasteurella multocida	Transmission demonstrated	Petrov (1975)
	Chlamydia spp.	Isolated from mites	Circella et al. (2011), Permin and Hansen (1998)
	Borrelia anserina	Unknown	Hoffmann (1987)
	Erysipelothrix rhusiopathiae	Isolated from mites	Chirico et al. (2003)
	Listeria monocytogenes	Isolated from mites	Grebenyuk et al. (1972)
	Coxiella burnetii	Transmission demonstrated	Zemskaya and Pchelkina (1967)
	Escherichia coli	Isolated from mites	Valiente Moro et al. (2009)
	Staphylococcus spp.	Isolated from mites	Valiente Moro et al. (2009)
	Streptomyces spp.	Isolated from mites	Valiente Moro et al. (2009)
	Spirochetes	Transmission demonstrated	Ciolca et al. (1968)
Viruses	Viruses Avian leucosis	Unknown	Hoffmann (1987)
	Newcastle disease	Isolated from mites	Arzey (1990)
	Fowl poxvirus	Transmission demonstrated	Shirinov et al. (1972)
	St. Louis encephalitis	Transmission not demonstrated	Chamberlain et al. (1957)
	Tick-borne encephalitis	Transmission not demonstrated	Wegner (1976)
	Eastern equine encephalitis	Transmission demonstrated	Durden et al. (1993)
	Western equine encephalitis	Transmission demonstrated	Chamberlain and Sikes (1955)
	Venezuelan equine encephalitis	Transmission demonstrated	Durden et al. (1992)

Of the pathogens associated, of note is the transmission of *Salmonella enteritidis.* Infection with *Salmonella enteritidis* causes diarrhoea, fever and abdominal cramps, occasionally leading to hospitalisation, and eggs and poultry are recognised as major importance in the transmission of the infection. The average cost of an infection with *Salmonella* is £1000, costing the UK £39 million per year (Lake, 2017; Santos et al., 2011). Mites can carry *Salmonella enteritidis* either through contact with the cuticle or by feeding from the blood of birds infected with the pathogen (Moro et al., 2010). PRM have also been found to be a reservoir for *Salmonella gallinarum,* the cause of fowl typhoid. Bacteria can reproduce within the mites and survive for up to four months, using mites as a reservoir (Pugliese et al., 2018; Zeman et al., 1982). The bacteria could be passed on from flock to flock by the PRM persisting in the poultry housing after one flock and transmitting *Salmonella gallinarum* to the new flock on their first blood feed (Moro et al., 2010). This represents the importance of PRM as a vector of disease.

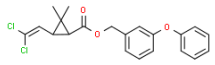
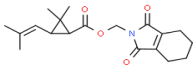
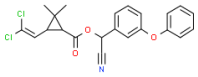
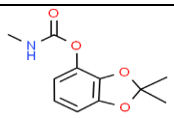
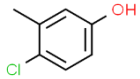
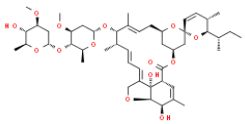
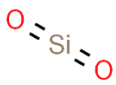
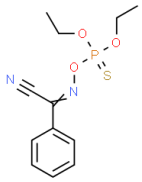
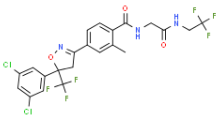
Infestation with PRM is being increasingly associated with dermatitis and gamasoidosis, namely in poultry workers and people living in close proximity to poultry housing (George et al., 2015). In one survey of 58 farms in Southern Italy, 19 % of farm workers reported pruritic skin eruptions after contact with PRM and is suggested as a cause for occupational safety concerns (Cafiero et al., 2011). In 1997, 7 male workers from the Mediterranean coastal plain of Israel reported severe itching and dermatitis after completing an early morning egg collection. Small, reddish lesions covered the whole body but were more intense on the arms, torso and back and disappeared without treatment after 2-6 days. Samples of mites, taken from the eggs collected by the workers as well as from the workers skin, were collected into small vials containing 70 % ethanol and later identified as *Dermanyssus gallinae* (Rosen et al., 2002). The reported number of cases of

dermatitis is increasing; in 2017 a 6 year old boy presented with a 4 day history of pruritic eruptions following several sparrow nests being found in the roof. Mite samples taken from the boy's bed were identified as *Dermanyssus gallinae* upon inspection (de Sousa and Filho, 2018). One report tested 26 samples taken from subjects living in Italy and showing clinical signs of dermatitis. The mite samples were analysed using transmission light microscopy and out of the 26 samples, 20 were confirmed to be *Dermanyssus gallinae* with abandoned bird nests (pigeon/ sparrow/ swallow) being close to the building (Cafiero et al., 2018). In one study, five members of a Serbian family were diagnosed with gamasoidosis after being in contact with PRM from a nearby dove's nest, further demonstrating the ability of PRM to feed from humans if lacking its natural avian host (Gavrilovic et al., 2015).

Control of PRM is typically achieved through programs incorporating biosecurity measures, cleaning and the use of synthetic acaricides with the latter remaining as the most effective method. Biosecurity measures include foot dips before entering poultry housing and a change of footwear before going from one housing unit to another, to avoid the spread of PRM. As well as changing footwear, limited exposure to outside birds is a measure taken to stop the spread of PRM, both within a farm and between nearby farming units (Knight-Jones et al., 2011). Manual cleaning of the sheds in between flocks is commonplace, but has been proven to have little effect on the long term population of PRM due to their ability to survive in cracks and crevices which are almost impossible to reach via cleaning. Whilst this may help reduce the population and allow acaricides to be more effective immediately after cleaning, it is not a long term solution to PRM control (Huber et al., 2011).

Control of PRM chiefly depends upon acaricide applications but despite this there are very few that are permitted to be used worldwide. Organophosphates, carbamates and pyrethroid-based acaricides are the most widely used across the globe but resistance has been reported against all of them in at least one country (Abbas et al., 2014). As well as increased resistance to the acaricides available, tighter legislation means that less products are coming on to the market in recent years and once popular products, such as the organophosphate fenitrothion, are now prohibited (Fiddes et al., 2005). Many unlicensed products are still being used to treat PRM but quantifying numbers is difficult (Table 2.3) (Marangi et al., 2012; Maurer et al., 2009).

Table 2.3: Pesticide use in European countries. *The family of pesticide, the specific pesticide , structure, commercial names and mode of action of commonly used pesticides in European countries (RSC, 2019). Also included are products that have not been approved but are in widespread use. Table adapted from Sparagano et al. (2014).*

Family of pesticide	Pesticide	Structure	Commercial product	Mode of action	Countries product is approved for use	Countries where product is not approved but widely used
Pyrethroids	Permethrin		Perbiochoc	Targets sodium channels	UK (permethrin)	Italy, The Netherlands, Belgium, Greece and Sweden
	Tetramethrin					
	Cypermethrin		Milben Ex			
			Fendona			
Carbamate	Bendiocarb		Ficam	AChE inhibitor		UK
Phenol	Chlorocresol		Interkokask	Lipid solvent		
Avermectin	Abamectin		CBM8	Glutamate chlorine channels	2012: UK	
Diatomaceous earth	Silica		Fossil shield	Desiccation		
			Hemexsan			
Neonicotinoid	Imidacloprid		Imidacloprid	Targets nicotinic acetylcholine receptor		
Organo-phosphate	Phoxim		Byemite	AChE inhibitor	2010: UK, France, Italy, Netherlands, Belgium, Denmark, Germany, Poland, Greece, Sweden	
Isoxazoline	Fluralaner		Exzolt	Targets GABA gated chloride channels	2017: EU wide approval	

Only two products have entered the European market which are licenced to target PRM in recent years, Byemite® (Bayer) and Exzolt (MSD Animal Health). Byemite® is an organophosphate (phoxim) which is an inhibitor of AChE and resistance to organophosphates is already noted in PRM, as well as the challenge of resistance, Byemite® is only registered in a few countries, not including ones with large populations of egg laying hens such as Germany, Poland, Spain and the UK (Flochlay et al., 2017; HPRA, 2016; Beugnet et al., 1997; Thind and Ford, 2007; Abbas et al., 2014). Exzolt is registered for use in the UK and is added to the drinking supply of the hens. The product is ingested by the hen and a percentage is detoxified by the bird, however a percentage remains in the blood stream unchanged and is taken up by the mite when blood feeding occurs. The aim is to have a high enough concentration in the hen's blood stream to be non-toxic to the hen but provide a toxic dose when absorbed by the mite. This entry differs to traditional spray acaricides which function by being absorbed through the cuticle of the mite when they are in contact with the spray. Exzolt, active ingredient fluralaner, targets γ-aminobutyric acid (GABA) gated chloride channels and has had 100 % efficacy up to 15 days after administration, but this drops to 74.8 % 19 days post treatment, requiring stringent application (Gassel et al., 2014; Brauneis et al., 2017).

A factor that could be contributing to pesticide resistance is the misuse of pesticides by farmers. Over using a product when a result is not seen immediately, or using products at a lower than recommended dosage in order to reduce costs, contributes to the exposure of PRM to the active compounds of the commercial products and could be allowing them to develop resistance. Many products which have not been approved for use are still being widely applied and misuse of acaricides in the treatment of PRM is widespread in

European countries (Table 2.3). An example of this misuse is fipronil, which was the cause of a food safety scare in 2017 when 26 of 28 European Union members found traces of fipronil in their eggs (Munoz-Pineiro and Robough, 2018). In Europe, fipronil is permitted for use as a veterinary medicine but is forbidden for use on animals intended for the food chain, such as poultry. The maximum residue level for fipronil in eggs is 0.005mg/ kg however, misuse of fipronil resulted in levels of up to 1.2mg/ kg found in European eggs (Munoz-Pineiro and Robough, 2018). This misuse is not only a risk for food safety but also risks under dosing (Beugnet et al., 1997; Marangi et al., 2009; Thind and Ford, 2007; Marangi et al., 2012). Under dosing provides a sub lethal dose to the mites and exacerbates the issue of resistance to products. As well as under dosing, uneven spraying of poultry housing can lead to sub lethal doses of product being applied due to the PRM living in cracks and crevices of the poultry housing and being difficult to access with spray.

Recently, as the efficacy of synthetic acaricides has reduced, studies have emerged into the use of plant essential oils as a control method for PRM. 56 plant oils have been tested for their toxicity in PRM and researchers investigated the influence of ‘time since last blood meal’ on the toxic effect of plant oils (Kim et al., 2004; George et al., 2010a; George et al., 2008b). However, the issue with using plant oils as a control method is that the oils can have differing chemical profiles which leads to different efficacies as acaricides. Many studies, which have used the same methodologies, have produced conflicting results for the same essential oils, which makes their future as a commercial control method for PRM unlikely unless more research is done to ensure their efficacy (Kim et al., 2004; Nechita et al., 2015; George et al., 2008a; George et al., 2010a; George et al., 2008b; George et al., 2009).

It is critical that the efficacy of existing acaricides for PRM be maintained to ensure their continued benefit to the industry. To achieve this goal, resistance must be monitored/managed wherever possible through considered application of these acaricides. A UK map of PRM resistance/susceptibility would be highly beneficial to this end as the application of acaricides could be controlled based on which areas are already showing resistance to a particular product. As well as this, a map would showcase the need for new treatment options in the UK and provide a baseline against which future resistance/susceptibility could be compared. A baseline is vital for monitoring the need for new treatments over time as well as the effectiveness of cleaning and biosecurity measures which are in place. The last time a survey of this nature was completed in the UK was in 2005 (Fiddes et al., 2005) and this survey included both layer and parent birds, of which layer represented 58% of the 43 responses. The industry would benefit from a more complete picture of resistance across the UK and other counties with high numbers of egg laying facilities, with geographical comparisons being made possible in order to establish the best treatment specific to an area.

This chapter presents the results of a survey, of acaricide usage and the occurrence of resistance across the UK, from a project aimed at addressing these issues in PRM. The survey was sent to farms across the UK and was answered by farmers who apply the products to which resistance is being seen. The survey questions farmers on products used as well as requesting information about the housing and feeding regime of the birds in order to draw further comparisons. The information obtained will allow for a map of perceived resistance to be generated for several regions across the UK that can be used to recommend optimal PRM treatment programmes in these areas for use by the farming industry. As well as this, knowledge of the products being used to

treat PRM means a more targeted approach can be undertaken when developing new products. The timing and method of usage of the products will be requested from farmers, which allows further study into the misuse of certain products and how to provide clearer instruction for use.

As well as a survey to gain information on perceived resistance, toxicity testing is essential to gain a fuller picture of the resistance in PRM. To assess the actual resistance of PRM to commonly used products, mites will be acquired from several poultry units from across the UK. These mites can then be tested under laboratory conditions in a standardised toxicity test in order to compare the actual resistance found in the PRM to the perceived resistance by farmers. This data will be the largest collection of PRM resistance surveys in the last ten years in the UK and will provide an up to date insight into perceived resistance across different geographical locations, and whether this perceived resistance is an accurate depiction of actual resistance found from toxicity testing.

2.3 Materials and Methods

2.3.1 Participant Recruitment

Contact details of egg producers from across the UK were provided by Dr. Mark Williams of the British Egg Council. Egg producers were initially contacted via email and asked for cooperation in a project that would monitor the distribution and perceived resistance of PRM across the UK.

2.3.2 Survey Design

A survey was designed which would be both short and easy to answer, based on colloquial farmer feedback on the type and length of questionnaire they would be willing to answer. The survey consisted of seven questions and was sent to those willing to cooperate. Questions 1-4 were aimed at gathering details of the farm location and name as well as contact details. Question 5 then asked for production methods such as flock size, number of buildings, size of buildings, others animals reared and lighting regimes. This question was an open box answer allowing the farmer to provide as much information as they could. Question 6 was designed as a grid to collect product information. It asked for the brand name of the product as well as who the product is manufactured by. It then went on to ask what form the product was used in, for example some products come in both a powder and spray form, and how frequently the product was applied. The next column asked for the timing of the products use, for example was it used on an inhabited or uninhabited shed, followed by the method used to apply the product; was it applied as a spot treatment to problem areas or was it applied to the entire shed. The final column asked the farmer to rate the perceived effectiveness of the product on controlling the PRM on a hedonic scale (0-10). The seventh question on the survey enquired which other preventative methods were used, such as applying grease to the roosts, as well as any extra comments

the participant wished to include (see appendix C for full survey). Surveys were emailed or posted back to Northumbria University where the data were collated and analysed.

2.3.3 Statistical Analysis

The minimum number of participants required was determined by an *a priori* power analysis. Power analysis revealed that in order for differences in samples to be detected (80 % chance) as significant at the 5 % level, a sample of 42 participants would be required.

$$N = \frac{0.6 \text{ X } 0.4\left[1.96 + 0.84\sqrt{\frac{0.8 \text{ X } 0.2}{0.6 \text{ X } 0.4}}\right]^2}{(0.8 - 0.6)^2}$$

$N = 42$

The data were analysed using the statistical package SPSS Version 22.0 (IBM, 2013). Analysis of a factor with more than two response options was done using an independent samples Kruskal-Wallis pairwise analysis (confidence interval = 95, significance level 0.05), with missing data excluded. For the North-South divide where there were only two response options, data were analysed using an independent samples Mann-Whitney test (confidence interval = 95, significance level 0.05).

2.3.4 Toxicity Testing

PRM were collected from poultry units for use in toxicity testing. Traps were sent out, originally consisting of plastic cylinders (each cylinder was 12 mm in diameter) fastened together and later corrugated plastic (each gap was 2 mm in

diameter), which were posted to participants. The traps were sent with plastic sealed containers which were used to post them back to Northumbria University as well as cable ties to secure the traps within the housing. A sheet of instructions detailing how to use the traps and the best place to secure the traps was also provided (see appendix D for instructions). Participating farms within the vicinity of the University were visited and mites collected from these facilities by hand. Plastic sealed bags were used to collect the mites, which were brushed into the bags using cardboard scrapers, and were transported immediately back to the University.

Mites were stored at 4 °C in complete darkness prior to use within the sealable plastic 'freezer bags' in which they had been initially collected or the sealed plastic container they had been posted in. Mites were used in experiments within 1 week of procurement. Before use in the assay, mites were assessed for percentage viability and engorgement. Samples of mites were taken at random and tested by agitation with an entomological pin, movement following agitation was deemed as viable. Around 20 % of mites were expected to be nymphs to ensure a healthy reproducing population was present. If over 50 % of the mites sampled were viable and over 25 % appeared to be engorged, the mites were used in testing. Average viability of the mites was ~70 % for mites arriving by post.

All Petri-dishes, once in use, were stored for the required period at 20 °C in complete darkness, after which mite mortality was assessed under magnification. A mite was considered dead if no movement was observed following repeated agitation with an entomological pin (George et al., 2010a). All products used for testing were stored in darkness at 20 °C prior to use.

Six well known PRM products were acquired for testing against *D. gallinae* from all farms; Milben Ex (Schopf), Interkokask (Hysolv), Ficam (Bayer), Perbio Choc

(Digrain), CBM8 (Hysolv) and Fendona (BASF). The products were made up according to manufacturer's instructions (see appendix E). Two local farms (Farm 51 and Farm 52) were used for extensive testing due to being able to collect mites from the facility in person and hence get a higher yield of PRM to work with. These PRM were tested using the active ingredients of commercial pesticides (cypermethrin, bendiocarb and permethrin) as well as the commercial products.

The concentration of these active ingredients used in testing was determined by the concentration of the active ingredient when used in commercial products at a working concentration; cypermethrin 4 mg/ml, bendiocarb 2.4 mg/ml and permethrin 6.4 mg/ml. The active ingredients were solubilised in 100 % ethanol to a stock concentration of 10 mg/ml before being further diluted with 100 % ethanol to the relative working concentration. The active compounds were tested with synergists (diethyl maleate, piperonyl butoxide and triphenyl phosphate) at the same concentration as the active product. Farm 51 was tested with synergists at 5 times the concentration of the active product. A negative control was prepared for each batch of PRM that were tested (100 % ethanol control for active ingredients solubilised in ethanol and water control for commercial products diluted in water).

Adult female PRM were tested using a contact assay. 100 µl of the solution to be tested was added to 14.2 cm^2 filter papers (Whatman No. 2) in a circular pattern and left to dry for 3 minutes in a fume hood. An ethanol negative control was included for every batch of mites collected. Approximately 25 adult female mites were added to each filter paper using a pooter and sealed in a glass Petri dish using polyethylene film and a glass lid. These dishes were stored at 20˚C in a dark storage cupboard. A minimum of 3 replicates were done for each concentration of product, with a maximum of 6 replicates if the yield of PRM was

high enough. The mites were taken out and counted after 24 hours. The mites were considered dead if there was no movement after agitation with a pin. Mortality was corrected for control using the Henderson-Tilton correction (Henderson and Tilton, 1955). Statistical analysis for comparison of product efficacy was done using a t-Test: Two-Sample Assuming Unequal Variances and was run in Excel.

2.4 Results and Discussion

2.4.1 Survey Data Analysis

Farmers are facing a lack of efficient products on the market to deal with the issue of PRM infestation. From colloquial conversation, it appears product labeling and marketing often leaves farmers confused about the product they are purchasing. If one product ceases to be effective, a new product is purchased to replace it, but the same active ingredient could be found in the new commercial product, therefore not improving the efficacy. The high prevalence of PRM on UK farms, as well as the lack of effective acaricidal products on the market, leaves farms facing large production losses. As well as this, using products with the same active compound as a product already showing signs of resistance could exacerbate the issue of resistance within PRM.

The data generated from the survey responses has been widespread across the UK (Figure 2.2) comprising data from 51 farms in 17 different counties. Of the two previous UK surveys on PRM, both completed over a decade ago, one survey was only done on farms in the North of England, with 29 farms participating, and so gave a limited overview of resistance (Guy et al., 2004). The latest study to be published in the UK is from 2005, and the prevalence rate of 60 % was used in the *a priori* power analysis to ensure enough surveys were completed for adequate study power (Fiddes et al., 2005). Both surveys previously completed found a high rate of PRM prevalence of 87.5 % (29 survey responses) and 60 % (43 survey responses) which supports the findings of this study where prevalence was found to be 98 % (Guy et al., 2004; Fiddes et al., 2005). Both studies also found high levels of reports of resistance to used pesticides, with 63 % of Northern farms reporting products becoming less effective over time. One of the largest concerns with the studies previously completed is the move to free range

systems since their production. The previous research surveyed farms that were 71 % caged systems or 45 % caged systems, compared to 0 % caged systems surveyed in this study. This is due to the shift in legislation (Directive 1999/74/EC) which has prohibited traditional cages and has led to more complex housing and a move to free range systems. This shift has increased incidence of PRM due to the increased areas of mite refuge and hence new data is required in order to gain an accurate picture of current PRM prevalence (Flochlay et al., 2017). Also, in one study, the pesticide most reported to be used was fenitrothion which has been banned for use since 2007 and hence survey responses are now outdated. In the survey completed in this study, areas such as Cumbria and North Yorkshire have a higher number of survey responses due to the prevalence of poultry farming in these regions, whilst areas such as South West England do not have a survey response as farming in this region focuses on cattle and sheep rearing due to its extensive grasslands (Union, 2014).

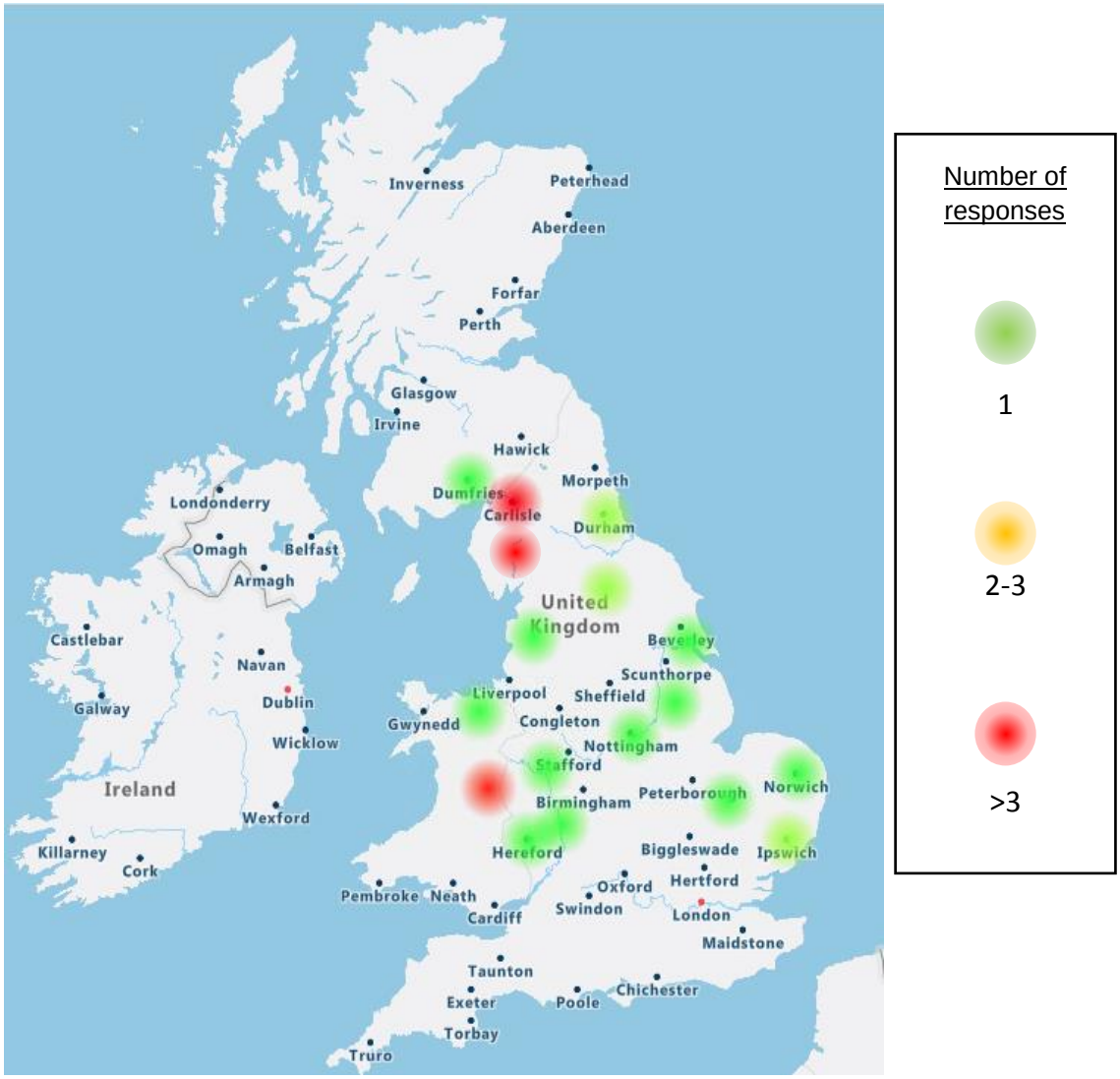


Figure 2.2: Heat map of the UK demonstrating spread of survey responses. *Heat map representing the number of survey responses per area (N=51). 1 response is represented by a green dot, 2-3 responses are represented by a yellow dot and >3 responses are represented by a red dot (N=51).*

Out of the 51 farms surveyed, 98 % reported the presence of PRM and used products to try and eradicate the pest species. The farm that did not report using any control products did not specify whether PRM were present, producing a prevalence rate of 98-100 %. This is a notable increase in prevalence when compared to a 2004 study of Northern farms which found the prevalence of PRM to be 87.5 % (Guy et al., 2004).

The use of surveys of PRM infestations have also been used worldwide, a survey of ectoparasites in China found 64.1 % of commercial layer hens to be infested with PRM with 77.8 % relying on the use of organophosphates or pyrethroids alone to control the ectoparasitic infestations (Wang et al., 2010).

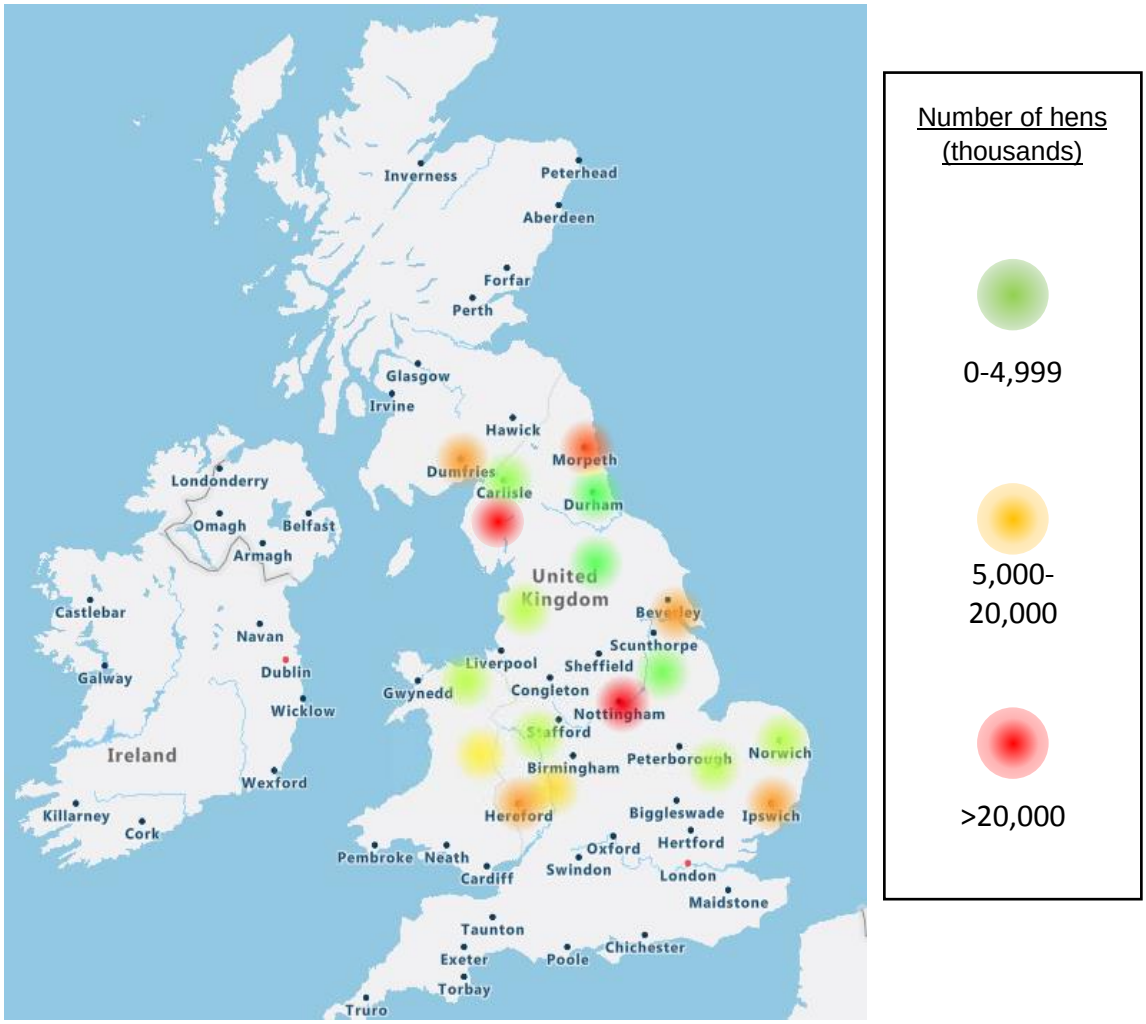


Figure 2.3: Heat map of number of hens per farm. *Heat map to show the number of hens kept on each farm surveyed. 0-4,999 hens are represented by a green dot, 5,000-20,000 hens are represented by an orange dot and >20,000 hens are represented by a red dot. Each dot covers a 30 mile radius surrounding the located farm. Where more than 1 farm response was present in the same 30 mile radius, the farm with the highest number of hens is represented (n=50).*

In order to establish the scale of farms responding to the survey, farmers were asked to provide the number of hens on the farm. This information can be viewed in Figure 2.3, where it is shown that the largest scale farms were found in Cumbria and Nottinghamshire. This also allowed an insight in to the scale of farms affected by PRM, from smaller scale facilities, to large facilities with over 50,000 birds. It also highlights the needs for tailored PRM control to fit different sized facilities with different product requirements.

The data was grouped as the UK as a whole for analysis of treatment application rather than per geographical location. It was revealed that there was no significant increase of the perceived effectiveness of each individual product if it was applied more often throughout the year or if it was applied by covering the shed, rather than doing a spot treatment on areas showing high infestation levels. This information could be used to help farmers reduce the money spent on acaricides as well as the time needed to apply products to the entire shed. Product could be wasted by doing 100 % coverage rather than spot treatment as well as by applying a product more frequently than required to achieve a high efficacy. It is, however, worth noting that 39 products out of the 110 listed by farmers were used ‘as required’ rather than specifying a frequency of use which makes comparisons between application frequencies difficult.

It was thought that applying products when the hens were housed could improve the efficacy of the product due to the increased likelihood of the mites being out of the refuge of the housing to blood feed from the hens. However, there was no significant difference in perceived product effectiveness when products were used whilst hens were housed in the shed or whether the product was used once the shed had been emptied and was in between flocks. This could be due to the large population of PRM still in the crevices when hens are present and therefore

only a small number are affected when products are applied whilst hens are housed and therefore this implies that the hens are effectively irrelevant in the subject of PRM infestations and resistance. It could also be that even though a product is more effective when the hens are present, the product is applied more thoroughly when the hens are not present which counteracts any improvement in efficacy. Due to this, it may be more time effective, and therefore cost effective, for products to be applied to an empty house due to the ease of application.

Interestingly, there was no statistically significant difference in the perceived effectiveness of commonly used pesticides dependent on the location of the farm surveyed. Location comparisons were made at a county level and also at a wider regional level but no statistical difference was observed in the effectiveness of the products as noted by the farmers. A comparison was also made between the North and South of the UK as a whole using the North/South divide line (Dorling, 2007). This divide defines Wales as Northern and results in no significant difference in perceived effectiveness. When a more colloquial North/South divide was used (from the River Humber to the River Mersey) one of the more commonly used pesticides, Milben Ex, was significantly more effective when used in the South of the UK than in the North.

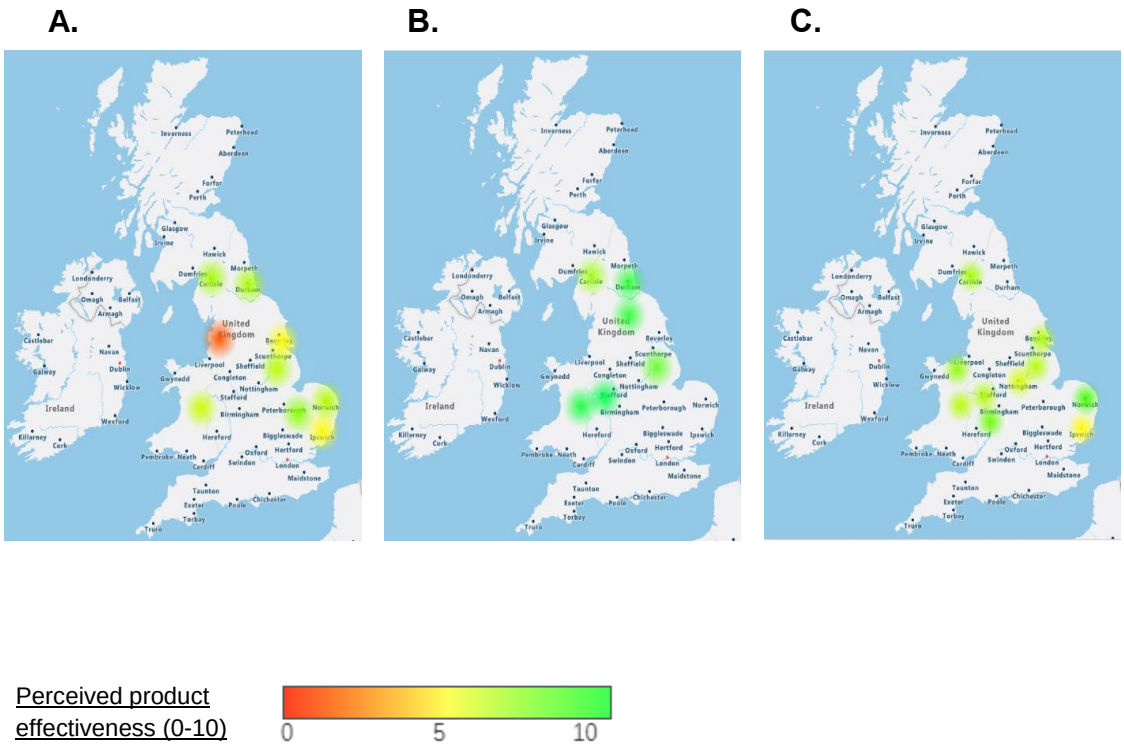


Figure 2.4: Heat maps of product effectiveness. *Maps showing the effectiveness of three products according to the survey responses which were ranked on a scale of 0-10 (0- no perceived effectiveness, 10- high perceived effectiveness). A. Ficam, active ingredient of bendiocarb (n=33). B. Milben Ex, active ingredient of cypermethrin (n=17). C. Diatomaceous earth (including survey responses for hemexsan, diatomaceous earth, dusting powder and fossil shield) active ingredient of diatomaceous earth (n=33).*

Heat maps (Figure 2.4) show the average effectiveness of Milben Ex, Ficam and diatomaceous earth by county, based on survey responses. Despite there being no significant difference in perceived pesticide effectiveness across the UK statistically, there are still observable differences in perceived effectiveness across the UK. These are noticeable when using average effectiveness results from each county per individual pesticide. For example, the average perceived

resistance to Hemexsan in Lancashire was 3/10 compared to Powys which had an average score for the same pesticide of 8/10. This score cannot be compared statistically as there was only one farm response from Lancashire which uses Hemexsan. Similarly, the average score for Ficam (active ingredient bendiocarb) from the East Riding of Yorkshire was 5/10 compared to Dumfries and Galloway which scored Ficam 10/10. Milben Ex, which contains the active ingredient cypermethrin, had a rating of 9.5/10 when used in Powys but a rating of 6.8/10 when used in Cumbria. These could not be statistically analysed due to only being one response from each county. The differences in perceived product effectiveness could represent a notable difference in the ability of PRM to metabolise different pesticides in different locations. This data could highlight the spread of pesticide resistance in PRM, with certain farms struggling to control PRM with products that are effective in other locations, furthermore, there seems to be no clear geographical trend in resistance with results varying greatly per county.

The lack of difference in effectiveness across different locations could highlight the persistently high levels of resistance in the PRM. 98 % of survey responses used PRM control products which is representative of the scale of PRM infestation in the UK compared to 60 % of farms in the previous study of Northern farms (Fiddes et al., 2005).

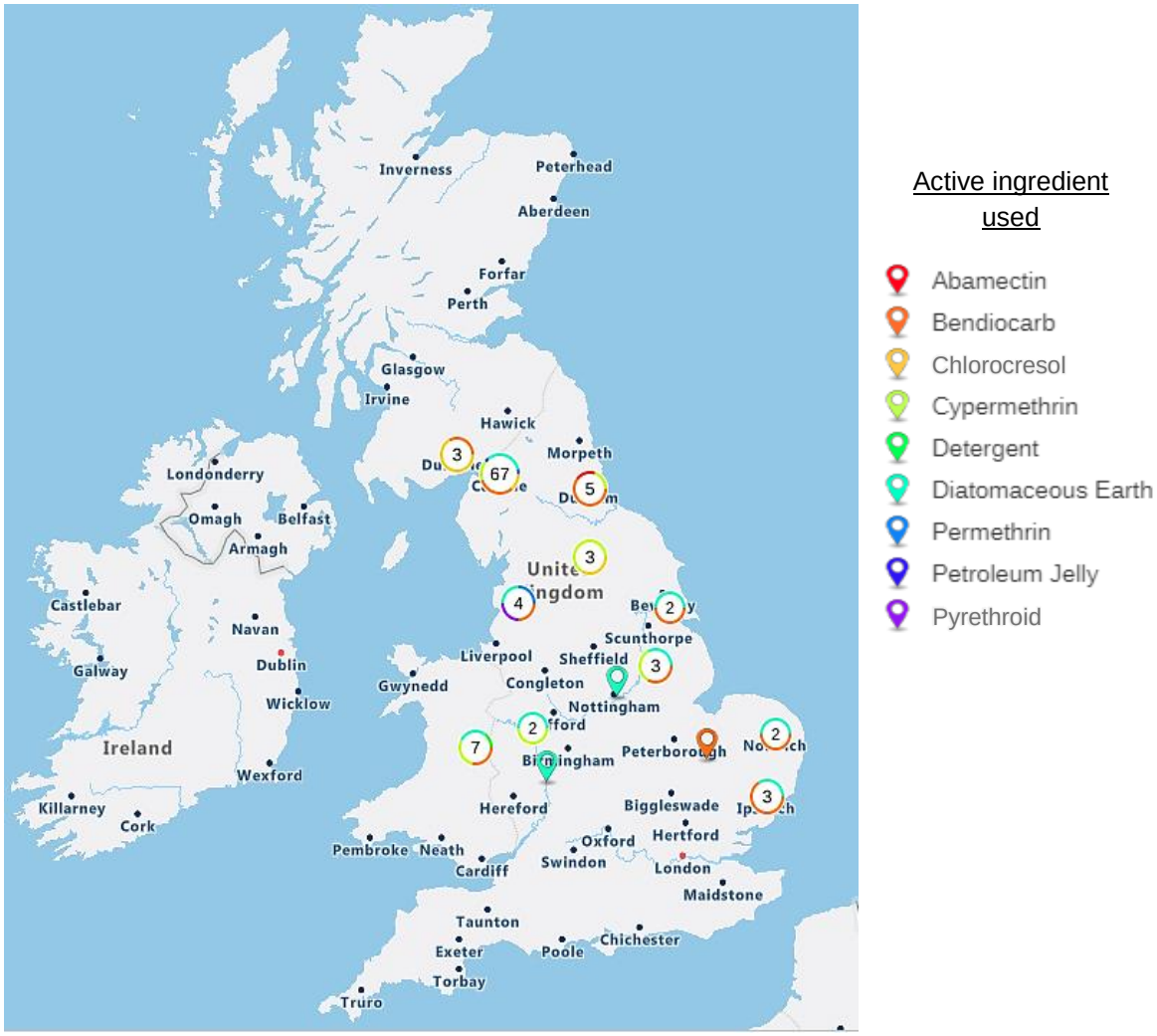


Figure 2.5: Active ingredients used across the UK. *Map to show the active ingredients used on farms across the UK based on survey responses. Active ingredients are mapped with a different colour, areas with multiple active ingredients used are represented on a circular chart (N=105).*

Multiple products contain the same active ingredient, making resistance harder to monitor. There is a wide spread of different active ingredients being used across the country, as can be seen in Figure 2.5, with bendiocarb, cypermethrin and diatomaceous earth among the most commonly used. Use of diatomaceous earth is also common across all farms, and is often the pesticide of choice when only one product is used.

Average product effectiveness across all products was 7.3/10 with one of the highest scoring products being petroleum jelly/grease (Table 2.4). Grease scored 9/10 on average which is noteworthy, as it is one of the only products which mites cannot form metabolic resistance to, as the action of grease is mechanical. The grease coats the exoskeleton of the PRM causing death by desiccation (Pritchard et al., 2015), although using this in a commercial setting poses difficulties with time consuming application. The highest rated product which is used by more than 3 farms is Milben Ex, active ingredient of cypermethrin. Milben Ex is currently rated 7.74/10 but is part of the pyrethroid family. Over reliance on an effective product, such as Milben Ex, could lead to future resistance in the same way that has happened with previously effective products. Pyrethroids, such as permethrin and flumethrin, are an example of a product which was once effective but resistance is now widespread. Pyrethroids have been used for many years as acaricides, but resistance has been reported against them in PRM in 6 different countries since as early as 1984 (Abbas et al., 2014). In addition to PRM, pyrethroid resistance has been noted in mosquito species such as *Aedes aegypti* and *Aedes albopictus,* as well as in other acari such as the North American cattle tick, *Rhipicephalus (Boophilus) annulatus,* which showed resistance to pyrethroid insecticides with elevated levels of P450s and GST complexes in resistant populations (Smith et al., 2016; Ziapour et al., 2017).

Table 2.4: Average perceived effectiveness of products. *The names of the products used, as taken from the survey, with the active ingredient of the product as well as the number of farms using the product and the average perceived effectiveness of that product from the survey. N/A= No score was given for that product.*

Name of Product Used	Active Ingredient	Number of Farms Using Product	Average of Perceived Effectiveness (0-10)
Red Mite Avian	Plant essential oils	2	9.00
Petroleum Jelly/Grease	Petroleum jelly	2	9.00
Milben Ex	Cypermethrin	17	7.74
Perbio Choc	Permethrin and tetramethrin	4	7.67
Fossil shield	Diatomaceous earth	5	7.60
Diatomaceous Earth	Diatomaceous earth	3	7.33
Hemexsan	Diatomaceous earth	25	7.30
CBM 8	Abamectin and permethrin	1	7.00
Fendona	Cypermethrin	1	7.00
Decimite	Propargite	1	7.00
Ficam	Bendiocarb	33	6.93
Interkokask	Chlorocresol	11	6.89
Poultry Shield	Surfactant	1	5.00
Ecofoam	Surfactant	1	N/A
Hi GAT Lambda Plus	Lambda-cyhalothrin	1	N/A
Gat Omega	Abamectin	1	N/A
None	N/A	1	N/A

The survey provides evidence that the active ingredient is the crucial determinant in mite mortality rates, and that the form in which the active ingredient is supplied is not a factor in the effectiveness of the product. No significant difference was found in perceived effectiveness when a product, such as Ficam, was used in the powder form or in the spray form. It would therefore be more cost effective for farms to use whichever form was the lowest cost to buy and apply to the shed.

2.4.2 Toxicity Testing Data and Analysis

In order to investigate if the perceived data is reliable as a true indication of resistance/effectiveness status, the next step was to perform standardised *in vitro*

toxicity testing, under laboratory conditions, to a range of actives on mite samples obtained from selected farms. Initially, traps were designed using card wrapped around plastic straws 12 mm in diameter. These traps were unsuccessful, likely due to the straws being too wide to provide refuge to PRM as previous studies have used traps with corrugated cardboard with gaps of 3 mm in diameter (Nordenfors and Chirico, 2001; Lundh et al., 2005; Barimani et al., 2016). Plastic traps with a corrugated plastic centre (gaps 2 mm in diameter) were sourced from Elanco and were sent out to participating farms which allowed for the collection of mites from 7 farms (Figure 2.6). Of the 7 farms, 6 sent a high enough yield of mites to allow for the testing of commercial products.

Figure 2.6: A map of mite collection points. *Each number on the map of the UK represents one farm that mites were collected from.*

The mite traps were sent out in staggered stages to compensate for the testing capacity of the laboratory. A range of popular commercial products were chosen to be tested on the adult female mites collected. Ficam, Milben Ex, Interkokask, Perbio Choc, CBM8 and Fendona which were used by 33, 17, 11, 4, 1 and 1 farms respectively. These products were tested at the manufacturer instructions for usage (see appendix E).

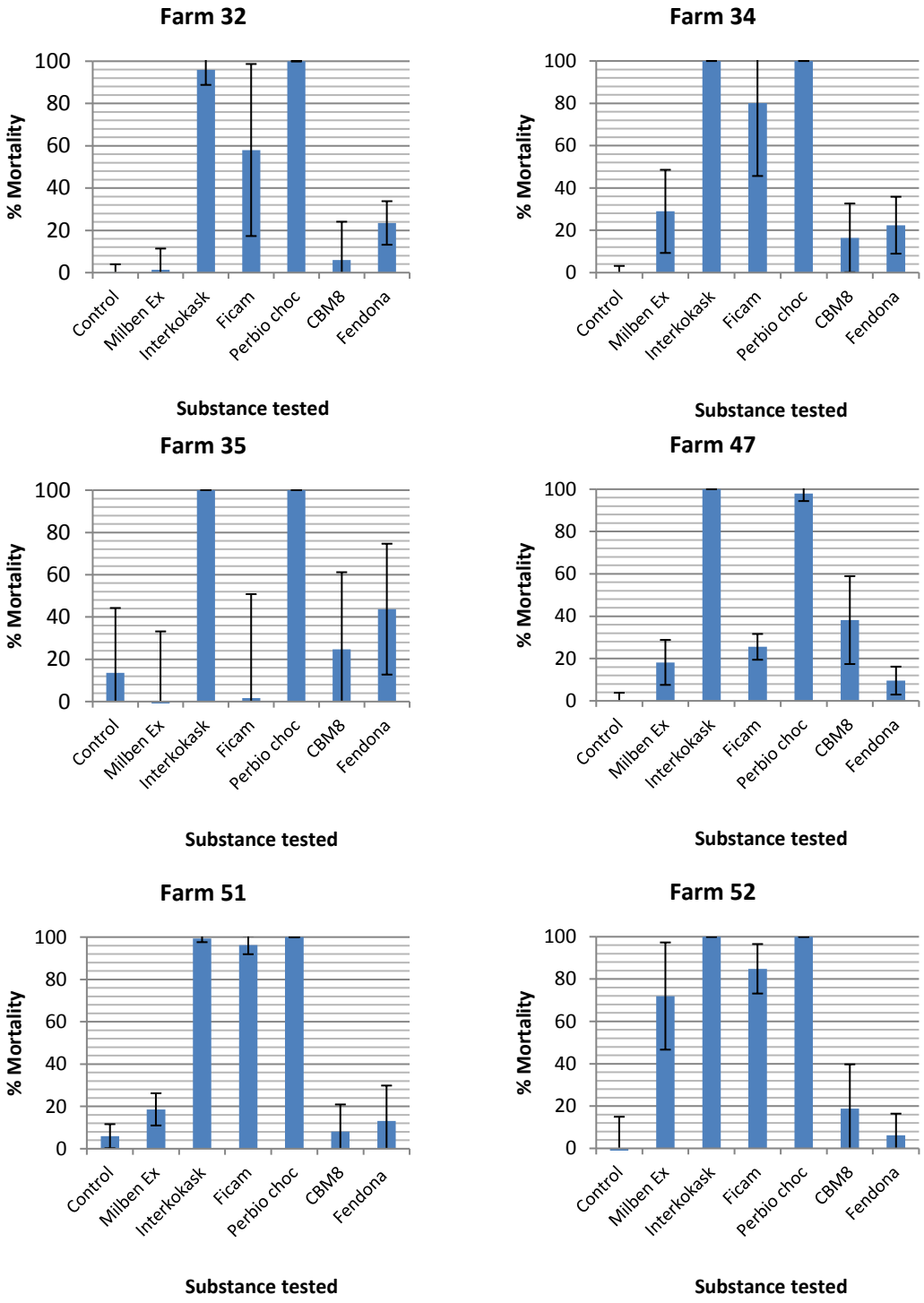


Figure 2.7: Results of the complete toxicity testing from 6 farms on adult female PRM. *Data represents the mean % mortality +/- SD, corrected using the Henderson Tilton correction. N=3 for farms 32, 34, 35 and 47. N=6 for farms 51 and 52 (control=water for all results shown).*

Across the six farms tested with commercial products (Figure 2.7), Fendona had a consistently low mortality and Milben Ex had low mortality in all farms other than farm 52 where it had a mortality of 72 %. Both Fendona and Milben Ex have cypermethrin as an active ingredient, part of the pyrethroid family, and provides strong evidence for resistance to cypermethrin. To support this, both Fendona and Milben Ex had an average score of 7/10 on the survey of perceived product effectiveness, indicating both products have the same level of efficacy. Milben Ex was rated 7.74/10 on the survey of perceived product effectiveness, which could be due to efficacy dropping off in recent months due to resistance (time gap between survey and testing was 12 months).

Interkokask and Perbio Choc have high mortality on all 4 farms. Perbio Choc, has both permethrin and tetramethrin as active ingredients which belong to the pyrethroid family, like cypermethrin. However, there are different types of pyrethroids, type I and type II, which differ by the addition of an α-cyano group in type II pyrethroids. Both permethrin and tetramethrin are type I pyrethroids and cypermethrin is a type II pyrethroid. Perbio Choc had a high average mortality of 99.7 % across the six farms, showing that resistance is unlikely to be across pyrethroids as a whole, but rather to the different types of pyrethroids, as type II pyrethroid cypermethrin (active product of Milben Ex) had much lower levels of toxicity across the farms. Interkokask has an active ingredient of chlorocresol, which is a derivative of cresol. Chlorocresol is a lipid solvent which breaks through the chitin exoskeleton of the mites and causes death of mites by desiccation. Due to the mechanical nature of the mechanism of Interkokask, metabolic resistance cannot occur, which could explain the high efficacy of Interkokask across all farms. Also, Interkokask requires contact with PRM to cause desiccation, which

occurs in the assay testing producing consistently high levels of mortality, but is less likely to be achieved in a commercial setting.

The results for Ficam are the most varied of all results, with 96.3% mortality on farm 51 and 1.6 % mortality on farm 35. The active compound in Ficam is bendiocarb which is a carbamate, to which resistance has been noted since 2004 (Abbas et al., 2014). The high variability in the results for Ficam highlight the need for farm testing on an individual basis, as general assumptions of product effectiveness based on one farms results cannot be consistently accurate per region or country wide. This agreement is supported by the following: Farm 34 had 80.1 % mortality using Ficam whereas farm 47, which is in the same county (Cumbria), had just 25.5 % mortality.

CBM8 is one of the newest products to market, being registered in 2014. The active ingredient is Abamectin, part of the avermectin class of pesticides which is a glutamate-gated chloride channel allosteric modulator and causes paralysis. This is combined with permethrin and microencapsulated to give a slow release of the active ingredients (Wolstenholme and Rogers, 2005). Despite being new to the market, and therefore theoretically less likely to be affected by issues of resistance, the product is still largely ineffective across all farms surveyed. This is likely due to one of the main actives being permethrin, to which resistance has already been reported in PRM (Abbas et al., 2014), and the other being Abamectin to which resistance has been found in similar acari such as the two-spotted spider mite, *Tetranychus urticae* (Kwon et al., 2010).

Whilst the results from the survey largely supported the toxicity testing results, a few discrepancies were present which highlight the need for per farm toxicity testing. Farm 32 scored Interkokask 7/10 and Ficam 8/10 whilst they scored 96 % and 58 % mortality rates on the toxicity testing respectively. However, Fendona

was also scored 7/10 despite only producing a 23 % mortality rate in toxicity testing. Farm 34 scored Perbio Choc 7/10 which is lower than the 100 % mortality rate in laboratory testing but farm 35 was accurate with the score of 0.5/10 for Ficam which scored just 2 % in toxicity tests on mites from the farm. Another mixed result was farm 47 which correctly scored Interkokask 8/10 in agreement with the 100 % mortality rate found in testing, but scored Ficam 9/10 despite only a 26 % mortality rate in toxicity testing. This could be due to an increased resistance in the mite in the time between the survey and testing, or the reduced mortality rate may have not yet been noticed by the farmer if the product had previously been effective.

Farms 51 and 52 were chosen for further toxicity testing due to the proximity to the University, which allowed for the PRM collection in person. The active ingredients of some of the popular products (Ficam, Milben Ex and Perbio Choc), which are likely to be metabolised by PRM detoxification pathways, were chosen for further testing on both farms. Ficam, Milben Ex and Perbio Choc were used by 33, 17 and 4 farms respectively. The active ingredient of Milben Ex is cypermethrin and the active of Perbio Choc is permethrin, which both belong to the pyrethroid family. Ficam contains bendiocarb which belongs to the carbamate family. PRM have been reported to be resistant to both pyrethroids and carbamates across the globe and a summary of this resistance can be seen in Table 2.5.

Table 2.5: Reports of drug resistance in PRM. *Both drug and class of drug are listed along with the country the resistance is reported in and the associated reference. Table adapted from Abbas et al. (2014).*

Drug Class	Drug	Country	Reference
Organochlorines	DDT	Czechoslovak	(Zeman, 1987)
	Fipronil	Korea	(Kim et al., 2007)
Carbamates	Carbaryl	Korea, Italy	(Kim et al., 2007; Marangi et al., 2009)
	Furathiocarb	Korea	(Kim et al., 2007)
Organophosphates	Trichlorfon	Czechoslovak, Sweden	(Zeman, 1987; Nordenfors and Hoglund, 2000)
	Fenitrothion	Korea	(Kim et al., 2007)
Pyrethroids	Flumethrin	Italy	(Genchi et al., 1984)
	Tetramethrin	Czechoslovak	(Zeman, 1987)
	Permethrin	Czechoslovak, France, Sweden, Korea, Italy	(Zeman, 1987; Beugnet et al., 1997; Nordenfors et al., 2001; Kim et al., 2007; Marangi et al., 2009)
	Deltamethrin	Iran	(Hadadzadeh et al., 2001)
	D-phenothrin	Korea	(Kim et al., 2007)
	Alpha-cypermethrin	Korea	(Kim et al., 2007)

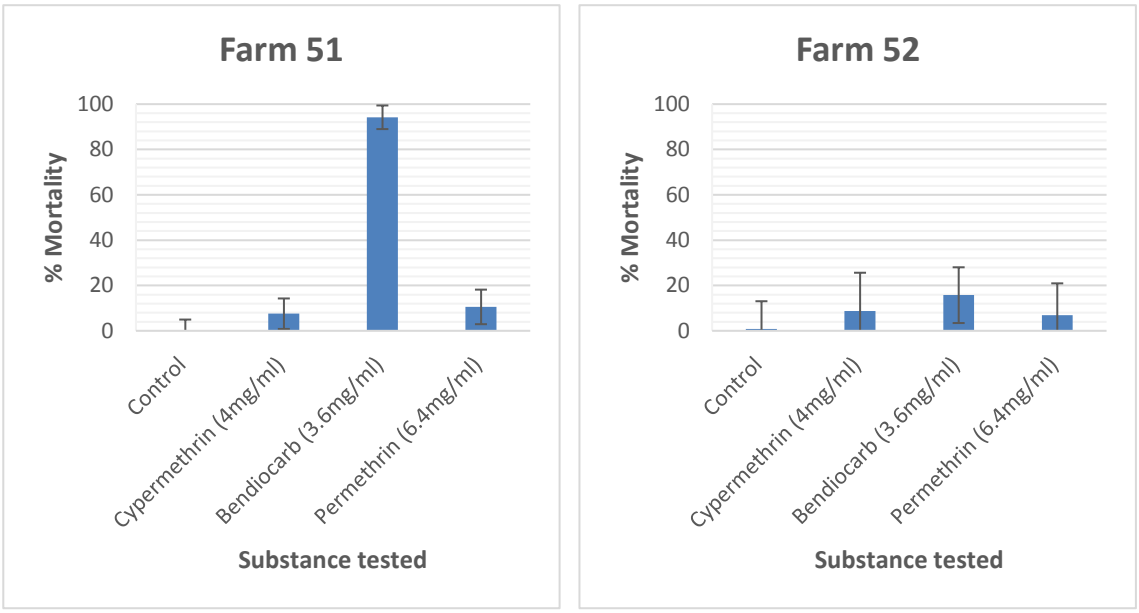


Figure 2.8: Results of the toxicity testing from 2 farms on adult female PRM. *Active ingredients were tested on both farm 51 and farm 52. Data represents the mean % mortality +/- SD of N = 6 (control= ethanol for all results shown) corrected using the Henderson Tilton correction.*

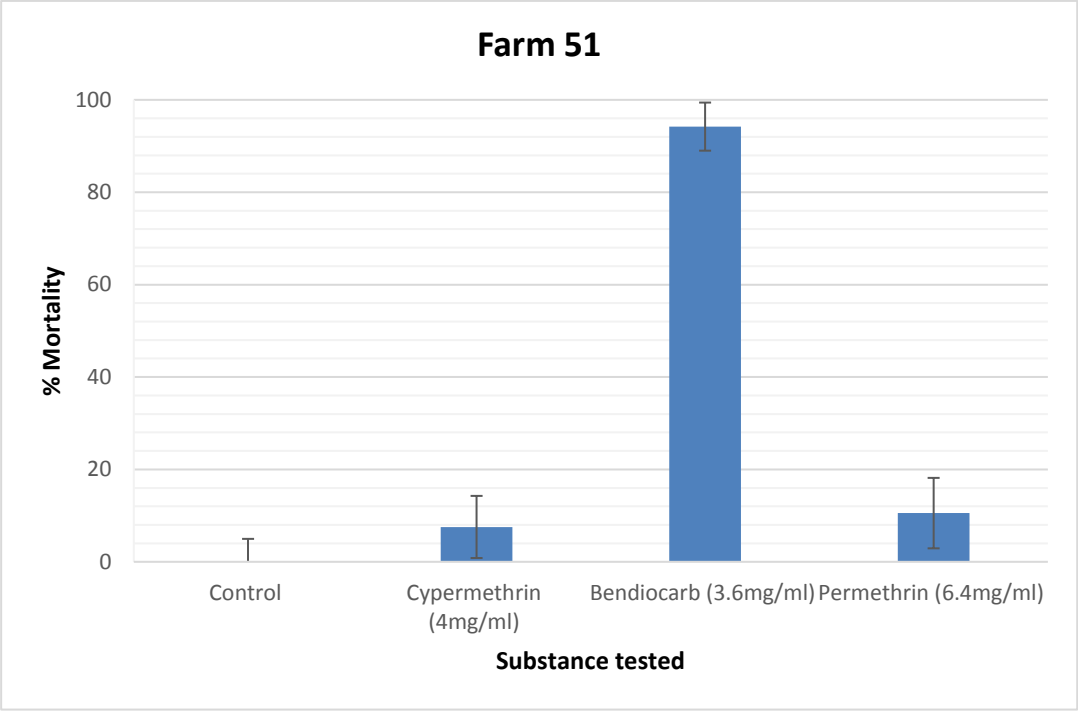
On farm 51, Milben Ex and Fendona were found to have 18.5 % and 13.1 % mortality respectively (Figure 2.8). The active ingredient of both these products is cypermethrin which had a mortality of just 7.5 % on the same farm, suggesting that testing of the active ingredient supports the results from the testing of the commercial product containing the same active ingredient. Ficam also supports this trend, with a mortality of 96.3 % on farm 51 and the active ingredient is bendiocarb which had a mortality of 94.2 % on the same farm. However, other results do not support this trend, on farm 52 cypermethrin had a mortality of 8.8 % whereas Milben Ex (active ingredient cypermethrin) had a mortality of 72 %. Also on farm 52 Perbio Choc (active ingredient permethrin) had a mortality of 100 % whereas permethrin alone had a mortality of 6.9 %. These results indicate that whilst the active ingredient is often the critical ingredient that determines

product effectiveness, occasionally other ingredients in the product may allow the active ingredient to be more effective than when used by itself at the same concentration. For example, Milben Ex contains 200 g/L cypermethrin which is a type II pyrethroid but also 1 g/L tetramethrin which is a type I pyrethroid. It is therefore possible that the addition of the different form of pyrethroid, which is potentially metabolised by a different P450, could allow a commercial product to remain effective where the main active ingredient is no longer effective.

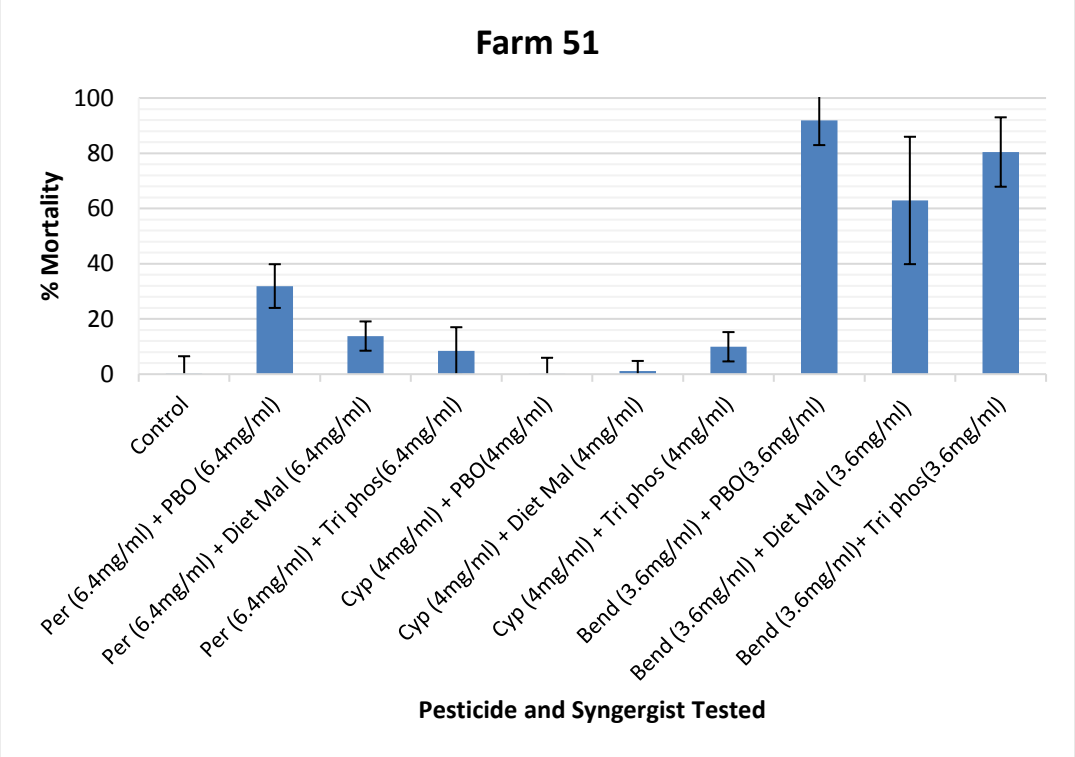
Due to the low level of toxicity of some compounds when used at the recommended concentration, synergist testing was completed to try and improve the efficacy of the active ingredients and identify the mechanisms underlying resistance. Synergists are commonly used in combination with pesticides to suppress metabolism-based resistance and increase the efficacy of the agents (Pasay et al., 2009). Synergism studies have been completed in other arthropod species in order to try and increase the efficacy of current control products. For example, PBO has been tested in *Hyalella Azteca* with pyrethrins, which found the LC50s for pyrethrins combined with PBO to be higher than the LC50 for pyrethrins alone at low ratios of PBO: pyrethrin (1:1) and much higher ratios (100:1) were required for a synergistic effect to be observed (Giddings et al., 2016).

The synergists chosen for this study were diethyl maleate (DEM), piperonyl butoxide (PBO) and triphenyl phosphate (TPP). These synergists inhibit different metabolic pathways; DEM inhibits glutathione S-transferase, TPP is an esterase inhibitor and PBO is a general inhibitor of P450s. The mechanism by which PBO inhibits P450s is well documented (Philippou et al., 2013; Hodgson and Philpot, 1974), however it is also worth noting that PBO can interact with esterases and inhibit their action in species such as the peach potato aphid, *Myzus persicae*

and the mosquito *A. aegypti,* and therefore effects seen in PBO studies are not conclusive of the role of P450 action (Philippou et al., 2013; Pereira et al., 2014).



A



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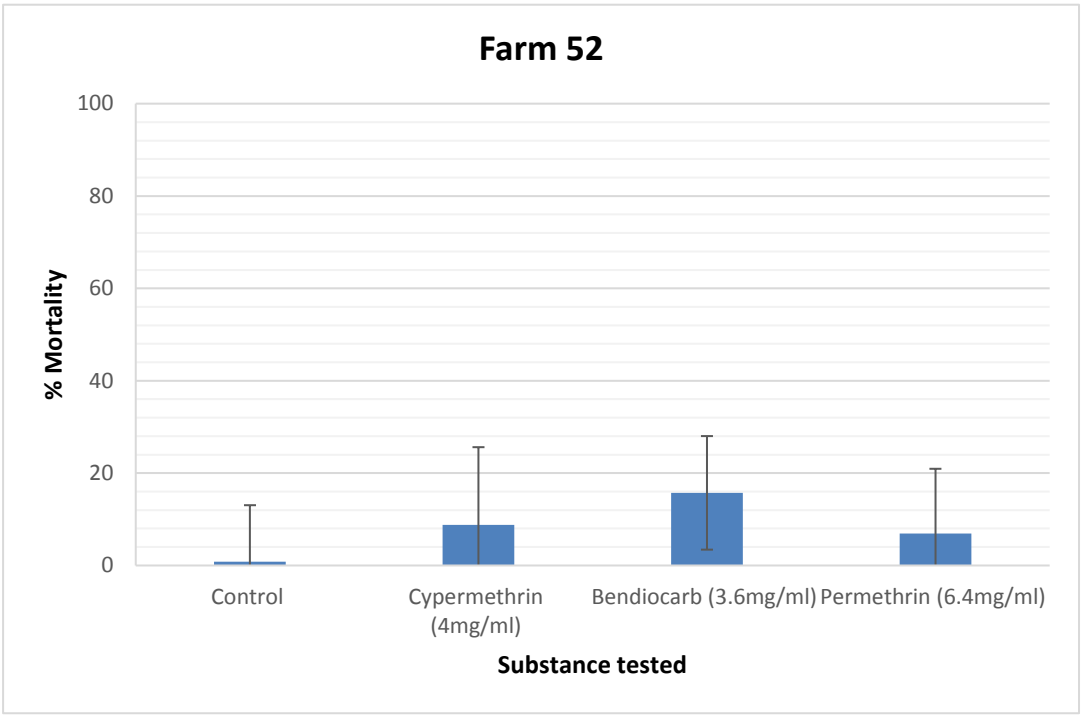
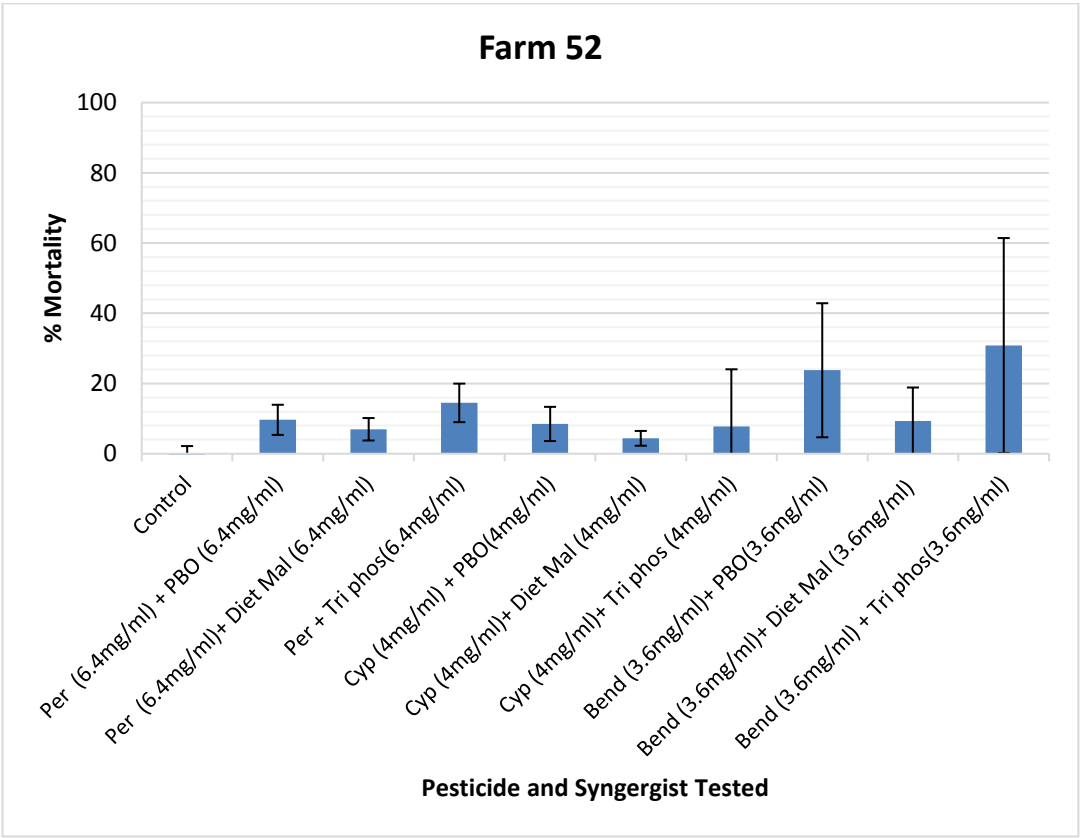
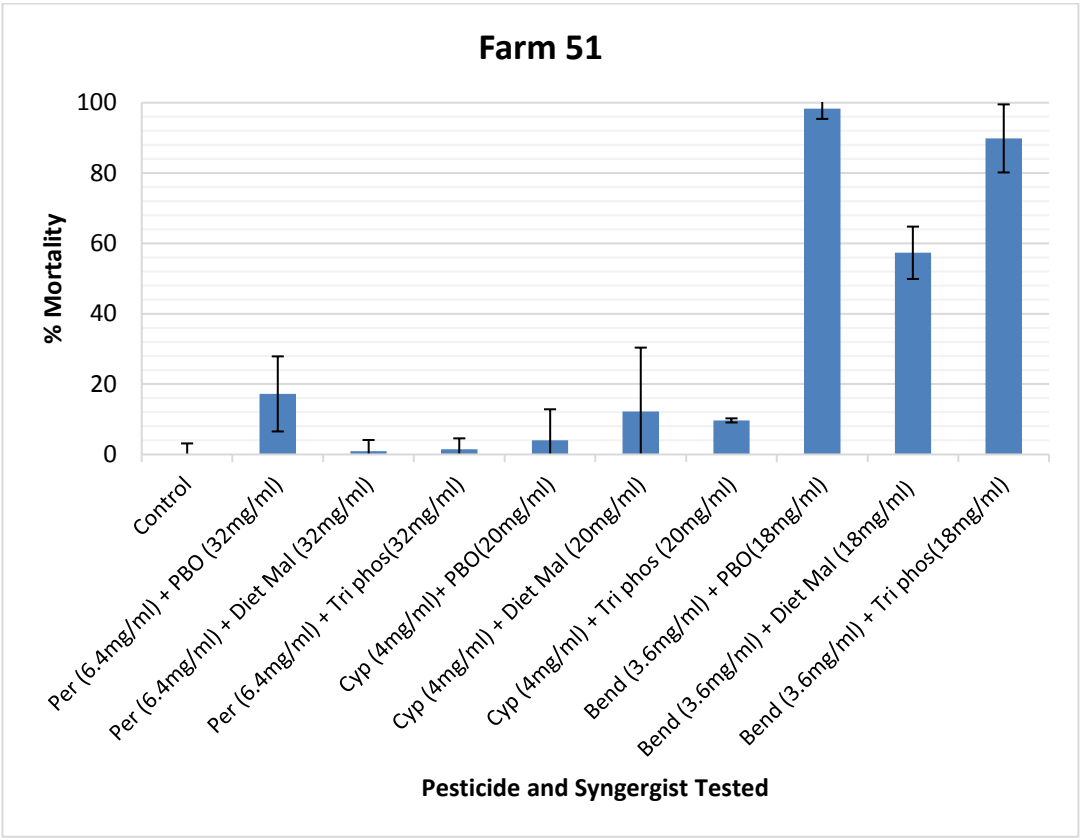

Farm 52
100
80
60
40
20
0
% Mortality
Control
Cypermethrin (4mg/ml)
Bendiocarb (3.6mg/ml)
Permethrin (6.4mg/ml)
Substance tested

C


Farm 52
100
80
60
40
20
0
% Mortality
Control
Per (6.4mg/ml) + PBO (6.4mg/ml)
Per (6.4mg/ml)+ Diet Mal (6.4mg/ml)
Per + Tri phos(6.4mg/ml)
Cyp (4mg/ml) + PBO(4mg/ml)
Cyp (4mg/ml)+ Diet Mal (4mg/ml)
Cyp (4mg/ml)+ Tri phos (4mg/ml)
Bend (3.6mg/ml)+ PBO(3.6mg/ml)
Bend (3.6mg/ml)+ Diet Mal (3.6mg/ml)
Bend (3.6mg/ml) + Tri phos(3.6mg/ml)
Pesticide and Syngergist Tested

D



E

Figure 2.9: Results of the toxicity testing from 2 farms (51 and 52) on adult female PRM. Figure 2.9: Results of the toxicity testing from 2 farms (51 and 52) on adult female PRM. *A= Farm 51, active ingredients tested. B= Farm 51, synergists were tested at 1 X concentration of active ingredients tested. C= Farm 52, active ingredients tested. D= Farm 52, synergists were tested at 1 X concentration of active ingredients tested. E= Farm 52, synergists were tested at 5 X concentration of active ingredients tested. Data represents the mean % mortality +/- SD of N = 3 (control= ethanol for all results shown), corrected using the Henderson Tilton correction.*

Using synergists on PRM from farm 51 and farm 52 (Figure 2.9) had little effect overall. The highest effects were seen using PBO; permethrin (6.4 mg/ml) and PBO (6.4 mg/ml) significantly increased mortality compared to permethrin (6.4 mg/ml) and DEM (6.4 mg/ml) (P = 0.0461). Permethrin (6.4 mg/ml) and PBO (6.4

mg/ml) also significantly increased mortality when compared to permethrin (6.4 mg/ml) and TPP (6.4 mg/ml) (P = 0.0253), leading to the assumption that P450s play a role in detoxification as inhibiting ESTs using TPP and inhibiting GSTs using DEM did not cause increased mortality, however using PBO as a general inhibitor of P450s did increase mortality. Permethrin (6.4 mg/ml) and PBO (6.4 mg/ml) significantly increased mortality compared to PBO (4 mg/ml) with cypermethrin (4 mg/ml) (P = 0.0050). However, PBO (3.6 mg/l) when used with bendiocarb (3.6 mg/ml) was more effective than both permethrin and PBO (6.4 mg/ml) and cypermethrin and PBO (4 mg/ml) (P = 0.0009 and P = 0.0006 respectively). DEM and PBO have recently been used along with s,s,s-tributylphosphorotrithioate (DEF), an inhibitor of esterases, to attempt to increase the efficacy of imidacloprid against *M. domestica.* All three showed significant synergism with imidacloprid in the resistant strain of flies and the activities of ESTs, GSTs and P450s were significantly higher than in the susceptible strain. Similar synergistic potential of DEF to imidacloprid between the susceptible and resistant strain suggested that GSTs and P450s played a more important role then ESTs in the resistant strain (Ma et al., 2017). This supports the results found in this study that PBO, an inhibitor of P450s, has significantly more of a synergistic effect than the inhibitors of GSTs and ESTs.

Identification of the specific P450 complexes in the PRM which are responsible for detoxification of pesticides is essential for targeting product ineffectiveness. Increasing the concentration of the synergists to 5 times the concentration of the active ingredient did not significantly improve the results compared to using the synergists at 1 times the concentration of the active ingredient. Overall, the addition of synergists made no significant difference to the effect of the active product alone on either of the farms tested and therefore despite significant

differences in the effect of synergists when compared to one another, they are not an effective option when looking to increase the efficacy of pesticides commercially.

The data generated is one of the first nationwide surveys carried out which is aimed at mapping acaricide resistance in PRM here in the UK or any European country. While the results are partly based on the perceived effectiveness of products by the egg-producing community, it is clear that this data has some guidance value in determining which products are potentially most effective on the whole, thus allowing the industry to take a more informed decision in their efforts to control PRM infestations and additionally facilitate efforts to limit the spread of resistance. However, toxicity testing data shows the survey cannot be solely relied upon as it often produces conflicting results. Resistance appears to be widespread and individual farms need to be toxicity tested in order to fully determine the best practice to use.

2.5 Conclusions and Further Research Plan

The conclusion of the study is that infestations with PRM are widespread across the UK, with 98 % of farms infested compared to 60-87.5 % of farms infested in previous studies, suggesting prevalence of PRM is on the rise in the UK (Fiddes et al., 2005; Guy et al., 2004). As well as increased prevalence, resistance appears present on many farms, with an overall perceived product effectiveness score of 7/10. The dramatic differences in perceived product effectiveness on farms in the same county leads to the conclusion that the best practice would be routine testing before application of acaricides on an individual farm level to ensure the efficacy of products in maintained.

Most often, farmers were aware of the issues of resistance on the farm and scored products being used on the farm a similar level to that found in toxicity testing in the laboratory. Any discrepancies found between the survey and the toxicity testing may be due to the 12 month time gap between them and hence may highlight the speed at which resistance could be developing.

In order to carry on with this work, more farms which have been surveyed would be allocated for mite collection in order to do further comparison of the perceived effectiveness compared to the actual product toxicity. A further survey in the future could allow for the comparison of the overall state of product effectiveness and resistance in the UK over time and would be a valuable insight for use in product development.

Chapter 3: Identifying and targeting resistance mechanisms in the Poultry Red Mite, *Dermanyssus gallinae*.

3.1 Abstract

Very little of the genetic structure of the PRM is documented and therefore efforts to isolate components of the detoxification pathways are hampered. To advance the understanding in the area of pesticide metabolism in the PRM, enzymes associated with metabolism need to be identified and isolated. 127 putative cytochrome P450 transcriptomic sequences were analysed to assign them a cytochrome P450 clade based on homology. Of the 127 sequences, 49 were potentially xenobiotic metabolising based on homology to *Anopheles gambiae.* These 49 sequences were then used in further analyses and 35 potential xenobiotic metabolising genes were identified based on homology to genetically similar species (*Musca domestica, Aedes aegypti* and *Drosophila melanogaster).* However, this will only allow the prediction of their potential metabolic substrates. A more targeted approach to identifying and isolating specific enzymes involved in the metabolism of acaricides is to use Activity Based Probes which require a preparation of microsomes from PRM which contain active cytochrome P450s. In this chapter, work has been carried out to generate viable microsomes for use in probe assays. However, the inactive P420 form of the cytochrome was detected and the assay requires further development in order to be used to identify the cytochromes responsible for acaricide detoxification. As well as identifying P450s, 2 potential P450 oxidoreductase gene fragments were found at the 5' and 3' end of the gene and attempts were made to isolate this sequence. A potential cytochrome b_5 sequence was identified which will be isolated and purified.

3.2 Introduction

One of the biggest issues that farmers face when trying to combat the problem of the poultry red mite (PRM) is the growing incidence of pesticide resistance. It has been estimated that €0.14 per hen is a direct cost spent on acaricides to target PRM (Van Emous, 2005), and from the survey completed in chapter 2 of this study, 98 % of farms reported infestations with PRM in the UK. Resistance to widely used products has been reported since 1984 in both of the major pesticide groups; organophosphates and pyrethroids (Abbas et al., 2014), and legislation means fewer new products are entering the market. Two products have been registered for use on PRM in the last 10 years (Byemite, Bayer and Exzolt, MSD Animal Health) and only Exzolt has been registered for use in the UK (HPRA, 2009). Exzolt, registered in 2017, has an active ingredient of fluralaner which is a systemic acaricide which is administered orally. Fluralaner selectively targets binding sites on nerve ligand-gated sodium channels which leads to paralysis and death (Thomas et al., 2018). This differs from the mode of action of other pesticides such as carbamates and pyrethroids but resistance could be forming to such a mechanism already, which requires a more in depth understanding in order to overcome it, a point which MSD Animal Health are unwilling to discuss (Gassel et al., 2014).

In the survey completed in Chapter 2 of this study, 53 % of the products reviewed were scored 7/10 or lower for product effectiveness, representing the low satisfaction in the products efficacy by the users. In the toxicity testing completed in Chapter 2, the great difference in product efficacy was demonstrated on an individual farm level. An example of this was Ficam which had a mortality of 96.3 % on Farm 51 and a mortality of 1.62 % on Farm 35, despite the farms being only 47 miles apart. This dramatic difference in efficacy, on farms which are so

geographically close together, illustrates the need to investigate resistance on an individual farm level. Identification of the cause of resistance in each individual case requires greater understanding of resistance mechanisms, and thus the enzymes involved, in order to explain this difference in mortality results from nearby farms.

Mosquitoes have been treated as a vector using pyrethrum from flower extract since the 1930s and resistance to pyrethroids and other insecticides such as Dichdichlorodiphenyltrichloroethane (DDT) and dieldrin has been reported since 1956 (Davidson, 1956; Ginsburg, 1937) . This resistance occurs through bio-degradation of the insecticide. In mosquitoes resistance is mainly associated with target site modification and metabolic resistance (David et al., 2013).

Target site resistance to both carbamates and organophosphates is conferred by a single point mutation which causes insensitivity of AChE and allows it to resist phosphorylation (Weill et al., 2004; Antonio-Nkondjio et al., 2016). This mutation, which is coded for by the *Ace-1*^{R} gene, involves a substitution from glycine to serine at position 119 (G119S) in certain species of mosquito; *Culex quinquefasciatus, Anopheles gambiae* and *Anopheles albimanus* (Scott and McAllister, 2012; Penilla et al., 1998; Djogbenou et al., 2008; Antonio-Nkondjio et al., 2016). *Rhipicephalus microplus* has been shown to have a 30 % insensitivity of AChE to propoxur (a pesticide of the carbamate family) which indicates that AChE also plays a role in resistant tick populations (Janadaree Bandara and Parakrama Karunaratne, 2017).

Pyrethroids target voltage sensitive sodium channels, and substitutions in this target site inhibit binding of the pyrethroid and hence inferring resistance. So far more than 50 sodium channel mutations have been identified in pyrethroid resistance insect pests and human disease vectors, many of which have been

functionally confirmed to be responsible for pyrethroid resistance (Dong et al., 2014; Rinkevich et al., 2013).

In *R. microplus*, a mutation in the domain II S4-5 linker region of the para sodium channel has been linked to synthetic pyrethroid resistance. The mutation causes a change from cytosine to adenine at position 190 in the tick sequence which causes a substitution from leucine to isoleucine (L64I). Homozygote allelic frequencies correlated highly with the percentage survival at the discriminating concentration of cypermethrin (0.3 % w/v) (Morgan et al., 2009).

However, a 2010 study found that the L64I mutation was not found in tick populations that were resistant to flumethrin. The domain II S4-5 linker region sequence identified a G-T non-synonymous mutation at position 214 that results in a glycine to valine substitution (G72V). The frequency of the G72V homozygous genotype was moderately related to the percentage survival at the discriminating level of flumethrin (0.02 % w/v) ($r = 0.74$). Though when both G72V and L64I mutations were present, there was a much stronger relationship between genotype and resistance to flumethrin ($r = 0.93$), suggesting a connection between the two mutations of the same gene (Jonsson et al., 2010).

The mutation V1014 of the voltage-gated sodium channel (VGSC) has been linked to knockdown resistance (*kdr*) in numerous insects (Soderlund and Knipple, 2003). It has been shown that the *kdr* phenotype is a key predictor of the resistance phenotype (Brooke, 2008; Donnelly et al., 2009; Silva et al., 2014). The V1014 mutation was first reported in *M. domestica* and conferred pyrethroid resistance and the mutation has subsequently been identified in several species (Williamson et al., 1996; Silva et al., 2014; Dong et al., 2014; Fang et al., 2019).

The V1016G point mutation in *A. aegypti,* the primary vector of dengue virus, has been found to significantly correlate with the survivability rates and resistant ratios in pyrethroid bioassays (Amelia-Yap et al., 2019). As well as a valine to glycine substitution (V1016G), a pheylalanine to cysteine substitution (F1534C) is also common in *A. aegypti* populations in Asia and is associated with resistance to type I pyrethroids (Saingamsook et al., 2017; Yanola et al., 2010).

Parasitic mites have also had VGSC mapped. *Varroa destructor* has had a significant worldwide impact on bee colony health and reports of pyrethroid resistance are widely reported (Rosenkranz et al., 2010; Gracia-Salinas et al., 2006; Bak et al., 2012; Elzen et al., 1998). A modification of leucine to valine at position 925 (L925V) of the VGSC has been correlated with resistance, with high mutation frequencies exclusively in hives with recent history of pyrethroid treatment (Gonzalez-Cabrera et al., 2013; Gonzalez-Cabrera et al., 2016; Hubert et al., 2014).

However, studies have shown that resistance to pesticides in arthropods is most commonly through the increased expression of enzymes which are capable of pesticide metabolism. Metabolism of a drug is divided into two main phases, phase I and phase II; phase I involves modification of the drug which prepares it for the phase II reactions by the addition or exposure of functional groups, phase II involves conjugation of these functional groups which allows the drug to be excreted (Gibson and Skett, 2001). These detoxification enzymes are namely enzymes such as glutathione-S-transferases (GST) (phase II detoxification), cytochrome P450 (CYP) (phase I detoxification) and esterases (EST) (phase I detoxification) (Wan et al., 2014; Istvan et al., 2017; Janadaree Bandara and Parakrama Karunaratne, 2017). P450s, as well as GSTs, have been highlighted

as principle agents in xenobiotic metabolism (Sun et al., 2018; Clements et al., 2017; Feyereisen, 2011).

GSTs are involved in phase II (conjugation) of the detoxification process. GSTs catalyse the conjugation of reduced glutathione (GSH) to the electrophilic xenobiotic (Bartley et al., 2015; Sheehan et al., 2001). The glutathione conjugate targets the xenobiotic to membrane transporters which allow it to be excreted form the cell during the final phase of the detoxification process (Bartley et al., 2015; Cole and Deeley, 2006). GSTs have been proven to be overexpressed in resistant species, with one study finding a ten-fold increase in GST activity in organophosphate resistant *Tetranychus urticae* (red spider mite) (Nauen and Stumpf, 2002). A five-fold increase in the expression of a GST transcript was found in *R. microplus* after exposure to the organophosphate coumaphos and in *Anopheles funestus* GSTe2 was the most upregulated detoxification gene in both DDT and permethrin resistant strains (Saldivar et al., 2008; Guerrero et al., 2012; Tchigossou et al., 2018). As well as the overexpression of GST genes, cytochrome P450 genes CYP6P9a and CYP6P9b were also overexpressed in resistant strains of the mosquito, with GSTd1-5 and GSTd3 more upregulated in strains resistant to DDT than in permethrin resistant strains (Tchigossou et al., 2018).

Metabolic resistance occurs most often via the over expression of detoxification enzymes such as P450s, GST's and Carboxy/cholinesterases (Hemingway and Ranson, 2000; Bartley et al., 2015; Tchigossou et al., 2018; Antonio-Nkondjio et al., 2017). P450s are the main detoxification enzyme family associated with the increased resistance to insecticides such as pyrethroids in mosquitoes, which is the most commonly used insecticide in vector control (David et al., 2013; Antonio-Nkondjio et al., 2017).

The P450 monooxygenase arrangement is an electron transport chain cycle which involves both hydroxylation and oxidation pathways in order to degrade and detoxify compounds (Deng et al., 2007). The P450 protein itself is the terminal oxidase of the cycle and is a super family of proteins with many clades. Arthropod P450s fall into four different clades; CYP2, CYP3, CYP4 and mitochondrial clade, based on evolutionary relationships (Feyereisen, 2006). The mitochondrial P450s and the CYP2 clades have been associated with vital roles in hormone biosynthesis, for instance CYP18A1, CYP307A1 and CYP306A1 in H*elicoverpa armigera* and CYP301A1 in *Drosophila melanogaster* (Gilbert, 2004; Zhang et al., 2016; Sztal et al., 2012; Zhang et al., 2018). However, the CYP3 and CYP4 clades have been associated with abiotic stresses including pesticide metabolism and detoxification roles, making them clades of interest in this project (Yu et al., 1984; Claudianos et al., 2006). Pyrethroid resistant mosquitoes have been observed to have higher levels of P450 activity than non-resistant mosquitoes (Amenya et al., 2008; Vulule et al., 1999). Commonly, the elevated P450 is linked to the CYP6 family (Hemingway et al., 2004; Fossog Tene et al., 2013; Antonio-Nkondjio et al., 2016). Resistance needs to be managed as soon as possible, removing the insecticide facing resistance to allow the susceptible phenotype to flourish again. To find the range of genes involved in resistance, a microarray was made from fragments of 230 *Anopheles gambiae* genes putatively involved in pesticide metabolism. This was then used to track the expression of detoxifying genes in resistant and susceptible strains leading to the identification of several P450s, as well as GSTs, associated with pyrethroid resistance in *A. gambiae* (David et al., 2005). In 2016, Antonio et al. (2016) proved both *A. gambiae* and *A. coluzzii* were resistant to bendiocarb and no AChE G119S mutation was detected, suggesting the resistance mechanism was metabolic rather than target site based. Microarray analysis showed over

expression of several cytochrome P450s including CYP6Z3, CYP6Z1, CYP12F2, CYP6M3 AND CYP6P4 (Antonio-Nkondjio et al., 2016). This principle has been expanded for use in other mosquito strains and now new initiatives are under development to provide monitoring tools for resistance. The Innovative Vector Control Consortium has developed a Vector Population Monitoring Tool which can collect deceased mosquitoes and measure their insecticide resistance status and malaria sporozoite infection rates (Hemingway et al., 2006). This major development has only been possible through the availability of the vector's genome sequence (David et al., 2013). Recommendations can now be made on an individual case level on the best practice for insecticide use in mosquitoes which manages the spread of resistance.

Genes found in the clade CYP3 are most numerous in insect P450s and this is the clade of interest in terms of pesticide metabolism as it contains the subfamilies CYP6 and CYP9 (Feyereisen, 2006). CYP6 and CYP9 subfamilies have been extensively linked to xenobiotic metabolism and therefore, pesticide resistance in many insect species including *Triatoma infestans, Aedes aegypti, Locust migratoria* and *Drosophilia melanogaster* and members of this subfamily are inducible by both pesticides and natural compounds, making them the families of interest in PRM (Grosso et al., 2016; Ishak et al., 2017; Huang et al., 2012; Chandor-Proust et al., 2013; Wan et al., 2014; Guo et al., 2016; Sun et al., 2018).

Data available for resistance mechanisms in PRM is mostly observational data that has been taken from studies of mite behaviour as well as toxicity testing experiments (Nechita et al., 2015; George et al., 2010b; George et al., 2010a; Brauneis et al., 2017). Very little of the PRM's genomic structure is documented

and therefore efforts to isolate components of the detoxification pathways are hampered.

The mechanism of P450 action in eukaryotes is localised to the smooth endoplasmic reticulum and is a process of an electron transport cycle. The substrate (pesticide) binds to the heme group of the P450 which induces an electron transfer from NADPH via POR. O_2 binds to the resulting ferrous heme which forms a highly reactive intermediate. A second electron is then transferred from a redox partner, this could be a second electron from POR or could be an electron provided by cytochrome b_5. Hydrogen atoms bind to the split O_2 to form H_2O and the remaining oxygen is temporarily bound to the iron before it is inserted into the substrate, to complete the monooxygenase reaction (Figure 3.1).

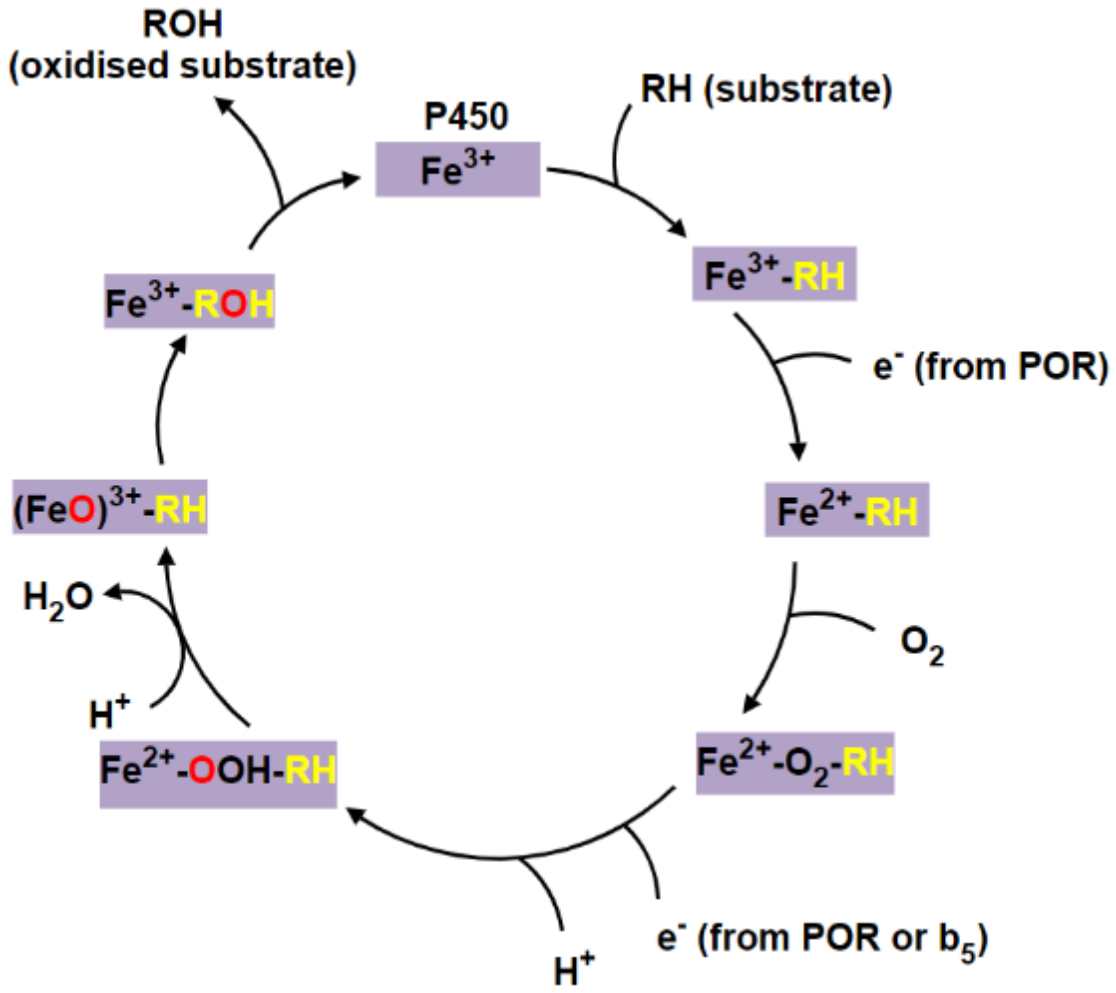


Figure 3.1: The catalytic cycle of Cytochrome P450. *RH represents substrate and is shown in yellow; POR represents cytochrome P450 oxidoreductase; b_5 represents cytochrome b_5.*

The ability to provide recommendations for treatment on a per case basis needs to be achieved in PRM as resistance is widespread and cannot be tracked to a geographical location, with resistance varying on an individual farm level (Chapter 2). PRM is also a haematophagous arthropod which may share similarly high levels of P450 activity in resistant strains to other insect species, such as the mosquito and the cattle tick. Transcriptomic sequence data has been provided, by Prof. Dr. Christina Strube, and will be analysed in this project to allow the identification of CYPs and assignment to families based on sequence

homologies. The same process will be carried out for the accessory proteins which are associated with P450 complexes, POR and cytochrome b_5, to allow for expression and characterisation of these proteins which is discussed in Chapter 4. Bioinformatics analysis of the P450s will only allow the prediction of their potential metabolic substrates. A more targeted approach to identifying and isolating specific enzymes involved in the metabolism of the acaricide of interest is to use Activity Based Probes (ABPs). In this approach the probe is a chemically modified form of the acaricide of interest such that it can bind to the enzyme, but not be metabolised by it. The probe is tagged to allow purification of the enzymes it is bound to, as well as allowing for visualisation on protein gels (Ismail et al., 2016). The use of probes requires a microsomal prep to be made from the PRM, which will be developed as part of this project, and for that prep to contain active P450s.

In this chapter, work will be completed to analyse the transcriptomic data from the PRM and assign sequences a putative xenobiotic metabolising role based on homology to sequences in similar species with known xenobiotic metabolising functions. This is based on a similar approach used by a previous PhD student, Kirsty Graham, who used it to successfully isolate a novel CYP, (CYP3006G8), cytochrome P450 oxidoreductase (POR) and cytochrome b_5 from *R. microplus* and cytochrome b_5 from *I. scapularis.* Based on this work, the PRM sequences will then be used to isolate POR and b_5 sequences in order for the accessory proteins to be expressed and characterised. Finally, microsomes will be prepared and an assay developed to determine the level of active P450s in the sample for use with APBs (Ismail et al., 2016).

3.3 Methods

3.3.1 Analysis of Transcriptome Using Bioinformatics

PRM transcriptomic data that has been acquired from collaborators in Germany (Prof. Christina Strube, Institute for Parasitology, University of Veterinary Medicine Hannover) was used to identify potential PRM acaricide metabolising P450s. The bioinformatics database used for this project was the National Center for Biotechnology Information (NCBI).

The 127 sequences sent were all putatively P450 sequences which had been pre-screened by Prof. Strube. These were entered into a Basic Local Alignment Search Tool via the National Centre for Biotechnology Information (NCBI) using a BLASTn search in the nucleotide collection (nr/nt) database to get a description of any similar sequences found and what clade the sequences belonged to (Figure 3.2). Any sequences presumed to be xenobiotic metabolising (49 sequences) were then taken and further researched with the aim of finding homology to cytochrome P450 clade 3 family (subfamilies monooxygenase 6 and cytochrome P450 monooxygenase 9) in *Anopheles gambiae*. All 49 sequences were also searched against each other in NCBI using a BLASTn multiple alignment search and sequences with a high homology were aligned using Clustal Omega Multiple Alignment Tool (ClustalW with character counts output with default settings). Of the 127 initial partial sequences, 37 were putatively found to metabolise xenobiotics based on P450 homology in *Anopheles gambiae* (sequences were only considered if the query cover was over 20 %). This process was also completed in reverse, with known pyrethroid metabolising enzymes from the mosquito (CYP6Z2; Accession number AF487780.1, CYP6P3; Accession number AF487534.1) being searched in the PRM database (tax id: 34641) to confirm homology. These PRM sequences were then searched against other

insect databases which contained known pyrethroid metabolising sequences (*Musca domestica;* tax id 7370*, Drosophila melanogaster*; tax id 7227 *and Aedes aegypti;* tax id 7159). This was to further verify the pyrethroid metabolising sequences found from the original homology to *A. gambiae*. Of the 37 matches to CYP6 and CYP9 in *Anopheles gambiae*, 35 sequences have homology across the three additional species searched.

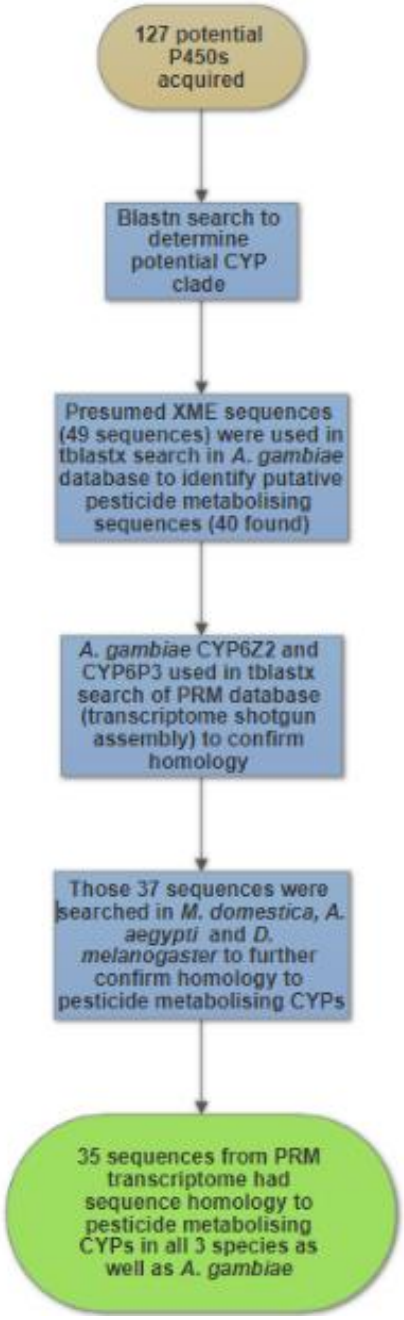


Figure 3.2: Flowchart of P450 sequences isolation process. *The chart begins with the 127 sequences that were sent as potentially PRM P450s (beige) and ends with the 35 sequences which are most likely to be drug metabolising sequences from the PRM (green). XME; xenobiotic metabolising enzyme.*

3.3.2 The Use of Bioinformatics to Identify Potential b_5 and POR Sequences

PRM transcriptomic data that has been acquired from collaborators in Germany (Prof. Prof. Christina Strube, Institute for Parasitology, University of Veterinary Medicine Hannover) was also used to identify potential PRM accessory proteins.

The known nucleotide sequence for cytochrome b_5 in *Rhipicephalus (Boophilus) microplus* (southern cattle tick) was used to complete a tBLASTx search, using NCBI, against the Transcriptome Shotgun Assembly (TSA) database of the *Dermanyssus gallinae* (tax id: 34641). This corresponded to a gene in the PRM transcriptome database with 72 % identity to the cattle tick b_5 (accession number GAIF01005602.1).

The cattle tick POR gene was also used to identify a homologous gene in PRM. The known POR gene from the cattle tick was searched in NCBI using tBLASTx search against the Transcriptome Shotgun Assembly (TSA) database of the *Dermanyssus gallinae*. This corresponded to two partial genes from the PRM. One gene section was at the 5' end of the POR gene (GAIF01009008.1) and the other was at the 3' end of the POR gene (GAIF01021004.1).

3.3.3 Production of a Phylogenetic Tree for Cytochrome b_5

A phylogenetic tree was created in order to view the relationship between the b_5 gene in acari as well as other insect and host species. This was created using the FASTA sequences from each species which were obtained from NCBI's nucleotide database. The FASTA were aligned using Clustal Omega (DNA input). The alignments were arranged into a phylogenetic tree using the R project for statistical computing by a fellow PhD student, Gregory Young.

3.3.4 RNA Extraction and the Isolation and Amplification of P450 oxidoreductase (RACE ready cDNA Synthesis)

RNA was extracted from 0.5 g of PRM and stored in RNAlater (Thermo Fisher, Paisley, UK). Mites were homogenised manually, using a pestle and mortar, before adding 1 ml of TRIzol® reagent (Thermo Fisher, Paisley, UK). The lysate was incubated for 5 minutes at room temperature, 0.2 ml of chloroform was added and the lysate was shaken vigorously. This was centrifuged for 15 minutes at 604 xg, 40 °C, in a benchtop centrifuge. The aqueous phase was then put through the PureLink RNA Mini Kit (Life Technologies, New York, USA) following manufacturer's instructions before being eluted in 30 µl of nuclease free water. The RNA quantity (ng/µl) and purity (260/ 280 value) was measured using the ND-100 (Nanodrop Technologies, Wilmington, USA) and integrity was checked using agarose gel electrophoresis. 2 µl of 2 X RNA loading buffer (20 % (v/v) Glycerol, 0.025 % (w/v) Bromophenol Blue, 1 X TAE) was added to 4 µl of RNA and the sample heated at 70 °C, then cooled instantly on ice. The samples were analysed by electrophoresis on a 1 % (w/v) agarose gel and were considered of good quality if no smearing was visible.

This RNA was used in a RACE (Rapid Amplification of cDNA Ends) reaction to provide a full-length POR sequence via a GeneRacer kit (Life Technologies, Paisley, UK). The protocol was followed according to manufacturer's instructions.

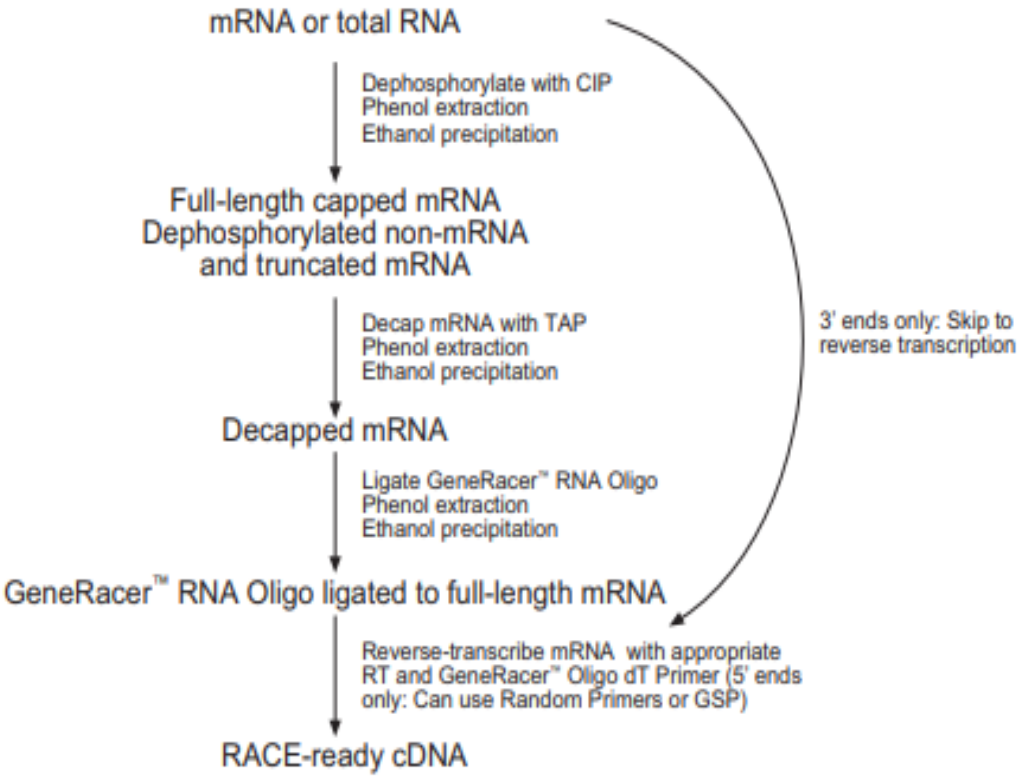


Figure 3.3: GeneRacer protocol overview. *Flow chart of the stages of the GeneRacer kit which begins with total RNA extracted from PRM and leads to RACE ready cDNA. Diagram adapted from manufacturer's protocol.*

cDNA prepared from the GeneRacer kit was then used to amplify fragments which corresponded to the 5' and 3' end of the putative POR gene. This was done using the gene specific primers (GSPs) which were designed as shown in Table 3.1. 5' and 3' race PCRs were carried out and the reactions were set up as follows for 5' RACE; 0.5 µl GeneRacer 5' primer (10 µM) (5' GGA CAC TGA CAT GGA CTG AAG GAG TA 3'), 0.5 µl GSP PRM POR 4 rev or GSP PRM POR 8 rev (10 µM), 0.5 µl RACE Ready cDNA template, 2.5 µl amplification buffer, 0.5 µl dNTPs (10 mM), 0.25 µl VELOCITY DNA polymerase, 7.75 µl sterile distilled water with a final volume of 12.5 µl.

For 3' RACE; 0.5 µl GeneRacer 3' primer (10 µM) (5' GCT GTC AAC GAT ACG CTA CGT AAC G 3'), 0.5 µl GSP PRM POR 4 fwd 1/2 or GSP PRM POR 8 fwd

1/2 (10 µM), 0.5 µl RACE Ready cDNA template, 2.5 µl amplification buffer, 0.5 µl dNTPs (10 mM), 0.25 µl VELOCITY DNA polymerase, 7.75 µl sterile distilled water with a final volume of 12.5 µl.

Table 3.1: Gene specific primers designed for use in RACE-PCR. *A set of primers was designed for each end of the POR gene. Two forward primers were designed for each pair of primers to increase chances of isolating the putative accessory protein.*

Primer I.D	**Forward primer 1 5'-3'**	**Forward primer 2 5'-3'**	**Reverse Primer 5'-3'**
PRM POR 4	GGA AGA TGA GGA TTC TTT GGC CGT G	ACT GAT CGA GAT GGC GGA AGA TGA G	CTC CGA CCA TAC GAG CGG CAT TG
PRM POR 8	TTA GCC ACT TGG AGT TTG CAG TGT G	CGT TTA GCC ACT TGG AGT TTG CAG T	GAT CGT GTT GTT CGT CCG CCC AC

5' and 3' RACE fragments were generated using the following PCR cycling conditions: 1 cycle of 98 °C for 2 minutes, followed by 40 cycles of 98 °C for 30 seconds, 61 °C (5' RACE) or 58 °C (3' RACE) for 30 seconds, 72 °C for 20 seconds, followed by 1 cycle of 72 °C for 10 minutes. These cycling parameters are the same as the parameters used in the positive GeneRacer control, the primers for which can be viewed in Table 3.2.

Table 3.2: Primers provided in the GeneRacer kit for use in RACE-PCR. *Two GeneRacer primers are provided, one 5' and one 3', as well as two control primers A and B.1.*

Primer I.D	Sequences	Bases	Tm
GeneRacer 5′ Primer	5′-CGACTGGAGCACGAGGACACTGA-3′	23	74 °C
GeneRacer 3′ Primer	5′-GCTGTCAACGATACGCTACGTAACG-3′	25	76 °C
Control Primer A	5′-GCTCACCATGGATGATGATATCGC-3′	24	72 °C
Control Primer B.1	5′-GACCTGGCCGTCAGGCAGCTCG -3′	22	76 °C

Both 5' and 3' RACE reactions were analysed by agarose gel electrophoresis using a 1 % agarose gel.

3.3.5 PRM Microsome Preparation

Microsomes were prepared using protocols recommended by Dr Hanafy Ismail (Ismail et al., 2016). Briefly, PRM were sourced from a farm in the North East of England and were stored at -80 °C after being snap frozen in liquid nitrogen. 1.25 g of thawed PRM were added to 10 ml of ice-cold PBS (2.7 mM potassium chloride, 137 mM sodium chloride, 10 mM potassium phosphate. pH 7.4 ± 0.2 (25 °C)). The PRM were homogenised using a handheld electric homogeniser (IKA T10 basic homogeniser workcenter) on ice. A cOmplete Protease Inhibitor

Cocktail Tablet (Roche) was added to the 10 ml solution. Insoluble material and debris was removed by centrifugation (Sorvall RC-5B Plus, SLA 1500 rotor) at 10,000 g, 4 °C for 10 minutes.

The clarified lysate was decanted and centrifuged in an ultracentrifuge (Beckman Coulter Optima L-100XP, rotor SW41 Ti) at 100,000 g for 45 minutes. Tubes used were thin walled ultra-clear tubes (Beckman Coulter). The microsomal pellet was resuspended in 1 ml of potassium phosphate buffer (50 mM potassium phosphate. pH 7.4 ± 0.2 (25 °C) containing 20 % glycerol), snap frozen in liquid nitrogen and stored at -80 °C until use.

3.3.6 Microsomal P450 Activity Testing

The total protein concentration of the microsomal preparation was determined using a Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher, Paisley, UK) using manufacturer's instructions to ensure a high level of protein was present in the sample.

In order to test the metabolic activity of the microsomal preparation, a protocol was adapted from McLaughlin et al. (2008). Briefly, microsomes were thawed on ice. 188 µL of potassium phosphate buffer (50 mM potassium phosphate. pH 7.4 ± 0.2 (25 °C) containing 20 % glycerol), was added to 15 wells of a Greiner flat-bottomed white 96 well plate. 10 µL of microsomes were added to each well, three were left clear of microsomes to be used as a control (10 µL of extra buffer was added to control wells). To examine metabolism, 2 µL of ethoxy-, methoxy-, benzyloxy- and pentoxy- resorufin (ER, MR, BR AND PR) (0.5 mM stock soluble in DMSO) were added to each well in triplicate. The plate was incubated for 10 minutes at 30 °C before being read on a plate reader (Biotek Synergy HT) for fluorescence (Excitation 530 nm; Emission 585 nm). 10 µL of 10 mM NADPH was added and the fluorescence readings were taken again.

To determine P450 content a method was adapted from Omura and Sato (1964a). Briefly, 7 µL of microsomes were added to 2 ml of spec buffer (200 mM Tris (pH 7.4), 20 mM CHAPS, 40 % glycerol, 2 mM EDTA). A small quantity of sodium hydrosulphite was added and gently mixed before dividing between 2 matched UV cuvettes. Absorbance was measured between 400-500 nm and a baseline was run on the spectrophotometer. One cuvette was taken as the sample cuvette and was flushed with CO for 30 seconds before reading on the spectrophotometer.

3.4 Results and Discussion

3.4.1 Gene Database and Cytochrome P450 Analysis

As well as resistance mapping, this project aims to characterise the mechanisms responsible for inferring resistance and to investigate novel ways of targeting such mechanisms. The value of such a task to the farming community cannot be understated. Farmers are facing low product efficacy and a lack of novel products on to the market (Gassel et al., 2014). An understanding of xenobiotic metabolism in PRM could allow for products to be produced which bypass the established route of xenobiotic metabolism and resistance in the PRM and hence improve product efficacy.

Many of the pesticides currently on the market which target PRM have an active ingredient from the pyrethroid family such as; Milben Ex (Schopf, Krölpa, Germany), Perbio Choc (Lodi, Kingswinford, UK) and Fendona (BASF, Cheadle Hulme, UK). Milben Ex was used by a third of the farms surveyed in Chapter 2 of the project and Perbio Choc and Fendona were used by 4 farms and 1 farm respectively. Resistance to pyrethroids is rising in PRM as well as resistance to other commonly used pesticides such as organophosphates and carbamates (Abbas et al., 2014). Due to the well-established role of cytochrome P450s in pyrethroid metabolism in other species, it was decided that focus would be on the isolation of the CYPs in PRM (Cossio-Bayugar et al., 2018; Smith et al., 2018; Walsh et al., 2018).

To begin this process, PRM transcriptomic data was provided by Prof. Christina Strube who had pre-screened the sequences and sent 127 which were potential cytochrome P450 sequences. The 127 provided sequences were individually entered into a Basic Local Alignment Search Tool via the National Centre for Biotechnology Information using the parameters outlined in section 3.3.1. This

was completed to get a description of the gene function of any similar sequences found in other species, so they could then be separated into whether or not the gene is capable of xenobiotic metabolism based on homology. Of the 127 sequences, 49 had high homology (over 50% identity) to known xenobiotic metabolising genes in other species. The 49 transcripts predicted to have a role in xenobiotic metabolism were assigned potential functions/P450 clades and were used for the next stage of analysis.

The 49 sequences were also run through a multiple alignment search using NCBI BLASTn to find homology to one another, in order to identify if any sequences could overlap and belong to the same P450 gene. Out of the 49 sequences, 7 different groups of sequences were found with high homology to one another (Table 3.3).

Table 3.3. Sequence alignments between sequences predicted to have a role in xenobiotic metabolism. *Sequences searched in NCBI BLASTn search against one another and matches with higher than 20% query cover and 50% percentage identity have been listed.*

Query Sequence	Percentage Identity (%)	Query Cover (%)	Matched Sequences
G9NSEKQ01BJ0HY	99	100	G9NSEKQ02HCZIP
	99	71	G9NSEKQ02J2W0U
	99	59	G9NSEKQ01C888X
G9NSEKQ02IHTVF	100	71	G9NSEKQ02IIAQI
	99	59	G9NSEKQ02GCXMA
	100	36	G9NSEKQ02GO1J4
isotig07728	99	100	isotig07729
	92	85	isotig03762
	92	85	isotig03761
	96	51	isotig07730
	92	55	isotig03766
	94	51	isotig07731
	89	38	isotig03765
	95	10	G9NSEKQ02IHP0I
G9NSEKQ02HLDP6	100	100	G9NSEKQ02JCWD2
	97	62	G9NSEKQ01BSUN6
G9NSEKQ02H37HD	89	100	isotig03762
	88	98	isotig03761
G9NSEKQ02GWOVH	99	54	G9NSEKQ02F57XU
G9NSEKQ02I4V33	99	85	G9NSEKQ02GMHEH

This could mean that these are sequences from different parts of the same P450 gene. Following isolation of a specific pyrethroid metabolising P450s using ABPs and identification of the corresponding nucleotide sequences from the database, these overlapping sequences would allow extension to potential obtain a full coding sequences for the individual P450s.Sequences which were presumed to be xenobiotic metabolising were then searched in a tBLASTx search (see section 3.3.1) in the *A. gambiae* database to find homology to XMEs.

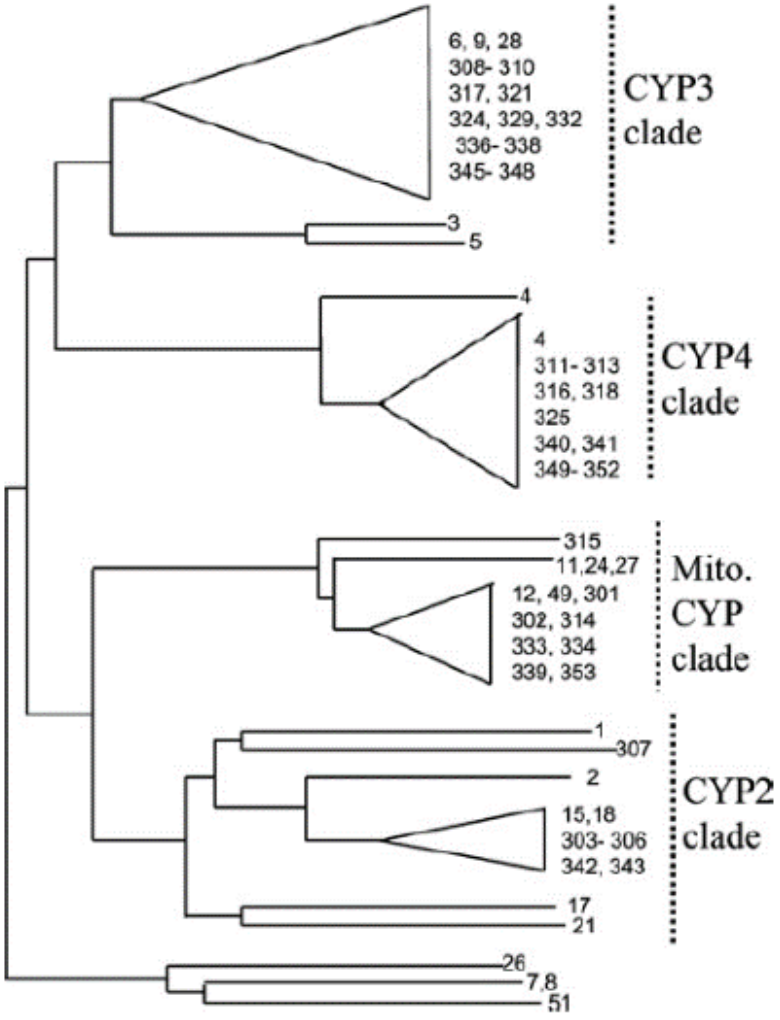


Figure 3.4: Insect P450 genes. *Insect P450 genes fall into four major clades which, as shown here, are CYP3, CYP4, mitochrondrial CYP and CYP2. Within each clade are the CYP family numbers attributed to insect P450 genes. Adapted from Feyereisen (2006).*

Insect P450s can be separated into 4 clades (Figure 3.4) which contain different subfamilies or clans. Of the 127 initial partial sequences, 37 were putatively found to metabolise xenobiotics based on P450 homology in *Anopheles gambiae.* All of the sequences searched in the *Anopheles gambiae* database had homology to the CYP3 or CYP4 clade (Table 3.4). Of the 37 sequences searched in the database 27 had homology to the CYP3 clade and 10 had homology to the CYP4 clade. Both of these clades are the clades to which xenobiotic metabolising P450s map to in nearly all insect species and have been frequently associated with resistance in mosquitoes (Feyereisen, 2006). CYP6Z1 and CYP6P3 in *A. gambiae* and CYP4G16 in *A. arabiensis* have all been identified as metabolising DDT, permethrin as well as deltamethrin respectively (Chandor-Proust et al., 2013; David et al., 2013). The 29 sequences with homology to the CYP3 clade were all of the clan Cytochrome P450 monooxygenase 6. This is responsible for pesticide metabolism in genetically similar species to the PRM such as CYP6 enzymes which cause resistance to pyrethroids in the fruit fly (*Bactrocera oleae*) and reduce mortality in response to deltamethrin in migratory locust (*Locusta migratoria*) (Pavlidi et al., 2018; Guo et al., 2016).

Table 3.4: PRM sequences with corresponding A. gambiae homology. *Column 1= PRM sequence name from the transcriptome data, Column 2= Max Score with A. gambiae sequence (NCBI tBLASTx), Column 3= Description of A. gambiae sequence that PRM sequence has homology to. Max Score of over 50 to mosquito XMEs have been shaded.*

PRM Sequence Name	Max Score (NCBI tBLASTx)	*Anopheles Gambiae* Sequence match	Associated P450 Clade
G9NSEKQ01BEANL	33.6	CYP6M1	CYP3
G9NSEKQ01BJ0HY	46.4	CYP6Y1	CYP3
G9NSEKQ02IHTVF	36.3	CYP6S2	CYP3
G9NSEKQ02IIAQI	36.8	CYP6S2	CYP3
G9NSEKQ02JXVSF	43.7	CYP6AA1	CYP3
isotig07728	56.1	CYP6P4	CYP3
isotig07729	56.1	CYP6P4	CYP3
isotig07730	58.8	CYP6P3	CYP3
isotig07731	60.6	CYP6P3	CYP3
isotig21871	42.3	CYP6P3	CYP3
isotig22816	98.7	CYP6AA1	CYP3
isotig23857	40.0	CYP6Z1	CYP3
isotig24478	67.5	CYP4AA1	CYP4
isotig30646	26.3	CYP6M1	CYP3
G9NSEKQ02IVX7L	50.1	CYP4C35	CYP4
isotig29635	63.8	CYP4G16	CYP4
G9NSEKQ02HLDP6	51.0	CYP4J9	CYP4
G9NSEKQ02JCWD2	51.5	CYP4J9	CYP4
G9NSEKQ01BSUN6	38.9	CYP4J9	CYP4
isotig12928	122	CYP4C26	CYP4
isotig12504	92.7	CYP6M4	CYP3
G9NSEKQ02H37HD	58.3	CYP325G1	CYP4
isotig03761	49.2	CYP6Y1	CYP3
isotig03762	49.2	CYP6Y1	CYP3
isotig03766	42.3	CYP6M1	CYP3
G9NSEKQ01CMVYR	39.6	CYP6Y1	CYP3
G9NSEKQ02J2W0U	31.3	CYP6N1	CYP3
G9NSEKQ02GWOVH	63.4	CYP6AA1	CYP3
G9NSEKQ02HCZIP	42.8	CYP6M4	CYP3
isotig20277	53.8	CYP6P4	CYP3
G9NSEKQ01DNWU8	30.9	CYP6S1	CYP3
G9NSEKQ02G7EKB	94.1	CYP4H16	CYP4
G9NSEKQ02GCXMA	28.6	CYP6S2	CYP3
G9NSEKQ02I4V33	50.1	CYP6P2	CYP3
isotig25683	55.1	CYP6Y1	CYP3
G9NSEKQ02IHP0I	30.9	CYP6R1	CYP3
G9NSEKQ01EFBC3	38.2	CYP314A1	CYP4

With a list compiled of sequences with a high homology to sequences in *Anopheles gambiae,* in clades and subfamilies which are capable of metabolising xenobiotics, the process could be completed in the reverse. Known pyrethroid metabolising enzymes from the mosquito were searched in the database to confirm homology within the PRM transcriptome. These sequences were; CYP6P3, confirmed to metabolise permethrin and deltamethrin in *A. gambiae* and CYP6Z2, confirmed to metabolise permethrin in *A. gambiae* (Muller et al., 2007; David et al., 2013). *A. gambiae* CYP6Z2 (AF487780.1) had 59 %, 41 % and 42 % identity to 3 reading frames of the PRM isotig25683 which had already been isolated as a potential pyrethroid metabolising enzyme. *A gambiae* CYP6P3 (AF487534.1) had 47 %, 47 %, 41 % and 38 % identity to PRM isotig20277 over 4 reading frames (with 30 % query cover) which had also been identified as pyrethroid metabolising in the mosquito. With the sequences having homology to known pyrethroid metabolising sequences in *A. gambiae*, the 37 sequences could be searched against other insect databases. This was to further verify the pyrethroid metabolising sequences found from the original homology to *A. gambiae*. *Musca domestica, Drosophila melanogaster* and *Aedes aegypti* were chosen to further search the genome database. *M. domestica* was chosen due to vast amount of research available and the huge genome research which has been completed as well as there being known pesticide metabolising gene sequences. A second mosquito species was chosen, *A. aegypti,* as an important pest species with high clinical significance and the ability to confirm results from *A. gambiae.* Finally, *D. melonagaster,* chosen due to it being extensively used in research and having a large genomic database. The results of this extensive search can be viewed in Table 3.5. Of the 37 PRM sequences found in *A. gambiae* to be potentially pyrethroid metabolising, 35 have P450 sequence homology across all three species (when only those with over 20 % query cover

in all species are considered). Of those sequences, 11 have a max score of over 50 in all three species. Many of the sequences have a high homology to CYP6A subfamily which are overexpressed in pesticide resistant *M. domestica* and have roles in detoxification in *Bactrocera dorsalis (Hojland et al., 2014; Huang et al., 2012).*

Table 3.5: PRM sequence identities with 3 other species. *PRM sequence names are shown with hits from 3 species as well as the corresponding Max Scores from NCBI as a measure of identity. Max scores of over 50 have been shaded. Results with less than 20 % query cover across all species have been removed.*

PRM Sequence Name	*Musca domestica* Sequence	Max Score	*Aedes aegypti* Sequence	Max Score	*Drosophila melanogaster* Sequence	Max Score
G9NSEKQ01BEANL	CYP6C2	35.9	CYP6Z8	33.1	CYP6A20	34.1
G9NSEKQ01BJ0HY	CYP6A37	69.3	CYP9M6v5	54.7	CYP6A19	56.5
G9NSEKQ02IHTVF	CYP6A37	34.5	CYP6AL1	32.7	CYP6A14	37.3
G9NSEKQ02IIAQI	CYP6A37	30.9	CYP6AL1	31.8	CYP28A5	31.8
G9NSEKQ02JXVSF	CYP6A4	45.5	CYP9J32	46.9	CYP6A14	44.6
isotig07728	CYP6A37	50.1	CYP9J32	50.6	CYP6A16	47.3
isotig07729	CYP6A37	50.1	CYP9J32	52.4	CYP6A16	49.6
isotig07730	(CYP6C1	52.4	CYP4	35.9	CYP6G1	54.2
isotig07731	CYP6C1	52.4	CYP4	35.4	CYP6G1	55.1
isotig21871	6A38v1	44.5	CYP9M6v2	43.2	CYP6A16	37.7
isotig22816	CYP6A5v2	96.8	CYP4H28v3	90.0	CYP6A16	105.0
isotig23857	CYP6G4	46.0	CYP9M6v4	46.4	CYP6D5	40.5
isotig30646	CYP6A37	28.1	CYP6Z8	24.9	CYP6A16	41.8
G9NSEKQ02IVX7L	CYP4D35	48.3	CYP4H44P	43.7	CYP311A1	38.2
isotig29635	CYP4D36	79.4	CYP4H31v2	79.4	CYP4C3	77.1
G9NSEKQ02HLDP6	CYP4G13v2	57.4	CYP4H44P	34.1	CYP4AC1	80.8
G9NSEKQ02JCWD2	CYP4G13v2	40.5	CYP4H44P	34.1	CYP312A1	41.4
G9NSEKQ01BSUN6	CYP4G13v2	42.1	CYP4H44P	34.4	CYP312A1	40.3
isotig12928	CYP4D4v2	80.3	CYP4H31v2	200.0	CYP4C3	179
isotig12504	CYP6A36	86.8	CYP9J28	101.0	CYP5A16	84.9
G9NSEKQ02H37HD	CYP6A4	54.6	CYP6CB1	62.9	CYP6A16	58.3
isotig03761	CYP6A4	55.6	CYP9J32	53.8	CYP6A16	57.9
isotig03762	CYP6A4	55.6	CYP9J32	53.8	CYP6A16	57.9
isotig03766	CYP6A37	42.8	CYP9J1	35.0	CYP6A16	42.8
G9NSEKQ01CMVYR	CYP6A25	41.8	CYP3A31	40.5	CYP6A16	44.1
G9NSEKQ02J2W0U	CYP6A37	48.7	CYP3A31	33.2	CYP9C1	40.5
G9NSEKQ02GWOVH	CYP6A5	50.1	CYP6AL1	50.6	CYP6A19	50.6
G9NSEKQ02HCZIP	CYP6A37	51.0	CYP9J	47.8	CYP6A2	49.6
isotig20277	CYP6A5v2	62.5	CYP9J24	54.2	CYP6A16	52.8
G9NSEKQ02G7EKB	CYP4D4v2	71.2	CYP9J26	75.8	CYP6A22	79.4
G9NSEKQ02GCXMA	CYP6A7	35.0	CYP6CB1	33.1	CYP6A14	37.3
G9NSEKQ02GMHEH	CYP6D3	27.2	CYP3A21	29.0	CYP9F3	25.4
G9NSEKQ02I4V33	CYP6C1	54.2	CYP3A21	59.7	CYP6D4	41.8
isotig25683	CYP6A40	47.8	CYP9J26	55.1	CYP6D5	49.2
G9NSEKQ01EFBC3	CYP6C1	27.2	CYP2j6	33.6	CYP305A1	39.1

As well as comparing the PRM sequence data to insect databases, the sequences were searched in an open database in order to analyse the top hits from the open search (Table 3.6). The majority of the cytochrome matches were from *Tropilaelaps mercedesae*, the Asian bee mite as well as *Galendromus occidentalis,* the western predatory mite. All cytochromes to which the PRM shows homology are from the CYP3 clade of cytochromes which gave further evidence of the pesticide metabolising properties of the PRM sequences isolated (Pavlidi et al., 2018; Guo et al., 2016).

Table 3.6: P450 homology to PRM sequences from NCBI database. *Results of BLASTx search in open database (non-redundant protein sequences nr) with no organism selected. Top cytochrome hits to which the PRM sequences have homology to are listed, as well as the species the cytochrome is from and the percentage identity (as taken from NCBI).*

PRM Sequence Name	Key NCBI Hits	Species	Percentage identity (%)
G9NSEKQ01BEANL	CYP P450 3A11	*Tropilaelaps mercedesae* (Asian bee mite)	53
G9NSEKQ01BJ0HY	CYP P450 3A31	*Tropilaelaps mercedesae* (Asian bee mite)	65
G9NSEKQ02IHTVF	CYP P450 3A21	*Galendromus occidentalis* (Western predatory mite)	65
G9NSEKQ02JXVSF	CYP P450 3A11	*Tropilaelaps mercedesae* (Asian bee mite)	53
Isotig07728	CYP P450 3A16	*Tropilaelaps mercedesae* (Asian bee mite)	63
Isotig07729	CYP P450 3A16	*Tropilaelaps mercedesae* (Asian bee mite)	62
Isotig07730	CYP P450 3A16	*Tropilaelaps mercedesae* (Asian bee mite)	56
Isotig07731	CYP P450 3A16	*Tropilaelaps mercedesae* (Asian bee mite)	56
Isotig21871	CYP P450 3A14	*Galendromus occidentalis* (Western predatory mite)	42
Isotig23857	CYP P450 3A14	*Galendromus occidentalis* (Western predatory mite)	48
Isotig30646	CYP P450 3A16	*Tropilaelaps mercedesae* (Asian bee mite)	54

Table 3.6: Continued.

Isotig12504	**CYP P450 3A21**	***Varroa destructor* (Varroa mite)**	**64**
G9NSEKQ02H37HD	CYP P450 3A6	*Tropilaelaps mercedesae* (Asian bee mite)	51
Isotig03761	CYP P450 3A16	*Tropilaelaps mercedesae* (Asian bee mite)	60
Isotig03762	CYP P450 3A16	*Tropilaelaps mercedesae* (Asian bee mite)	60
Isotig03765	CYP P450 3A16	*Tropilaelaps mercedesae* (Asian bee mite)	56
Isotig03766	CYP P450 3A16	*Tropilaelaps mercedesae* (Asian bee mite)	65
G9NSEKQ01CMVYR	CYP P450 3A31	*Galendromus occidentalis* (Western predatory mite)	38
G9NSEKQ02J2W0U	CYP P450 3A24	*Varroa jacobsoni* (Varroa mite)	62
G9NSEKQ02GWOVH	CYP P450 6D5	*Galendromus occidentalis* (Western predatory mite)	44
G9NSEKQ02HCZIP	CYP P450 3A24	*Varroa jacobsoni* (Varroa mite)	48
Isotig20277	CYP P450 3A19	*Centruroides sculpturatus* (Arizona bark scorpion)	38
G9NSEKQ02GCXMA	CYP P450 3A21	*Galendromus occidentalis* (Western predatory mite)	48
G9NSEKQ02GMHEH	CYP P450 3A56	*Varroa destructor* (Varroa mite)	49
G9NSEKQ02I4V33	CYP P450 3A56	*Varroa destructor* (Varroa mite)	57
Isotig25683	CYP P450 3A56	*Varroa jacobsoni* (Varroa mite)	54

Of the sequences searched in the open database, one of the highest homologies (65 %) was between the PRM sequence G9NSEKQ01BJ0HY and CYP P450 3A31 in the Asian bee mite (Figure 3.5). The alignment shows high homology across the length of the coding sequence.

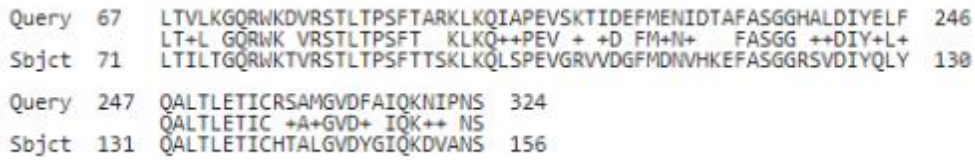


Figure 3.5: Alignment of PRM G9NSEKQ01BJ0HY and CYP P450 3A31 from the Asian bee mite. *Alignment created in NCBI. Query= PRM sequence, Subject= Asian bee mite sequence.*

From this analysis, a database has been created of potential pesticide metabolising P450 sequences which can be compared to future peptide data obtained from the ABPs. Any peptides isolated using the ABPs can be compared to the database and provide confirmation of gene function. This production of a library of sequences with probable pesticide metabolising functions is novel in the PRM and allows a wealth of future work to be done which could allow greater understanding of pesticide metabolism in PRM.

3.4.2 Bioinformatics Analysis of b_5 and POR from PRM

The importance of understanding the role of accessory proteins in the function of metabolising enzymes is vital to developing control methods for the pest species PRM. Firstly b_5 and POR sequences needed isolating using the transcriptomic data provided. Using the b_5 sequence from *R. microplus* as bait (see section 3.3.2), a highly similar sequence was identified from the PRM transcriptomic database (Figure 3.6).

Figure 3.6: Alignment of b_5 in cattle tick and PRM. *Comparison of the predicted PRM b_5 protein sequence against the known Cattle tick cytb_5 sequence, showing high similarities (72 % identity using NCBI) (GAIF01005602.1). Key heme associated residues have been coloured blue.*

The gene appeared highly conserved, missing just 4 amino acids from the start of the gene and 20 from the end of the sequence. To obtain the full sequence, the transcript sequence was taken from the PRM database and searched for a start and stop codon, to facilitate the incorporation of predicted start and stop codons and part of the untranslated 3' and 5' regions, which allowed the extension of the gene by 123 nucleotides at the 5' and 120 at the 3' end of the gene. Confidence in the correct identification of the b_5 gene was increased due to the presence of key heme associated residues, shaded blue in the alignment, which are conserved across all cytochrome b_5 sequences including mammalian (Schenkman and Jansson, 2003). These histidine residues co-ordinate with the pocket of the b_5 which contains the heme iron. This putative b_5 sequence could then be used for expression and characterising of the gene which is vital for the increased understanding xenobiotic metabolism in PRM (see Chapter 4).

Figure 3.7: An alignment between the cytochrome b_5 gene in Dermanyssus gallinae, Rhipicephalus microplus and Ixodes scapularis and their host species. *Alignment created between PRM b_5 as well as mosquito (A. gambiae), domestic fowl (G. gallus), human (H. sapien), cattle (B. Taurus), southern cattle tick (R. microplus) and deer tick (I. scapularis). Key heme associated histidine residues have been highlighted in black. The high sequence homology between host species and how this differs from acari is highlighted in green. Conserved region in tick species highlighted with dashed line.*

The cytochrome b_5 gene has been isolated from two veterinary important acari, *Rhipicephalus (Boophilus) microplus* (southern cattle tick), *Ixodes scapularis* (the black-legged tick or deer tick) as well as from *Dermanyssus gallinae* (poultry red mite). This allows comparison at the sequence level with other arthropods and their host species (Figure 3.7). Comparison of the tick and mite b_5 sequences to determine the degree of homology among haematophagous arthropods confirmed the conservation of key haem binding residues and highlights the high

homology around these residues. The green highlighted sequence shows the high sequence homology of host species and how this differs from acari. The presence of a histidine and proline residue in this position in the host but not in the acari could cause a slight change in structure which could become evident during 3D modelling. Also, the tick species have a conserved region of GVAAAAS, found near the C-terminus (highlighted with a dashed line in Figure 3.7) which is specific to the tick and is not found in host species.

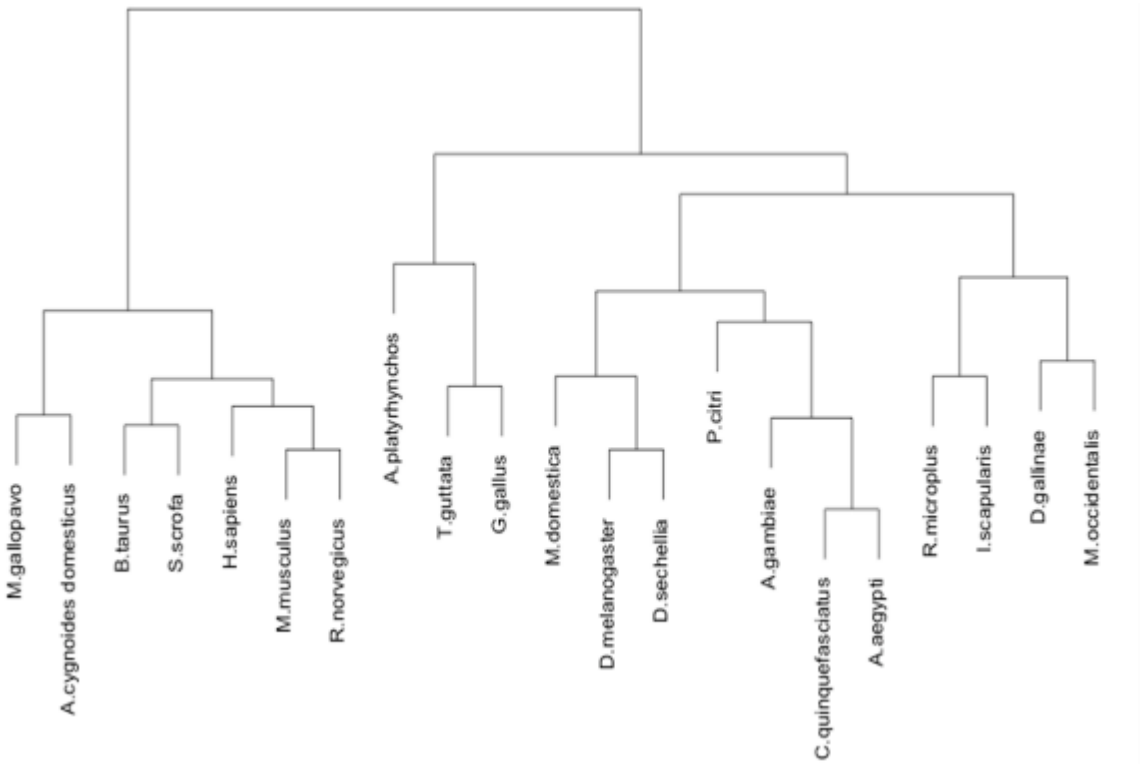


Figure 3.8: Phylogenetic tree of cytochrome b_5 sequences. *Sequences were aligned using Clustal Omega using FASTAs for each sequence from NCBI before being used to create a phylogenetic tree using 'R' statistical computing program.*

A phylogenetic tree was created to identify potential differences in cytochrome b_5 that could be exploited in order to design new targeted b_5 acaricides (Figure 3.8).

High homology was seen between the acari species as expected, with clustering to a distinct branch among the arthropods. The acari have clustered separately from haematophagous insects, who are also clustered distinctly from the other insects. Interestingly *Gallus gallus,* host of *D. gallinae,* has clustered to the right hand side of the tree whilst other mammalian species have clustered on the far left of the tree on a branch that splits early in the tree, implying greater sequence divergence. This suggests that there are differences enough in the $Cytb_5$ sequence to allow separation despite the high sequence homology. Accession numbers can be found in Table 3.7.

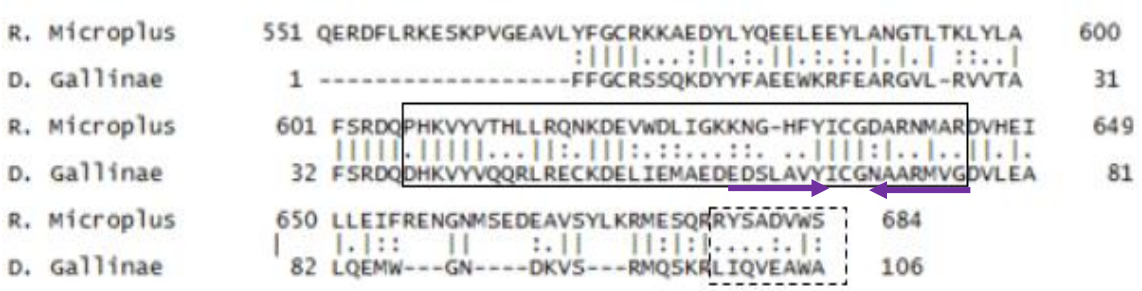
Table 3.7: Species included in phylogenetic tree. *The species are listed with both latin and common names as well the accession number for the cytochrome b_5 sequence. FASTA sequences for R. microplus and I. Scapularis were provided by a previous PhD student, Kirsty Graham.*

Accession Number	Latin name	Common name
XP_003748384	*Metaseiulus occidentalis*	Western orchard predatory mite
AHZ12900	*Panonychus citri*	Citrus red mite
XP_308640	*Anopheles gambiae*	African malaria mosquito
XP_001867082	*Culex quinquefasciatus*	Southern house mosquito
XP_001660580	*Aedes aegypti*	Yellow fever mosquito
NP_001274474	*Musca domestica*	House fly
ABW37749	*Drosophila melanogaster*	Fruit fly
XP_002032763	*Drosophila sechellia*	Fruit fly
NP_001025752	*Gallus Gallus*	Red jungle fowl
NP_001232778	*Taeniopygia guttata*	Zebra finch
XP_003204966	*Meleagris gallopavo*	Domestic Turkey
XP_012960808	*Anas platyrhynchos*	Mallard
XP_013036578	*Anser cygnoides domesticus*	Domestic goose
NP_776458	*Bos Taurus*	Cattle
P00167	*Homo Sapien*	Human
NP_080073	*Mus musculus*	House mouse
NP_071581	*Rattus norvegicus*	Brown rat
NP_001001770	*Sus scrofa*	Wild boar

Finally, the aim is to express the b_5 gene from *D. gallinae, R. microplus* and *I. scapularis* (Chapter 4). This will be done by using a recombinant baculovirus to express the cytochrome b_5 gene in cultured insect cells. By doing this, the effect

of changes in the expression of b_5 within cells can be determined and their susceptibility to pesticides can be monitored, thus assessing the possible role of b_5 in resistance. The information obtained from sequence comparisons and future 3D modelling will provide a molecular basis for the design of new acaricides or synergists targeting b_5 and thus provide a means of controlling populations of acari that have become resistant to the acaricides currently in use.

As well as isolating b_5 from the PRM transcriptome, POR needed to be identified as a key accessory protein for P450 complexes. POR was also found using the *R. microplus* sequence as a bait to search the PRM database (section 3.3.2). Unlike with b_5, no clear sequence was identified using this method, however two separate sequences which potentially represented both the 3' and 5' end of the POR sequence were identified.



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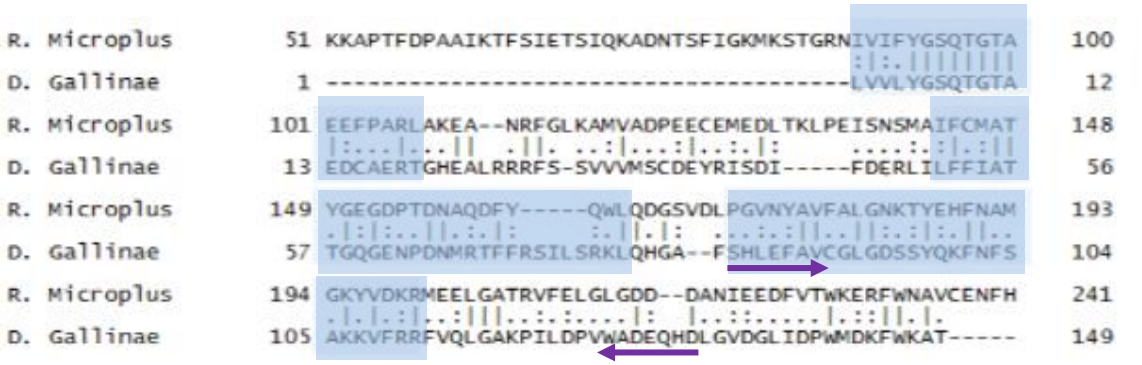


B

Figure 3.9: POR alignment of 3' and 5' end of the gene. *A= Comparison of predicted section of PRM POR sequence against the known cattle tick sequence at the 3' end of the gene (GAIF01021004.1). FAD domain boxed with a dashed line and NADP(H) domain boxed with a solid line. B= Comparison of predicted section of PRM POR sequence against the known cattle tick sequence at the 5' end of the gene (GAIF01009008.1). Key co-factor binding regions are shaded blue. Gene specific primer sequences for both forward and reverse primers used in GeneRacer reactions are represented by purple arrows for both sequences.*

These PRM sequences showed high homology to *R. microplus* POR at both the 3' and the 5' end of the gene. FAD and NADP(H) domains were highly conserved between the species (Figure 3.9) but the conservation of key co-factor binding regions, which vary little between acari species, was less conserved than expected (Wang et al., 1997) . This could result in a sequence being amplified

from PRM which was genetically similar to, but not, POR. Any new sequences which could be amplified from PRM and have homology to P450s or P450 accessory proteins would be of value to future PRM research and so the partial sequences were used for downstream applications. These sequences were used to create RACE ready cDNA which was required to amplify a full length PRM POR gene.

3.4.3 Amplification of P450 Oxidoreductase from *Dermanyssus gallinae*

The sequence that was isolated as potentially being PRM POR was found in two sections, the 3' end and the 5' end, as described in section 3.3.2. In order to find the full-length sequence, RACE (Rapid Amplification of cDNA Ends) ready cDNA was prepared using the GeneRacer Kit. This was to generate full length, RNA ligase-mediated rapid amplification of 5' and 3' ends of the POR sequence. Primers were designed to both ends of the putative POR fragment, with 2 forward primers being designed for each set to maximise chances of successful amplification, see Table 3.1. The protocol was completed, as described in section 3.3.4, using the second RNA extracted from the PRM which can be viewed in Figure 4.7 of section 4.4.1 after being run on an agarose gel.

As well as using the GeneRacer kit on the PRM RNA, HeLa total RNA, provided with the GeneRacer kit, was used as a positive control to confirm the kit's viability. Control PCRs were then performed on the HeLa cDNA before the PCRs were carried out on the PRM cDNA. Control primers to the β-actin gene are included for use in the positive control PCRs (Table 3.2) and when the control primers are used with the 5' or 3' GeneRacer primers, the 5' or 3' end of the β-actin gene is amplified. When the GeneRacer 5' primer and Control Primer B are used with the HeLa template, a band of around 850 bp is amplified, when the 3' GeneRacer and the control Primer A are used with the HeLa template, a region of around

1750 bp is amplified and, finally, when Control Primer A and Control Primer B are used a region of around 750 bp is amplified (Figure 3.10).

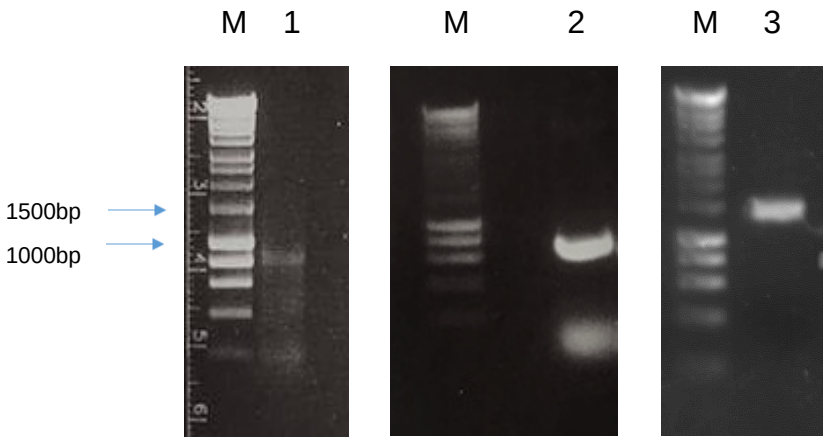


Figure 3.10: GeneRacer positive control. *M= molecular markers, Lane 1= GeneRacer 5' primer + Control Primer B.1 + HeLa template, Lane 2= Control Primer A + Control Primer B.1 + HeLa template, Lane 3= GeneRacer 3' primer + Control Primer A + HeLa template.*

PRM POR was expected to be around 2200 bp long in total, based on the length of the RM POR sequence. Initial PCR using VELOCITY DNA Polymerase (Bioline, London, UK) was unsuccessful, but optimisation of the PCR using cycling parameters of: 1 cycle of 98 °C for 2 minutes, followed by 30 cycles of 98 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 60 seconds, with a final step of 72 °C for 10 minutes produced a more promising result, as seen in Figure 3.11.

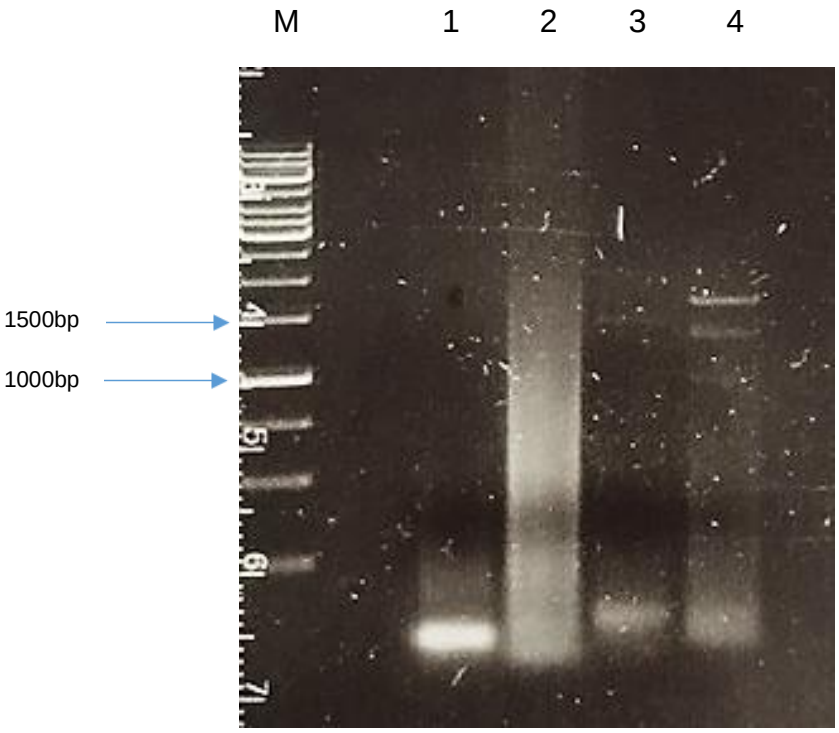


Figure 3.11: Agarose gel of the RACE PCR. *M= molecular markers, Lane 1= 3' RACE with PRM POR 4 (forward 1), Lane 2= 3' RACE with PRM POR 8 (forward 1), Lane 3= 5' RACE with PRM POR 4 (reverse), Lane 4= 5' RACE with PRM POR 8 (reverse).*

The sequence produced from the 3' RACE primers and the 5' GSP was expected to be around 500bp and the sequence produced from the 5' RACE and 3' GSP was expected to be approximately 1700bp long based on the position of the aligned sequences. This 5' RACE PCR produced a band of ~1700 bp long which was the expected size for the gene of interest. POR in total is expected to be around 2.2 kb based on POR in similar species such as *R. microplus* and the aim is to produce two overlapping RACE products which can be joined to produce a full-length POR sequence.

The PCR was scaled up from a 12.5 µl reaction to a 50 µl reaction to allow the excising of the band from the agarose gel. This scaling up failed to produce a band and after several attempts it was decided to do four 12.5 µl replicas of the

PCR and pool them together. This was unsuccessful, and several PCR optimisation steps were undertaken which also failed. This approach was used by a previous PhD student, Kirsty Graham, who also failed to scale up a previously successful small scale PCR in tick species *R. microplus* and *I. scapularis.* This could be an issue which is common to acari and leads to difficulties with amplification.

A new PRM RNA extraction was carried out using the methods described in section 3.3.4. This RNA quantity was 1208 ng/µl and had a 260/280 reading of 2.2 on the nanodrop and was considered pure enough to continue. This RNA was put through the GeneRacer kit and was used in RACE PCR reactions.

The first RACE PCR with the latest PRM RNA extraction was completed using the same conditions and parameters that were used when the 5' RACE product was previously produced. After this was unsuccessful, PCR optimisation was carried out, including using a high-fidelity polymerase (Q5, NEB) in order to increase the chances of accurate replication of the template, using higher concentrations of primer stocks (100 mM stock rather than 10 mM) to ensure primer concentration was not a limiting factor, using 3 varying concentrations of $MgCl_2$ (0.125 µl, 0.250 µl and 0.500 µl in a 12.5 µl reaction) as a cofactor for the polymerase as well as 3 varying concentrations of DMSO (0.062 µl, 0.125 µl and 0.250 µl in a 12.5 µl reaction) to lower the Tm of the PCR to improve amplification of GC regions. The cycling parameters were adapted numerous times, with both a two-step and three-step PCR being trialled as well as cycle numbers ranging from 30-40 cycles. Different annealing temperatures were also attempted for both sets of primers ranging from 58-70 ºC. After this optimisation was unsuccessful, a final RNA prep was extracted from the PRM. This prep contained 278.6 ng/µl of RNA and had a 260.280 value of 1.95 and was put through the GeneRacer kit.

This RACE ready cDNA was used to carry out the optimisation steps as described above in RACE PCR (example gel in Figure 3.12, conditions: 1 cycle of 98 °C for 2 minutes, followed by 40 cycles of 98 °C for 30 seconds, 60 °C for 30 seconds and 72 °C for 60 seconds, with a final step of 72 °C for 10 minutes), including using the secondary set of forward primers which had been designed.

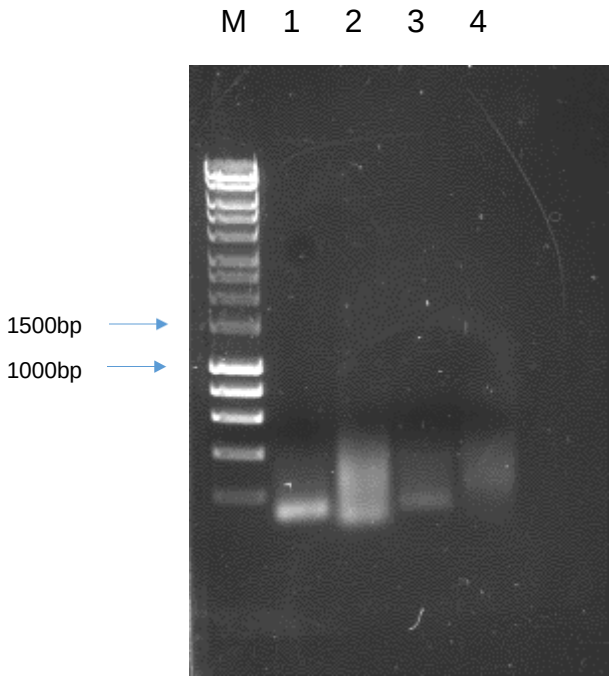


Figure 3.12: Example agarose gel of RACE PCR. *M= molecular markers, Lane 1= 3' RACE with PRM POR 4 (forward 1), Lane 2= 3' RACE with PRM POR 8 (forward 1), Lane 3= 5' RACE with PRM POR 4 (reverse), Lane 4= 5' RACE with PRM POR 8 (reverse).*

Final amplification resulted in no bands of the appropriate size, as seen from the example gel in Figure 3.12, and the amplification of POR remains to be achieved.

The next step would be to redesign the primers in order to try maximise success with POR, and to return to the initial polymerase which obtained a 5' RACE product. Since this project has been undertaken, a draft genome assembly of *D. gallinae* has been completed which would allow further analysis of sequences

from PRM which correspond to the POR gene in similar insect species (Burgess et al., 2018). The work completed in this project will be of great value in the future when isolating the POR from PRM as it provides a wealth of background detail on PCR optimisation. 3 RACE ready preps of cDNA from the PRM are now stored in -80 °C and allows future researchers to have a quick start on the process of PCR optimisation.

3.4.4 Microsome Preparation and Assay Development

PRM P450 sequences can be assigned a predicted function based on homology, but to identify and obtain the specific sequences of P450s involved in the metabolism of acaricides in PRM, Activity Based Probes were required. ABPs allow a targeted approach to amplifying P450s. In the approach used by Kirsty Graham, P450 sequences from the relevant species (*R. microplus*) were provided. Sequences were chosen to be cloned based on predicted xenobiotic metabolising functions, as determined by homology to known pyrethroid metabolising P450s from *A. gambiae*. Therefore this was not a targeted process as it was not certain if a cloned P450 would indeed have this functionality. In the targeted approach undertaken in this project, an ABP could isolate a P450 based on specific acaricide binding activity and thus clone a P450 certain to have a role in the metabolism of an acaricide.

In order to use ABPs, and to make use of the library of putative pesticide metabolising sequences, a preparation of microsomes is required which contains active P450s for the ABPs to bind to. The process of extracting the microsomes from the PRM was challenging and many stages of assay development were completed. ABPs work in a mechanism dependant manner to covalently label the P450s (Figure 3.13). This label is able to be detected by adding a fluorescent reporter group, using copper-catalyzed azide-alkyne cycloaddition (“click

chemistry"), on to the probe. This can then be used to detect the P450 once the probe has bound to them (Wright and Cravatt, 2007). ABPs have previously been used to gain greater insight into the potential mechanisms of artemisinin (ART)-based antimalarial action. It is essential to maintain the efficacy of ART as it has significantly reduced the number of malaria deaths in the last decade (Ismail et al., 2016). ART Activity Based Probes were used to identify proteins within the malaria parasite that are alkylated by ART, hence offering a strategy for investigating resistance mechanisms to ART-based drugs (Ismail et al., 2016). If used in PRM, these probes could assist in the isolation of acaricide metabolising P450s and pave the way to combatting resistance to commonly used pesticides, by creating a fuller picture of the relevant metabolic pathway within the PRM.

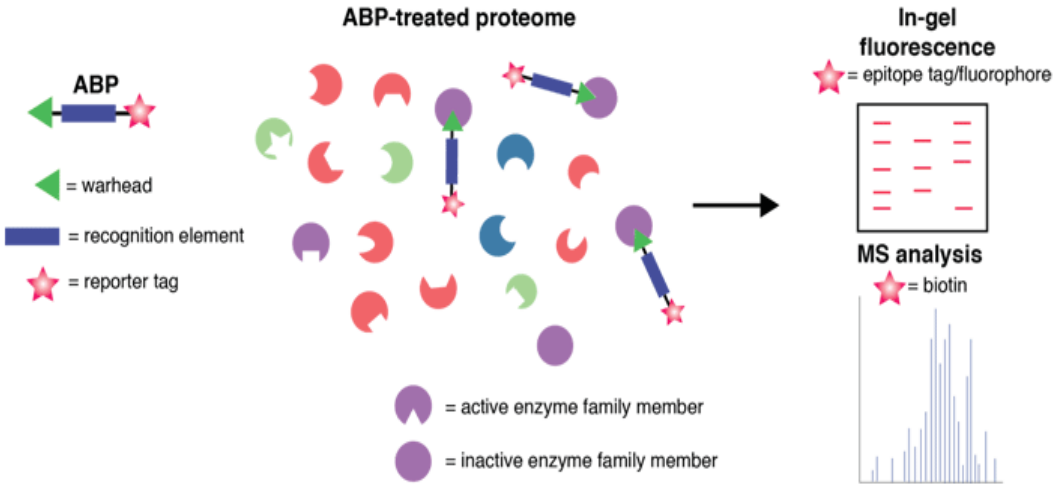


Figure 3.13: Activity Based Probes (ABPs). *ABPs consist of a chemically reactive warhead, a recognition element and the reporter tag. The recognition element is optimised for the P450 enzyme family and delivers the probe to the catalytic site of the enzyme. In the case of PRM ABPs, deltamethrin would be used as the recognition element. The reporter group allows visualisation by SDS-PAGE or identification using mass spectrometry. Diagram adapted from MRC Protein Phosphorylation and Ubiquitylation Unit (2016) and Brauneis et al. (2017).*

ABPs function by being a similar structure to the acaricide in question, hence being metabolised by the same pathways in the host species. ABPs based on the deltamethrin scaffold have been used to identify pyrethroid-reactive P450s and related detoxification enzymes in rat liver microsomes. This highlights the probes potential for directly assessing pyrethroid-metabolizing enzyme activity in the PRM (Ismail et al., 2013).

The use of ABPs requires active P450s found in microsomal preparations. The term ‘microsomes’ describes the high speed centrifugation of the post-mitochondrial supernatant of the insect tissue (Figure 3.14). The PRM homogenate can be spun at increasingly higher speeds which will separate the homogenate into its components based on their size and density. Whole cells, nuclei and components of the cytoskeleton pellet first, followed by mitochondria at around 10000 xg. Once these larger and denser cell components have formed a pellet; the supernatant, containing soluble enzymes, can be spun at 100000 xg. This produces a sediment which is mainly comprised of material from the endoplasmic reticulum of the insect cells (Siekevitz, 1963). This pellet contains an isolated and concentrated stock of cytochrome P450s, which are located on the endoplasmic reticulum, known as microsomes. The family of cytochrome P450s which are present in microsomes are responsible for Phase I biotransformation of xenobiotics. Incubation of test material with microsomal preparations in various species is the usual method by which the Phase I biotransformation of xenobiotics are determined.

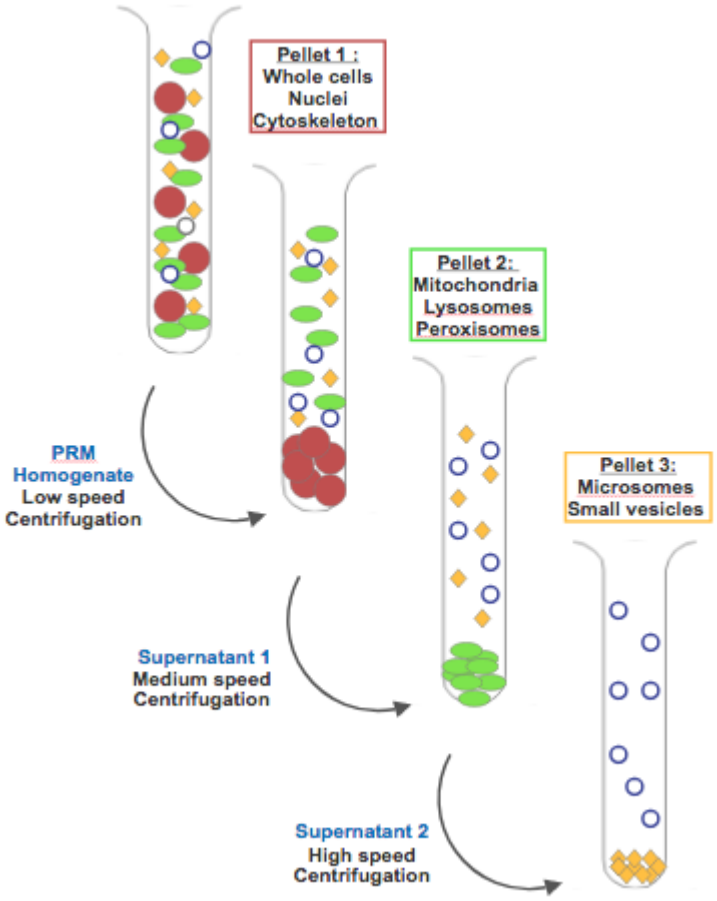


Figure 3.14: Diagram showing differential centrifugation. *Repeated centrifugation at increased speeds separates the PRM homogenate into its components based on size and density (larger and denser compounds centrifuge into a pellet quickest as they experience the largest centrifugal force). Smaller components stay in the supernatant until the centrifugal force increases enough to form a pellet. This allows the small microsomes to be separated into a concentrated microsomal prep.*

In order to determine the amount of protein in the microsome prep, a BCA assay was completed (as described in section 3.3.6). 12.80 µg/µl of protein was present in the microsome sample and this preparation was then used in a resorufin assay in order to determine whether active P450s were present. The resorufin assay was adapted from research by Mclaughlin in 2008 and examines the metabolism

of ethoxy-, methoxy-, benzyloxy- and pentoxyresorufin (ER, MR, BR AND PR) in order to identify fluorogenic substrate probes for the P450 genes. Whichever resorufin showed the highest specific activity would be further examined with respect to the kinetic parameters of that particular resorufin (McLaughlin et al., 2008). Resorufin assays were measured as described in section 3.3.6, at Excitation 530 nm and Emission 585 nm and the results can be viewed in Figure 3.15.

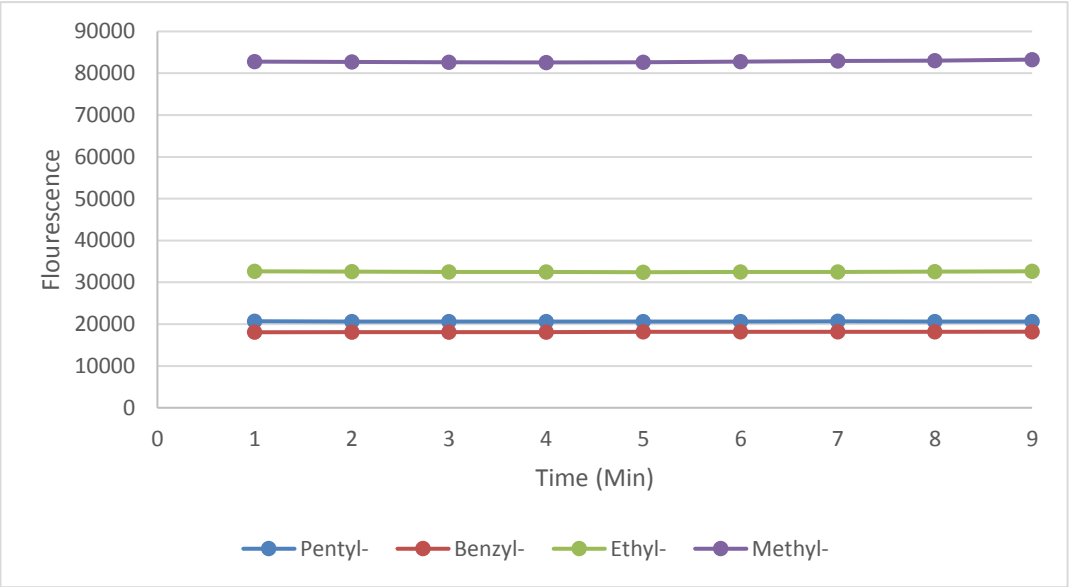
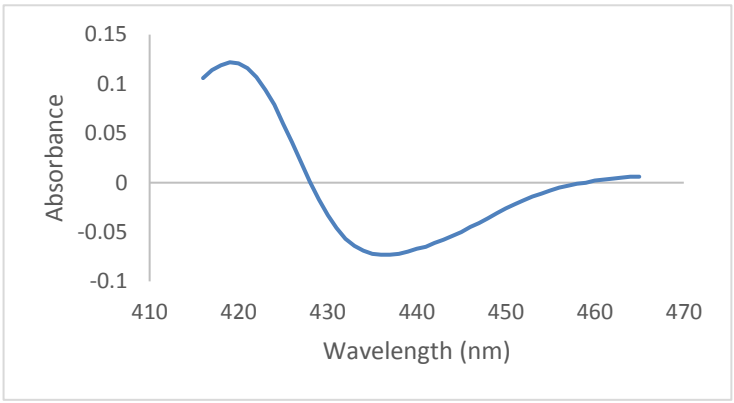


Figure 3.15: Results of resorufin assay in graphical form. *Ethoxy-, methoxy-, benzyloxy- and pentoxyresorufin are represented by different colours. Readings were taken once a minute for 9 minutes and florescence at Excitation 530 nm and Emission 585 nm is recorded. Results corrected for control (no microsomal preparation present).*

With results from the plate reader showing no difference in metabolism of ER, MR, BR and PR, a new microsomal prep was made. This time, a handheld electric homogeniser (IKA T10 basic homogenizer workcenter) and a manual dounce homogeniser were used to create two separate preparations for comparison, to ensure that the electric homogeniser was lysing the mites to a high enough

degree that active microsomes were prepared. A BCA was carried out to ensure there was a high level of protein present. The sample prepared using an electric homogeniser contained 34.31 µg/µl and the sample prepared using a manual homogeniser contained 38.83 µg/µl of protein. The small difference in protein content between the methods of extraction was promising, as electric homogenisation is quicker and therefore a preferred method, and both preparations were tested using a P450 spectrophotometer assay to view the peak in absorbance at 450 nm as a quantifier of P450 content (Figure 3.16).



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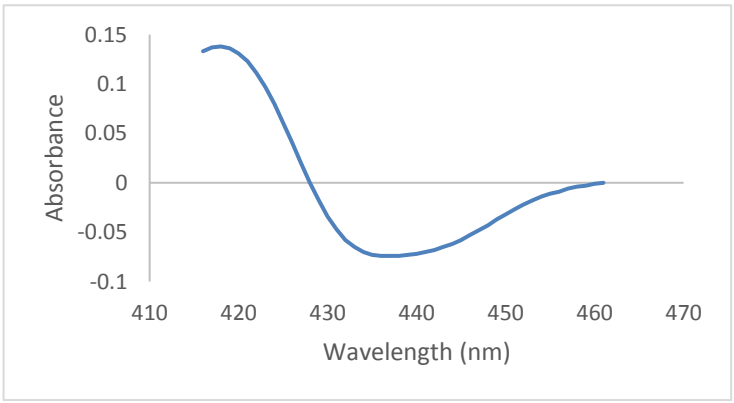


B

Figure 3.16: P450 spectrophotometer assay homogenised microsome prep from PRM. *A= manually homogenised sample. B= Machine homogenised sample. Wavelength was measured from 400-500 nm.*

Ferrous P450 reacts with carbon monoxide to form a complex which produces a spectrum that peaks at 450 nm when the P450 is active. The peak at 420 nm implies the presence of P450 in the sample but that which is enzymatically inactive (Omura and Sato, 1964b; Omura and Sato, 1964a). This conversion of P450 to the P420 form is common in cytochrome P450s but usually occurs after being exposed to high temperatures (of up to 60 ºC) or high pressure (2 X 10^8 to 2.4 X 10^8 Pa) (Hui Bon Hoa et al., 1989; Martinis et al., 1996). P450s can also convert to this form if exposed to proteases, detergents, acetone, neutral salts or denaturants (Imai and Sato, 1967; Yu and Gunsalus, 1974; Sasaki et al., 2005). The result of this microsome preparation was a high protein sample containing P450s, but those which were not in the active form. Due to this, they could not be used with APBs as they would not bind to the probe which was mimicking the xenobiotic. The cause of this could be the difficulties in producing a homogenised preparation of microsomes without the preparation being exposed to heat. Electrically homogenised samples are kept on ice throughout the protocol but the friction from the homogeniser generates heat. Manual homogenising produces a higher protein content (38.83 µg/µl) than electrically homogenising and also generates less friction and heat, although manual homogenisation is difficult to complete entirely on ice. Alternatively, the issue could be due to the amount of protease inhibitor (A cOmplete Protease Inhibitor Cocktail Tablet (Roche)) added to the microsome sample. A higher concentration of protease inhibitor may stop the conversion of P450 into the inactive P420 form.

Progress has been made in developing the protocol for microsome preparation, originally developed for use in the mosquito, and the sample now contains P450 protein. Work to ensure active P450 is included in the microsomal preparation would be the next step in the protocol development and would allow for the

interesting stage of using Activity Based Probes with PRM samples for the first time.

3.5 Conclusions and Further Research Plan

The pesticides used today to target PRM are being metabolised by the same mechanisms in many arthropod species. Once this pathway is discovered in the PRM, products on the market can be adapted to ensure efficacy is maintained and new products can be brought to market which are metabolised by different pathways entirely. An argument has been presented that alternative compounds should be developed/used, leading to increased research into the use of bio-control products such as essential oils, with the view that these compounds will be less toxic to the environment (George et al., 2010a). This research however has had limited success regarding the killing efficiency of these compounds and in some cases generated conflicting results. It also must be borne in mind that "natural" compounds are metabolised in the same way as synthetic compounds and are thus prone to the same issues e.g. development of resistance. The preservation of product efficacy against PRM is crucial to maintain the viability of egg farming within the UK.

In order to maintain product efficacy, in depth knowledge of the mechanisms of resistance in PRM, such as cytochrome P450s and their accessory proteins, is required for this mechanism to be bypassed in future products. In this chapter, the PRM transcriptomic database has been analysed and potential xenobiotic metabolising P450s have been identified. In addition to this, the transcriptomic data has also been used to identify PRM cytochrome b_5 which can now be used in a baculovirus expression system in order to characterise its role in xenobiotic metabolism (Chapter 4). POR requires further research in order to purify the accessory protein and characterise it using the baculovirus expression system and the recent draft genome assembly of PRM may aid future efforts to isolate POR (Burgess et al., 2018).

Finally, using ABPs to identify the pathways of pesticide metabolism is essential for this preservation of efficacy. This project has attempted to develop/adapt a protocol for microsomal preparation containing active P450 proteins similar to that used for several insect species. Further work needs to be completed for those P450s to remain active in the sample, which will be aided by the knowledge of techniques developed in this project. Temperature is likely to be a factor in the degradation of the P450 sample and repeat preparations should be made with a concerted effort of the sample remaining at 4 ºC, potentially with the protocol being completed in a cold room.

When an active P450 microsomal preparation has been achieved, any peptide data from the use of probes can be compared with the database that has been created of potential pesticide metabolising P450 sequences and is able to provide confirmation of gene function, a novel step within the PRM. P450s isolated can be expressed using a recombinant expression system to allow for characterisation of the gene. As well as this, further optimisation of the POR gene PCRs could allow for further expression of the accessory protein, which, when combined with the expression of cytochrome b_5 in chapter 4 of this project, allows for a new understanding of the P450 complex as a whole in PRM.

Chapter 4: The Isolation and Expression of P450 Accessory Proteins in the Poultry Red Mite, *Dermanyssus gallinae*.

4.1 Abstract

Drug detoxifying mechanisms in *Dermanyssus gallinae* are yet undocumented, unlike detoxifying mechanisms in other insect species of clinical significance. An important step, in order to advance understanding of poultry red mite (PRM) resistance, is to isolate cytochrome P450s from *D. gallinae* which are putatively involved in the metabolism of pesticides and additionally to isolate the accessory proteins which have a role in the functioning of the P450 complexes themselves. In this project, RNA was isolated from PRM and, using sequence data previously analysed, gene specific primers have been developed to both cytochrome b_5 and P450 oxidoreductase to allow isolation of these accessory proteins. The successful isolation and expression of cytochrome b_5 from PRM allowed the testing the cross-reactivity of mosquito b_5 antibody which could detect 5 ng of b_5 protein. This led to the *in vitro* expression of b_5 in *Spodoptera frugiperda* insect cells. As well as the *in vitro* expression of cytochrome b_5, Glutathione S-transferase (GST) from PRM was amplified and expressed *in vitro* as comparison of detoxification enzyme functions. The ability of cells expressing GST to catalyse the conjugation of reduced glutathione to 1-chloro-2,4-dinitrobenzene was assessed by measured absorbance at 340 nm at 1 minute and after 20 minutes. The results of the assay show a clear increase in absorbance in the samples transfected with the GST baculovirus versus control at both 1 minute ($P = 0.0225$) and 20 minutes ($P = 0.0083$) and indicates the increased metabolic functioning of those cells. From the *in vitro* testing, Glutathione S-transferases appear to have the ability to increase cell viability when exposed to common pesticides (deltamethrin, permethrin and bendiocarb). The role of b_5 is still unidentified but

purified b_5 is now ready to be used in P450 assays, once P450s have been isolated, to determine the effect of b_5 on specific pesticide metabolism. Purified GST is also ready for co-expression assays with P450s, which is one step closer to a greater understanding of pesticide metabolism and resistance in *D. gallinae.*

4.2 Introduction

Drug detoxifying mechanisms in the poultry red mite (PRM) *Dermanyssus gallinae,* are yet undocumented, unlike detoxifying mechanisms in other insect species of clinical significance, such as the common house fly, *Musca domestica*. House flies can transmit a number of infections to humans due to their repeated visiting of both faeces and human food. Of the 100 different pathogens transmitted by house flies, 65 can be transmitted to humans including anthrax, cholera and salmonella (Service, 2012). Due to this clinical significance, cytochrome P450s have been isolated that are overexpressed in pesticide resistant strains of house fly. CYP6A1 is a phenobarbital-inducible P450 of *M. domestica* which is highly expressed in pesticide resistant strains and has low levels of expression in highly susceptible strains (Carino et al., 1994). Chemicals such as DDT, known to induce other P450s in house flies, did not induce CYP6A1, which shows that metabolic resistance in the house fly involves the regulation of expression of more than one P450 gene. It also shows that the induction mechanism of P450 genes by pesticides is selective for some P450 genes (Carino et al., 1992). These advances make efforts to control the pest species more targeted.

Another pest of clinical significance is *Anopheles gambiae,* transmitter of malaria in sub Saharan Africa. It is estimated that 1 million children a year die from malaria, meaning that research into detoxification mechanisms in the vectors of the disease is vast (Lehane, 2005). It is thought that 68 % of the 663 million cases of malaria worldwide have been prevented using long-lasting insecticide treated bed nets (LLINs) (Bhatt et al., 2015; Churcher et al., 2016). The only class of insecticides which are currently used to treat LLINs is pyrethroids, and resistance to pyrethroids is widespread in African malaria vectors, such as *A. gambiae*

(Ranson and Lissenden, 2016; Gleave et al., 2018). Increased production of P450s is considered the most effective mechanism of pyrethroid resistance in malaria vectors and studies have shown up regulation of 5 out of 6 cytochrome P450s in *A. gambiae* in response to infection with plasmodium. These over expressed P450s belong to CYP4, CYP6 and CYP9 families of cytochromes which are associated with detoxification rather than biosynthetic pathways (Felix et al., 2010; Feyereisen, 2006). Similarly, parasite infection resulted in the overexpression of a GST gene (GSTD5), making GST expression in PRM of interest to this study (Felix et al., 2010). GSTs are also a major contributor to pesticide resistance in *Anopheles funestus* from Kpome, with GSTe2 being the most upregulated detoxification gene in both DDT and permethrin resistant mosquitoes, also a blood feeding species (Tchigossou et al., 2018).

Without advances in the knowledge of specific drug metabolising mechanisms in the PRM, it is difficult to develop new acaricides which will have lasting efficacy over a prolonged period of time, as they are likely to be metabolised by the same mechanisms that cause resistance to previous products (Flochlay et al., 2017). An important step in such an advance, is to isolate cytochrome P450s from the PRM which are putatively involved in the metabolism of pesticides, and additionally to isolate the accessory proteins which have a role in the functioning of the P450 complexes themselves. P450s have been identified as having a leading role in drug metabolism in humans since the late 1940s (Mueller and Miller, 1948). More recently, P450s have been recognised as having a role in pesticide metabolism and resistance in insects such as the fruit fly and mosquito (both from the order diptera) (Pavlidi et al., 2018; Mo et al., 2018). *Culex pipiens pallens,* northern house mosquito, is also a blood-feeding arthropod showing increasing signs of pesticidal resistance. Significant increases in P450 enzyme

activity, specifically in the CYP6 family, are observed in the mosquito following a blood meal which leads to increased resistance to both organophosphates and pyrethroids (Chang et al., 2017).

This overexpression of P450s in resistant species, which are genetically similar to the PRM, leads to the conclusion that P450 complexes are of key interest in the study of resistance in the mites. To isolate and characterise P450s in the PRM, in order to develop new acaricides which bypass the P450 complex mechanism, all components of the P450 complex must be identified and isolated.

Cytochrome P450 found in bacteria and eukaryotic mitochondria are type I P450s and those found localised to the endoplasmic reticulum of eukaryotic cells are type II P450s. These type II enzymes are commonly found to be involved in xenobiotic metabolism (Werck-Reichhart and Feyereisen, 2000).

Cytochrome P450s have an approximate molecular weight of 50 - 55 kDa and function with two accessory proteins, 15 kDa cytochrome b_5 and 77 kDa cytochrome P450 oxidoreductase (POR) (Figure 4.1). P450s are so named on account of their characteristic as hemoproteins and their unusual spectral properties in the presence of carbon monoxide, which on reducing the heme produces an absorption maximum at 450 nm.

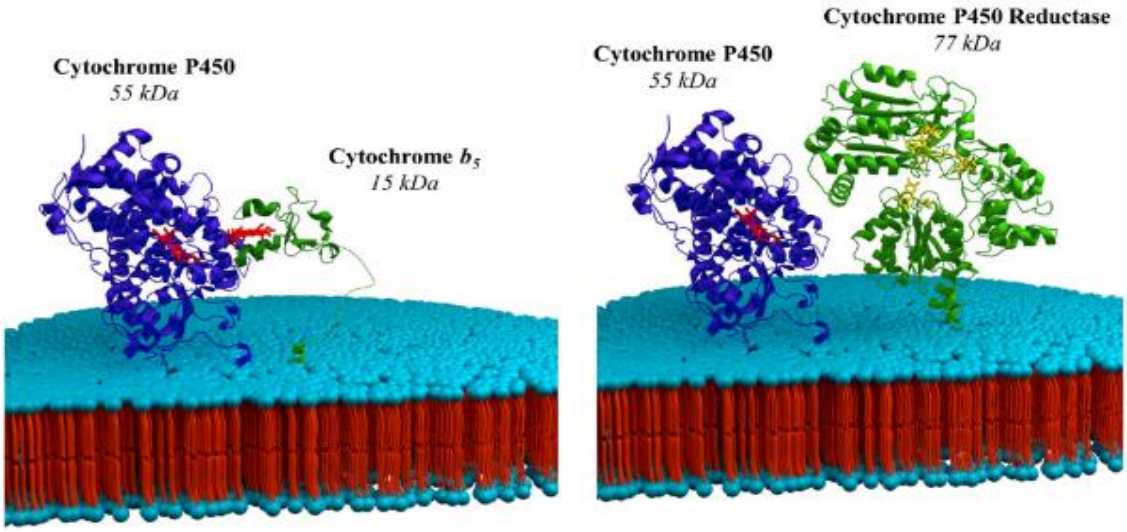


Figure 4.1: Model structures of P450 complex. *Model structures showing cytochrome P450 with cytochrome b_5 on the left and cytochrome P450 with cytochrome P450 oxidoreductase on the right. Figure taken from Barnaba et al. (2017).*

P450s catalyse the incorporation of a single atom of oxygen into the substrate and the remaining oxygen atom is reduced to water (Bernhardt, 2006). This is shown by the equation:

$$RH + O_2 + NAD(P)H + H^+ \longrightarrow ROH + H_2O + NAD(P)^+$$

This reaction is known as a monooxygenase reaction and RH depicts the substrate. Cytochrome P450s belong to external monooxygenases which implies they have an absolute requirement for an external electron donor to transfer the electrons required for oxygen activation and substrate hydroxylation from a reducing equivalent i.e. NADPH (Bernhardt, 2006). Cytochrome P450 requires two electrons to be sequentially delivered from its redox partners, POR and b_5, in order to carry out the oxidation of molecules and POR is capable of donating both electrons whilst b_5 is only capable of donating the second electron not the first (Zhang et al., 2015; Guengerich, 2006; Gruenke et al., 1995; Zhang et al., 2008;

Im and Waskell, 2011). Cytochrome b_5 has a varied role in the activity levels of P450s and hence on the levels of detoxification and resistance to a product. It has been shown to both stimulate and inhibit the action of P450s, as well as having no effect at all on the activities of P450s *in vivo* and *in vitro* in both human and mice studies (Finn et al., 2008; McLaughlin et al., 2010; Im and Waskell, 2011; Gruenke et al., 1995). The stimulatory effect is due to its ability to produce active oxidising species more quickly than POR and its inhibitory effect comes from competing with POR for a binding site on the proximal surface of the P450 (Im and Waskell, 2011; Zhang et al., 2008), with no effect being shown when the two events cancel each other out. It has also been suggested that the presence of different substrates can cause different affinities between b_5 and the P450 (Zhang et al., 2005; Gruenke et al., 1995).

The role of POR in the detoxification process is more established. POR is a member of a relatively small family of diflavin redox proteins which consists of two lobes, one has an FMN domain and one has an FAD domain (Hrycay E and Bandiera, 2015). Eukaryotic P450s are located in the endoplasmic reticulum and the electrons flow from NADPH to FAD to FMN and then reach the P450 (McLean et al., 2005; Munro et al., 2007). In most organisms only one 680 amino acid POR encoding gene exists, suggesting POR can reduce many different P450s in one species (Cordova et al., 2017). POR is folded to allow the FMN and FAD domain to be located close together to facilitate the quick and efficient passing of one electron after another. POR allows for the transfer of electrons to the P450 protein, but also allows for electrons to pass to other proteins, such as cytochrome b_5 (Laursen et al., 2011). POR transports the electrons from NADPH to all the microsomal (type II) cytochrome P450 enzymes.

POR has a specific change of shape for its role as an electron donor. The FAD domain of the POR accepts electrons from NADPH which prompts a conformational change in the POR (Figure 4.2). This change allows FAD to be aligned with the FMN domain of POR and the electrons can flow to the FMN domain, which causes POR to return to its previous configuration. This return of shape allows the FMN domain to dock with the P450 via electrostatic interactions.

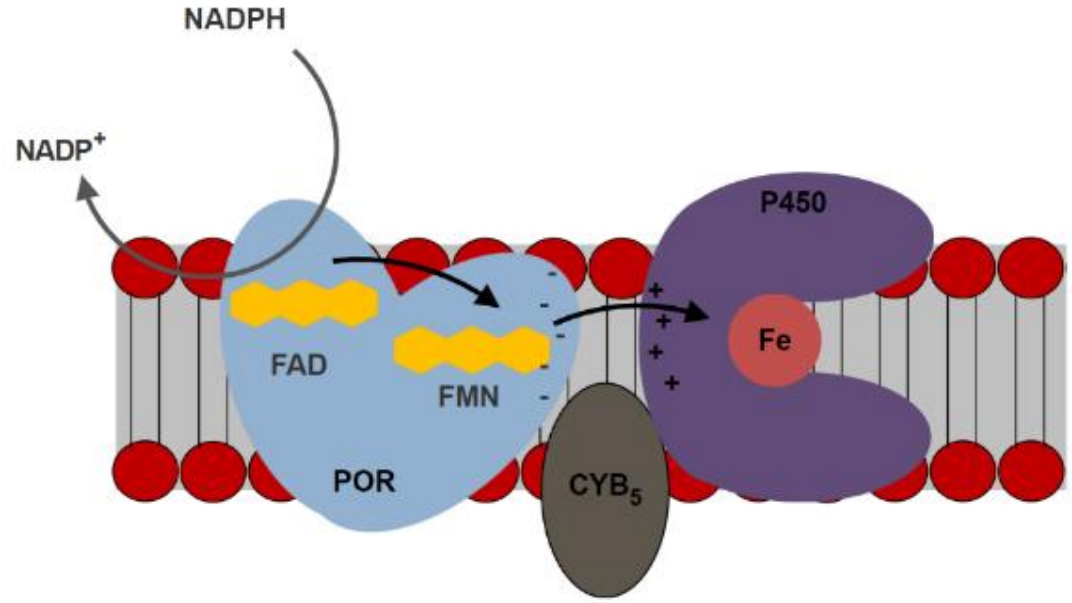


Figure 4.2: Schematic of type II P450 complex. *The lobes of the POR (blue protein) containing flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN). P450 is represented by the purple protein and cytochrome b_5 as the grey protein. The arrows represent the flow of electrons in the chain.*

POR has been isolated from many pest species due to its indisputable role in the functioning of the P450 complex (Chen and Zhang, 2015; Liu et al., 2014; Etzerodt et al., 2017; Tsou et al., 2018). Both POR and b_5 have been characterised as playing a role in resistance mechanisms of species such as *Plutella xylostella* (diamondback moth). Both genes were found to be overexpressed in cypermethrin resistant strains and low concentrations of cypermethrin could induce expression of both P450 redox partners (Chen and

Zhang, 2015). In *Helicoverpa armigera* (cotton bollworm), the overexpression pattern of b_5 was identical to the overexpression of CYP6B7, which highlighted an involvement of b_5 in CYP6B7 mediated pyrethroid resistance (Ranasinghe and Hobbs, 1999). Similarly, a recent study showed *H. armigera* was significantly more susceptible to a pyrethroid after knockdown of CYP6B7 as well as POR and b_5 (Tang et al., 2012). Knockdown of the POR gene in *Nilaparvata lugens* (brown planthopper) increased susceptibility to both cypermethrin and imidacloprid, a neonicotinoid (Liu et al., 2015). Despite the role of redox partners in P450 mediated resistance mechanisms, few have been isolated from arthropod species. The first hematophagous acari species to have POR and b_5 isolated in order to create a full functional cytochrome P450 is *R. microplus,* although work has not yet been done to characterise the genes in order to gain clues into the mechanisms of resistance in the arthropod species (Graham et al., 2016).

Other detoxification mechanisms of interest include the Glutathione S-transferases (GSTs). P450s are involved in phase I of detoxification by catalysing the addition of polar groups to xenobiotics. GSTs are often associated with phase II metabolism and involve the conjugation of reduced glutathione (GSH) to the xenobiotic (Bartley et al., 2015; Sheehan et al., 2001). GSTs are of importance to the metabolism of xenobiotics in haematophagous parasites and so are of interest to this project to target the mechanisms of pesticide metabolism in PRM (Bartley et al., 2015; van Rossum et al., 2004; Toh et al., 2010).

The aim of this chapter is to express and purify the PRM cytochrome b_5, which has been identified using bioinformatics in Chapter 3, and use this to create a baculovirus expression system which can be used to monitor the effects of b_5 on pesticide metabolism. As well as this, cytochrome b_5 from two other tick species, *R. microplus* and *I. scapularis* will be expressed and purified for use in further

downstream applications. A PRM GST has been provided by collaborators and the aim is to also use this in the expression system, which could provide comparison of its effect on xenobiotic metabolism with cytochrome b_5.

4.3 Methods

4.3.1 RNA Extraction and standard production of cDNA from *Dermanyssus gallinae*

RNA was extracted from PRM using the method described in section 3.3.4. In order to carry out PCR and generate the b_5 gene, cDNA was generated using Precision Reverse Transcription all-in-one Premix (Primer Design Ltd, Hampshire, UK), according to the manufacturer's protocol. Briefly, 9 µl of the RNA (2 µg) extracted was added to 20 µl of the RT premix and was incubated for 20 minutes at 42 °C before being incubated for a further 10 minutes at 72 °C. The final volume of cDNA was 29 µl.

4.3.2 Amplification of Cytochrome b_5 gene using PCR

The putative b_5 sequence had been identified using transcriptomic sequence data from the PRM with b_5 from the *R. microplus* as bait (see section 3.3.2). The aligned sequence was extended by 123 and 120 nucleotides in the 5' and 3' direction respectively to facilitate the incorporation of the predicted start and stop codons and parts of the untranslated 3' and 5' regions. Primers were designed to this region, to ensure the whole coding region was present in the sequence amplification and a standard PCR was used. Standard PCR was done using the primers in Table 4.1.

Table 4.1: PCR primers b_5. *Two sets of primers were designed, known as set 1 and set 5, in order to have the best chance of isolating PRM b_5.*

	PRM b_5 Set 1 5'-3'	**PRM b_5 Set 5 5'-3'**
Forward	TCC CAT CCA GGT CGG AAG AT	CCA CCA GAA CGC TGA CTG AT
Reverse	TAT AGC CCA CTC GTG GTG GA	ATG ACG ATG GAT AGC CCA CC

PCR reactions were set up as follows using VELOCITY DNA Polymerase (Bioline, London, UK) with a total reaction volume of 12.5 µl; 7.5 µl distilled water, 2.5 µl 5x Hi-Fi reaction buffer, 0.5 µl dNTPs (10 mM), 0.5 µl of each forward and reverse primer (10 µM), 0.25 µl $MgCl_2$ (50 mM), 0.5 µl of PRM cDNA, 0.25 µl polymerase enzyme. The following PCR conditions were used; 1 cycle of 98 °C for 2 minutes, followed by 35 cycles of 98 °C for 30 seconds, 54 °C for 30 seconds and 72 °C for 20 seconds, with a final step of 72 °C for 10 minutes.

PCR products were run on a 1 % agarose gel and correct size bands were extracted from the gel using a gel extraction kit (Qiagen, West Sussex, UK) following the manufacturers protocol and the final elution step was carried out in 50 µl of sterile distilled water.

4.3.3 Cloning Cytochrome b_5 into pCR Blunt and Transformation into Competent Cells

The purified gel extraction sample was cloned using Zero Blunt PCR Cloning kit (Life Technologies, Paisley, UK) and One Shot TOP10 Chemically Competent *E. coli* cells (Life technologies, Paisley, UK).

5 µl of the purified PCR fragment was ligated with 1 µl of pCR Blunt vector, 2 µl of 5 X ExpressLink T4 DNA ligase buffer, 1 µl ExpressLink T4 DNA Ligase and 1 µl of sterile distilled water to give a reaction volume of 10 µl. This was incubated at room temperature for 1 hour.

3 µl of ligation mixture was added to 50 µl of chemically competent TOP10 cells and gently mixed. The mixture was incubated on ice for 30 minutes before heat shocking at 42 °C for 45 seconds. The cells were returned to ice for 2 minutes and 450 µl of sterile LB broth was added. The cells were shaken at 45 minutes at 37 °C and 225 rpm and then plated on LB selective plates with the appropriate antibiotic (for pCR Blunt 50 µg/ml Kanamycin). The plates were incubated overnight at 37 °C to allow growth of colonies.

4.3.4 Colony Screen via Restriction Digest and Sequencing Confirmation

Colonies visible on the plates were deemed to be kanamycin resistant and were removed from the plate and added to 10 ml of LB broth with 50 µg/ml Kanamycin followed by incubation in a shaking incubator (225 rpm) at 37 °C overnight. Plasmid DNA was purified from 3 ml of culture for each colony using a Qiagen QIAprep spin Miniprep Kit (Qiagen, West Sussex, UK) according to the manufacturer's protocol with plasmid eluted in 30 µl of sterile distilled water. An *EcoRI* (NEB, Hertfordshire, UK) digest was done to confirm the presence of appropriate sized PCR product. A restriction digest was set up as follows; 5 µl of plasmid DNA, 1 µl of 10 X reaction buffer, 0.5 µl of enzyme and 3.5 µl sterile water. The digest was incubated for 2 hours at 37 °C before being analysed on a 1 % agarose gel.

Colonies which appeared to have the appropriate size insert were sent for sequencing using M13 primers.

4.3.5 Cloning Cytochrome b_5 into pET15b

To allow the successful expression of b_5 in bacterial cells, the coding DNA sequence (CDS) was amplified to include the restriction sites for *NdeI (5' CAT ATG 3') a*nd *XhoI* (5' CTC GAG 3') (NEB, Hertfordshire, UK) and cloned into pCR Blunt to confirm correct incorporation of the restriction sites. The primers used can be viewed in Table 4.2.

Table 4.2: Gene specific primers for b_5. *Forward and reverse primers for b_5 designed to ligate with pET15b with the restriction sites underlined.*

	Forward 5'-3'	**Reverse 5'-3'**
PRMb$_5$pET	GCC ATA TGA CGA CGA CGA CGC CAG C	GCC TCG AGT TAT CGG GAA TGG TAC AGG A

PCR was completed using *Pfu* DNA polymerase (Promega, Madison, USA); 0.2 µl of plasmid, 1.5 µl of each primer, 0.5 µl of enzyme, 5 µl of 10 X buffer, 1 µl of dNTPs (10 mM), 40.3 µl of sterile distilled water. The conditions were as follows; 1 cycle of 95 °C for 3 minutes, followed by 35 cycles of 95 °C for 30 seconds, 63 °C for 30 seconds and 72 °C for 1 minute, with a final step of 72 °C for 10 minutes. PCR products were run on a 1 % agarose gel.

PCR products were gel extracted and cloned into pCR Blunt as described in the above sections 4.3.2-4.3.4. Three colonies (C1, C4, C10) showing bands of the correct size were confirmed using sequencing with M13 primers (see section 4.4.2).

Both the pCR Blunt *NdeI/XhoI* b_5 (with restriction sites) and the pET15b were digested with *NdeI* followed by *XhoI* as follows: 50 µl of plasmid DNA, 6 µl of 10 X reaction buffer, 2 µl enzyme and 2 µl sterile water. The digest was incubated

for 2 hours at 37 °C before being analysed on a 1 % agarose gel. Bands of the appropriate size were excised from the gel using a gel extraction kit as described in 4.3.2 before being ligated using T4 DNA ligase. Briefly; 1 µl of 10 X DNA ligase buffer, 1 µl T4 DNA ligase, 3 µl pET15b plasmid DNA, 3 µl b_5 DNA and 2 µl sterile distilled water were mixed and incubated at 16 °C overnight.

This ligation was then transformed into TOP10 cells as described in section 4.3.3 and the transformation was spread on ampicillin (50 µg/ml) plates and miniprepped as per section 4.3.4. An *NdeI* and *XhoI* (NEB, Hertfordshire, UK) double digest was done to confirm the presence of appropriate sized b_5 product. A restriction digest was set up as follows; 6 µl of plasmid DNA, 1 µl of 10X reaction buffer, 0.5 µl of each enzyme and 2 µl sterile water. The digest was incubated for 2 hours at 37 °C before being analysed on a 1 % agarose gel.

Colonies (C3, C4) which appeared to have the appropriate sized insert were sent for sequencing using T7 primer; T7 (20) TAA TAC GAC TCA CTA TAG GG, to confirm the insertion of the b_5 in frame with the His-tag.

4.3.6 Expression and Purification of Cytochrome b_5 from *Dermanyssus gallinae, Ixodes scapularis* and *Rhipicephalus microplus*

To express cytochrome b_5, the PRM-b_5-pET15b construct was transformed into *E. coli* cells (BL21(DE3)pLysS) using the transformation protocol from 4.3.3. As well as transforming the PRM b_5 gene, b_5 from two other tick species (*Ixodes scapularis* (deer tick) and *Rhipicephalus microplus* (cattle tick)) were also transformed into BL21(DE3)pLysS cells. Transformants were grown on selective LB agar plates with 50 µg/ml ampicillin and 34 µg/ml chloramphenicol. Colonies that were grown on the plates were then put into LB broth (50 µg/ml ampicillin and 34 µg/ml chloramphenicol) and grown at 37 °C overnight, at 225 rpm. 5 ml

of the overnight culture was then added to 500 ml of TB (terrific broth) media (50 µg/ml ampicillin and 34 µg/ml chloramphenicol) and incubated at 37 °C, 225 rpm until the O.D.600 reached a value between 0.6 and 0.8. 1 ml samples were then taken (before the samples were induced) for analysis using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Expression was induced by adding 1 mM IPTG and 1 mM ALA to the culture and incubation continued overnight at 30 °C, 225 rpm. Post incubation, 1 ml samples were taken for analysis on an SDS-PAGE gel. Uninduced and induced samples from each species were then run on a 15 % SDS-PAGE gel. The culture was then spun at 3795 xg (Sorvall RC-5B Plus, SLA 1500 rotor), at 4 °C.

Pellets were resuspended in 25 ml of 2X TSE (100 mM Tris acetate, 500 mM sucrose and 0.5 mM EDTA), followed by 30 ml of cold, sterile distilled water. 4.8 ml of 0.26 mg/ml lysozyme was added to each pellet and then the resuspended pellets were spun at 4 °C for 1 hour on an automatic tube spinner. The fractions were then spun at 3795 xg (Sorvall RC-5B Plus, SLA 1500 rotor) for 20 minutes at 4 °C and the pellet resuspended in 60 ml spheroplast resuspension buffer (100 mM potassium phosphate pH 7.6, 6 mM magnesium acetate with 20 % v/v glycerol). 1 cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail tablet was dissolved in 1 ml sterile water and added to the fractions before sonication for 30 seconds 3-4 times. The suspension was diluted 1:2 with 100 mM spheroplast resuspension buffer and centrifuged at 15,180 xg (Sorvall RC-5B Plus, SLA 1500 rotor) for 15 minutes at 4 °C. The supernatant was then spun at 273,620 xg (Beckman Coulter Optima L-100XP, rotor SW41 Ti) for 60 minutes at 4 °C and the pellet resuspended in 1 ml of binding buffer (20 mM Tris-HCL (pH 7.4), 500 mM KCl and 20 % v/v glycerol). Membranes were stored at -70 °C.

For purification, membranes were solubilised with 40 ml solubilisation buffer (20 mM Tris (pH 7.4) 500 mM KCl, 20 mM CHAPS, 5 mM imidazole and 20 % v/v glycerol) at 4 °C on a magnetic stirrer overnight. Samples were centrifuged at 273,620 xg (Beckman Coulter Optima L-100XP, rotor SW41 Ti) at 4 °C for 45 minutes. A 5 ml HisTrap column (GE, Buckinghamshire, UK) was equilibrated with 50 ml of solubilisation buffer before the b_5 membranes were loaded on to the column. The column was then washed with 50 ml solubilisation buffer and a red band was visible at the top, confirming the b_5 protein had bound to the column. The column was then washed with 15 ml of solubilisation buffer with increasing concentrations of imidazole (15 mM, 30 mM, 50 mM) before being eluted in Ni-agarose elution buffer (20 mM Tris (pH 7.4), 500 mM KCl, 20 mM CHAPS, 500 mM imidazole and 20 % v/v glycerol). 1 ml of every fraction eluted was stored for analysis on a 15 % SDS-PAGE gel. The final fraction, which was red in colour, was buffer exchanged (using a PD10 column) into a low salt storage buffer (20 mM Tris (pH 7.4), 100 mM KCl, 10 mM CHAPS and 20 % v/v glycerol) and were stored at -70 °C before running on a 15 % SDS-PAGE gel (for protocol, see appendix B).

Protein was quantified using a b_5 spectrophotometer assay. 50 µl of protein was added to 2 ml KPh (100 mM) pH 7.4. This was divided equally between 2 matched UV cuvettes and absorbance was read between 380-580 nm. The spectrophotometer was zeroed and a baseline was run before 10 µl of 30 % H_2O_2 was added to the sample cuvette. The spectrophotometer was zeroed again and a scan run of the oxidised spec. A small quantity of sodium hydrosulphate was added to the sample cuvette and mixed and the absorbance at 409 and 426 nm was read. Concentration of b_5 was calculated using the equation:

nmoles/ µl= [(Abs 426 – Abs 409)/185] X dilution factor

4.3.7 Testing the Cross Reactivity of Mosquito Antibody Using a Western Blot

The presence of PRM b_5 was further checked using a Western blot. Firstly, a dot blot was completed to ascertain a suitable dilution of the primary b_5 specific antibody (provided by Dr Mark Paine). A piece of nitrocellulose paper was taken and split into 6 sections using a pencil. 2.77 µl of PRM b_5 (50 ng) was taken and added to each section of the nitrocellulose. The blot was blocked with Tris-buffered saline with tween 20 (TBS-T) containing 5 % NFM (non-fatty milk) for 1 hour at room temperature under rocking conditions. 6 different concentrations of primary were then added to the blot (1:625, 1:1250, 1:2500, 1:5000, 1:7500, 1:10,000) in the TBS-T milk for 1 hour at room temperature under rocking conditions. Following the incubation, 3 X 5 minute washes were carried out in TBS-T before incubation with the secondary antibody (Goat Anti-Rabbit Immunoglobulins/HRP, Agilent, Dako, California, USA) for 1 hour at room temperature under rocking conditions in TBT-T milk. Three, 5 minutes TBS-T washes were then carried out before enhanced chemiluminescence (ECL) was added (Amersham ECL Western blotting reagent, GE, Buckinghamshire, UK). Images were taken using a Syngene G:Box.

A Western blot was completed in order to check the sensitivity of the primary antibody at a specific dilution, 1:2500 and 1:5000 were used after confirming they provided clear detection of 50 µg of protein on the dot blot. A 15 % SDS-PAGE gel was then run (see appendix B) with varying amounts of b_5 protein (5 ng, 10 ng, 25 ng, 40 ng, 50 ng and 100 ng). Nitrocellulose was cut to the size of the gel and placed on top of the gel in between two filter papers. This sandwich was then placed inside the immunoblotting cassette and loaded into a running tank with 1 X transfer buffer (25 mM Tris, 190 mM glycine, 20 % methanol). The blot was run

for 1 hour at 120V before being blocked with Tris-buffered saline with tween 20 (TBS-T) containing 5 % NFM (non-fatty milk) for 1 hour at room temperature under rocking conditions. The blot was then placed in the primary antibody (b_5 specific 1:2500 and 1:5000) in the TBS-T milk overnight at 4 °C under rocking conditions. Following the overnight incubation, 3 X 5 minute washes were carried out in TBS-T before incubation with the secondary antibody (1:2000) (Goat Anti-Rabbit Immunoglobulins/HRP, Agilent, Dako, California, USA) for 1 hour at room temperature under rocking conditions in TBT-T milk. Three 5 minutes TBS-T washes were then carried out before enhanced chemiluminescence (ECL) was added (Amersham ECL Western blotting reagent, GE, Buckinghamshire, UK). Images were taken using a Syngene G:Box.

4.3.8 Production of Baculovirus Expression System for Cytochrome b_5 and Glutathione S-transferases

High level expression of the recombinant b_5 and GST proteins was achieved using the Bac-to-Bac Baculovirus Expression System (Thermo Fisher, Paisley, UK). The genes were cloned into pFastBac 1 vectors according to the manufacturers protocol (Figure 4.2). Briefly, b_5 and pFastBac were grown from glycerol stocks and mini prepped before being run on a 1 % agarose gel and gel extracted. They were then digested using *XhoI* followed by *BamHI* (NEB, Hertfordshire, UK) in the following reaction: 50 µl of plasmid DNA, 6 µl of 10 X reaction buffer, 2 µl enzyme and 2 µl sterile water. The digest was incubated for 2 hours at 37 °C before being analysed on a 1 % agarose gel and bands of the appropriate size were gel extracted and ligated using T4 DNA ligase and grown overnight as described in 4.3.2. Colonies were checked for the presence of b_5 using a restriction digest with *XhoI* and *BamHI,* as described in section 4.3.4, and bands showing the correct molecular weights were confirmed with sequencing

using Polyhed fwd (18) AAA ATG ATA ACC ATC TCG. This recombinant plasmid was then transformed into DH10Bac *E.coli* cells and grown on selective plates (50 µg/mL kanamycin, 7 µg/mL gentamicin, 10 µg/mL tetracycline, 100 µg/mL Bluo-gal and 40 µg/mL IPTG). A white phenotype was indicative of successful transformation and white colonies were screened using PCR. PCR primers were as follows: M13 forward 5′- TGT AAA ACG ACG GCC AGT -3′ and PRM b_5 pET rev 5' GCC TCG AGT TAT CGG GAA TGG TAC AGG A 3'. PCR was completed using Taq DNA polymerase (NEB, Hertfordshire, UK); 1 µl of plasmid, 1.25 µl of each primer (10 mM stock), 0.5 µl of enzyme, 5 µl of 10 X buffer, 1 µl of dNTPs (10 mM), 1.5 µl 50 mM $MgCl_2$ and 38.5 µl of sterile distilled water. The conditions were as follows; 1 cycle of 93 °C for 3 minutes, followed by 30 cycles of 94 °C for 45 seconds, 63 °C for 45 seconds and 72 °C for 2 minute, with a final step of 72 °C for 10 minutes. PCR products were run on a 1 % agarose gel. A Plasmid DNA Midiprep Kit (Thermo Fisher, Paisley, UK) was made of successful colonies and was then used to transfect insect cells of the cell line Sf9 (Thermo Fisher, Paisley, UK), using Cellfectin II Reagent.

In order to create a GST expression system, GST required amplification. GST primers were designed in order for it to be cloned into pCRBlunt; Forward GST: 5'GCC ACC AGT TCG TGA TGG GC 3' and reverse GST: 5' GCC CAT CAC GAA CTG GTG GC 3'. PCR was completed using Q5 DNA polymerase (NEB, Hertfordshire, UK). 2 µl of plasmid, 1 µl of each primer (100 mM stock), 0.25 µl of enzyme, 5 µl of 5 X buffer, 0.5 µl of dNTPs (10 mM), 5 µl of 5 X GC enhancer and 10.25 µl of sterile distilled water. The conditions were as follows; 1 cycle of 98 °C for 30 seconds, followed by 30 cycles of 98 °C for 10 seconds, 72 °C for 20 seconds, with a final step of 72 °C for 2 minutes. PCR products were run on a 1 % agarose gel. GST was ligated with pCRblunt using T4 DNA ligase and

transformed into TOP10 cells (see section 4.3.3). The presence of GST was checked using an *EcoRI* restriction digest as set up in section 4.3.4 before being sent for sequencing with M13 primers; M13 uni (21) 5’ TGT AAA ACG ACG GCC AGT 3’ and M13 rev (29) 5’ CAG GAA ACA GCT ATG ACC 3’.

GST and pFastBac were then digested with *BamHI* followed by *KpnI* (both NEB, Hertfordshire, UK) in a restriction digest, excised from a 1 % agarose gel and ligated using T4 DNA ligase before being transformed into TOP10 cells as described in section 4.3.4.

Colonies were screened for the presence of GST fragment using a restriction digest with both *KpnI* and *BamHI* as well as screening by PCR. Colonies were screened using GST specific forward and reverse primers. Forward GST: 5’GCC ACC AGT TCG TGA TGG GC 3’ and reverse GST: 5’ GCC CAT CAC GAA CTG GTG GC 3’. PCR was completed using Q5 DNA polymerase (NEB, Hertfordshire, UK). 2 µl of plasmid, 1 µl of each primer (100 mM stock), 0.25 µl of enzyme, 5 µl of 5 X buffer, 0.5 µl of dNTPs (10mM), 5 µl of 5 X GC enhancer and 10.25 µl of sterile distilled water. The conditions were as follows; 1 cycle of 98 °C for 30 seconds, followed by 30 cycles of 98 °C for 10 seconds, 72 °C for 20 seconds, with a final step of 72 °C for 2 minutes. PCR products were run on a 1 % agarose gel. The positive colonies confirmed by sending for sequencing with Polyhed fwd (18) AAA ATG ATA ACC ATC TCG. These colonies were then transformed into DH10 cells (the same protocol as pFastBacb$_5$ colonies) and screened on antibiotic plates as well as screening by PCR. PCR primers were as follows: M13 forward 5′- TGT AAA ACG ACG GCC AGT -3′ and reverse GST: 5’ GCC CAT CAC GAA CTG GTG GC 3’. PCR was completed using Taq DNA polymerase (NEB, Hertfordshire, UK); 1 µl of plasmid, 1.25 µl of each primer (10 mM stock), 0.5 µl of enzyme, 5 µl of 10X buffer, 1 µl of dNTPs (10 mM), 1.5 µl 50 mM $MgCl_2$

and 38.5 µl of sterile distilled water. The conditions were as follows; 1 cycle of 93 °C for 3 minutes, followed by 30 cycles of 94 °C for 45 seconds, 69 °C for 45 seconds and 72 °C for 2 minute, with a final step of 72 °C for 10 minutes. PCR products were run on a 1 % agarose gel. A Plasmid DNA Midiprep Kit (Thermo Fisher, Paisley, UK) was made of successful colonies and was then used to transfect insect cells of the cell line Sf9 (Thermo Fisher, Paisley, UK), using Cellfectin II Reagent.

For the purpose of producing recombinant Baculovirus, Sf9 cells were routinely cultured as a cell monolayer in Sf-900 II serum free media (Thermo Fisher, Paisley, UK) containing 1 % v/v penicillin (10,000 U/mL) and 1 % v/v Amphotericin (Thermo Fisher, Paisley, UK). Cells were routinely split 1:4 every 3-4 days at 90 % confluency, fed every 2-3 days and grown at 27 °C in a controlled humidity incubator. Cells had a density of 1.6 X 10^6 before proceeding with transfection.

To transfect the cells, for both b_5 and GST, cells were seeded at 1.6 X 10^6 in a 6 well plate and allowed to attach for 15 minutes at room temperature before 2.5 ml of plating medium was added per well (0.375 ml Supplemented Grace's Insect Medium and 2.125 ml of Unsupplemented Grace's medium). Cells were then transfected with 8 µl of Cellfectin reagent and 1 µl baculovirus DNA and left to incubate for 3 hours. Transfection mixture was then removed and 2 ml of Sf-900 II serum free media was added per well. Cells were incubated for 7-10 days until signs of viral infection were apparent and the medium was collected (P1 stock) and stored with 2 % final concentration fetal bovine serum at 4 °C.

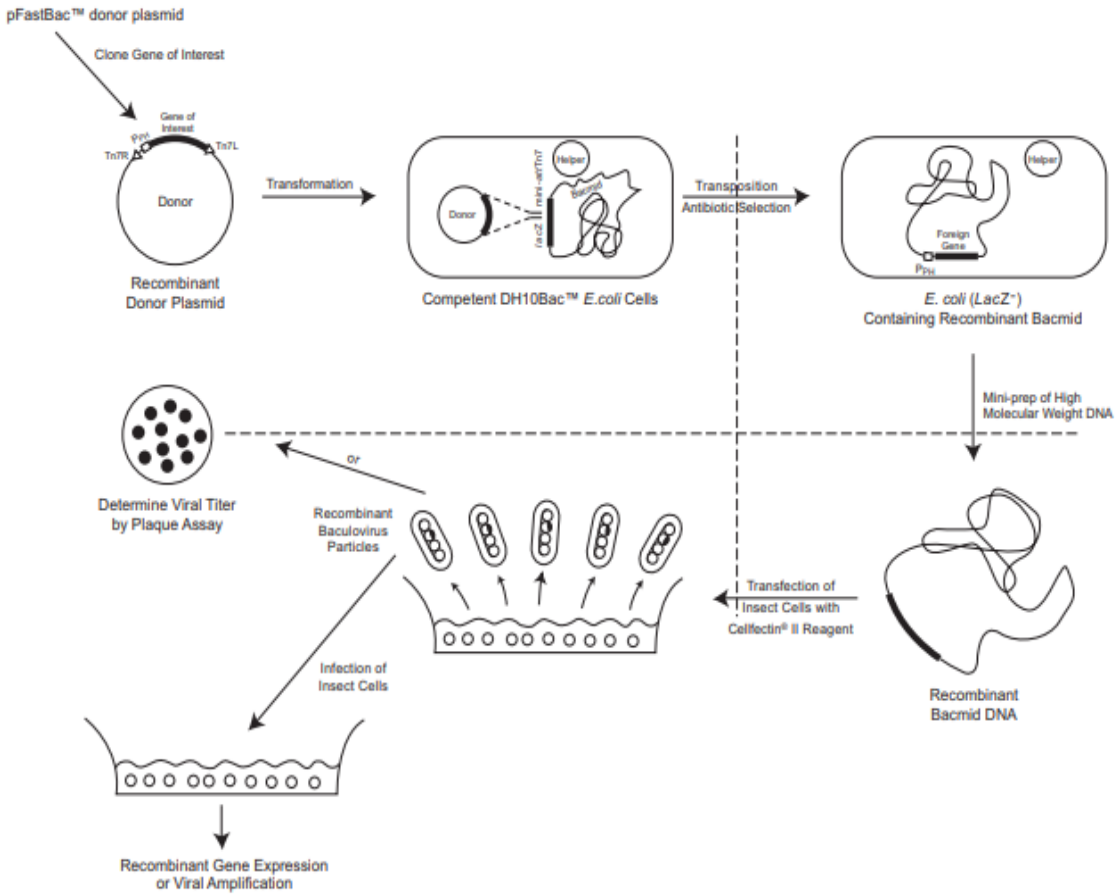


Figure 4.3: Baculovirus protocol overview. *The generation of the recombinant baculovirus and the expression of b_5 and GST using the expression system. Diagram adapted from manufacturer's protocol.*

Once Sf9 cells had been transfected with the recombinant bacmid DNA, the viral titre was amplified twice in order to ensure a high level of protein expression. Briefly, cells were seeded at 2 X 10^6 in a 6 well plate. Cells were incubated for 1 hour before DNA was added (18 µl for b_5 or 7 µl for GST based on protein yield) Cells were incubated for 7-10 days until signs of viral infection were apparent and the medium was collected (P2 stock). This process was then repeated in order to collect P3 stock which was stored at 4 °C until use.

4.3.9 Pesticide Assay and Confirmation of Protein Expression

Healthy cells, at 95 % confluency were used to seed 6 well plates (density 5 X 10^5). These plates were treated with 1 µl, 5 µl, 10 µl, 20 µl and 50 µl of the b_5 and GST viral P3 stock and one well was left as a control. This plate was monitored for cell viability over 5 days, to ensure a high level of virus would not cause cell damage. No excessive damage was observed, even in the highest viral concentration (1:40) and so three 96 well plates were seeded with Sf9 cells (density 5 X 10^5) in log phase of growth and 2.5 µl (1:40) of P3 viral stock added per well. 1 plate had P3 cytochrome b_5 viral stock added, one had P3 GST viral stock added, and one plate was used as a control plate with no viral stocks added to the cells. After 72 hours of exposure to the virus, media was changed and pesticides were added to the cells. Pesticides used were permethrin, bendiocarb and deltamethrin at final concentrations of 1 µM, 10 µM, 50 µM, 100 µM (Boonsuepsakul et al., 2008). Pesticides were dissolved in 1 % Dimethyl sulfoxide (DMSO) due to their relative insolubility in water. Six replicates were done of each concentration of each pesticide as well as 8 X 1 % DMSO control and 4 X 0 % DMSO control wells. Each of the three 96 well plates were treated identically and incubated at 27 °C for 48 hours.

All three plates underwent a MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay (Vybrant MTT cell viability assay kit, Thermo Fisher, Paisley, UK) on three of the six replicates. This was carried out following the manufacturer's 'quick protocol' option. Briefly, the media was removed from the cells and replaced with 100 µl of fresh culture medium. 10 µl of 12 mM MTT stock solution was added to each well and incubated for 4 hours at 37 °C. 85 µl of media was removed from each well and 50 µl of DMSO was added. This was then incubated at 37 °C for a further 10 minutes before mixing and reading the

absorbance at 540 nm on a FLUOstar Omega (BMG LABTECH). The remaining cells needed collecting to test for protein expression. 12 wells of cells expressing b_5 and 12 wells expressing GST were collected and stored in 1 X laemmli and GST buffer (10 mM Tris, 0.5 M NaCl pH 7.4) respectively. GST samples were snap frozen and stored at -80 °C and b_5 samples were stored at -20 °C.

The assay for GST activity was adapted from Bartley et al. (2015). 99 µl of phosphate buffer (100 mM potassium dihydrogen phosphate, 1 mM EDTA (pH 6.5) and 2 mM GSH) was added to each well followed by 10 µl of Tris buffer (10 mM Tris, 0.5 M NaCl (pH 7.4)). 10 µl of GST sample was added to three wells and 10 µl of control cells not treated with GST baculovirus was added to another three wells. 1 µl of 100 mM 1-chloro-2,4-dinitrobenzene (CDNB) was added to each well and the absorbance was read at 340 nm.

Cytochrome b_5 activity was tested via a Western blot using b_5 specific antibodies as per section 4.3.7.

4.4 Results and Discussion

4.4.1 Extraction of RNA from *Dermanyssus gallinae*

To isolate and express the putative accessory proteins, as found in section 3.3.2, RNA needed to be extracted from the PRM. RNA extraction was carried out as described in section 4.3.1. The samples were analysed via Nanodrop to determine the quality and purity of the samples and run on an agarose gel as described in section 4.3.1. The first sample taken had a 260/280 reading of 2.13 which is deemed 'pure' for RNA and was run on a gel as seen in Figure 4.4.

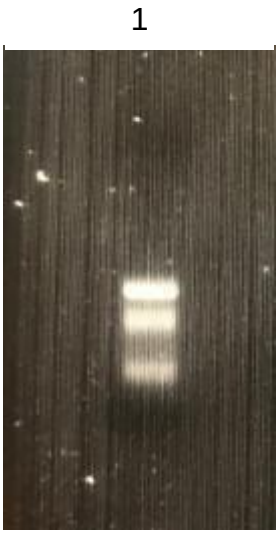


Figure 4.4: RNA extraction from PRM. *1 % agarose gel showing the RNA integrity. Lane 1 contains 5 µl of PRM RNA.*

Results from the RNA extraction show three bands on the agarose gel, which was unexpected, due to RNA normally showing two bands in mammalian RNA and one band in other tick species. Mammalian RNA has two bands because as the RNA is denatured it splits into two, which are the 18s and the 28s subunit. Ticks and other insects commonly have one band on an RNA agarose gel because when the RNA is heated and denatured, the 28s subunit splits into two

further units which are both the same size as the 18s subunit, meaning only one band can be viewed on a gel (Winnebeck et al., 2010)(Figure 4.5).

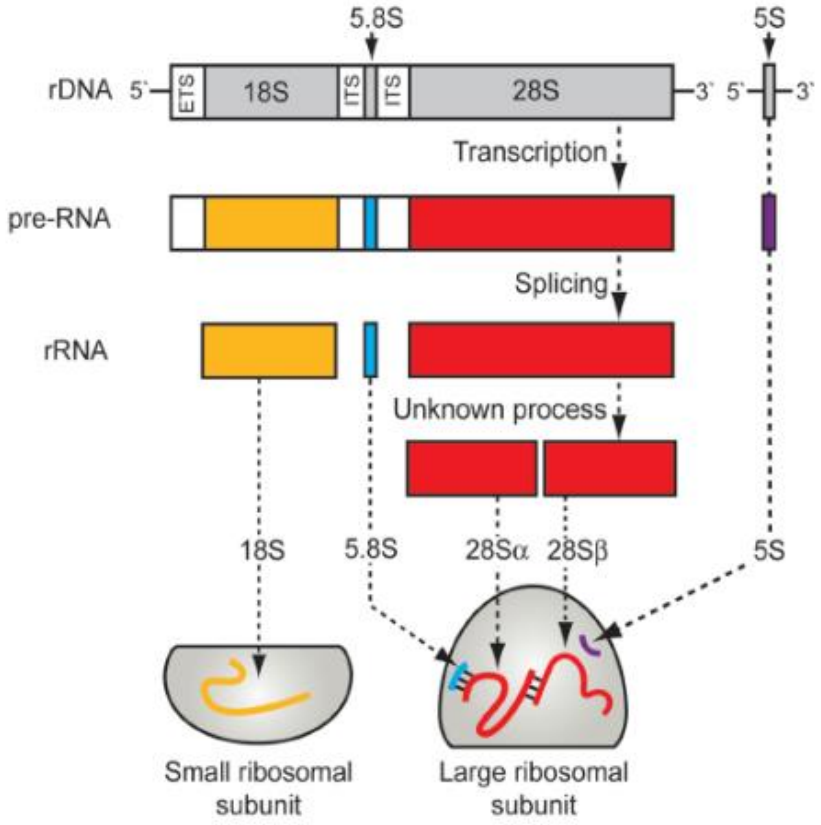


Figure 4.5: Assembly of rRNA into its subunits in insects. *In Eukaryotes each ribosome is comprised of 4 different molecules of RNA, two larger named 18s and 28s (after their sedimentation properties) and two smaller named 5s and 5.8s. The smaller ribosomal subunit is made from 18s and the larger unit is comprised of the other three units (5s, 5.8s and 28s). 5.8s, 18s and 28s rRNA are transcribed as a single precursor molecule and post-transcriptional processing of this molecule yields separate rRNAs which combine with ribosomal proteins to form ribosomal subunits. Insects have an additional processing step which cleaves 28s rRNA into α and β fragments. ETS: externally transcribed spacer region, ITS: internally transcribed spacer region. Diagram taken from (Winnebeck et al., 2010). (Gillespie et al., 2006).*

The band visible on the agarose gel (Figure 4.4) had very little smearing and so the RNA was converted into cDNA (see section 4.3.1). Several PCRs were completed in order to amplify cytochrome b_5 which resulted in empty gels. It was decided that a comparison gel should be run, using RNA extracted by a previous PhD student (Kirsty Graham) comparing the RNA sample from the *I. scapularis* and also a practice RNA she had completed using PRM (Figure 4.6).

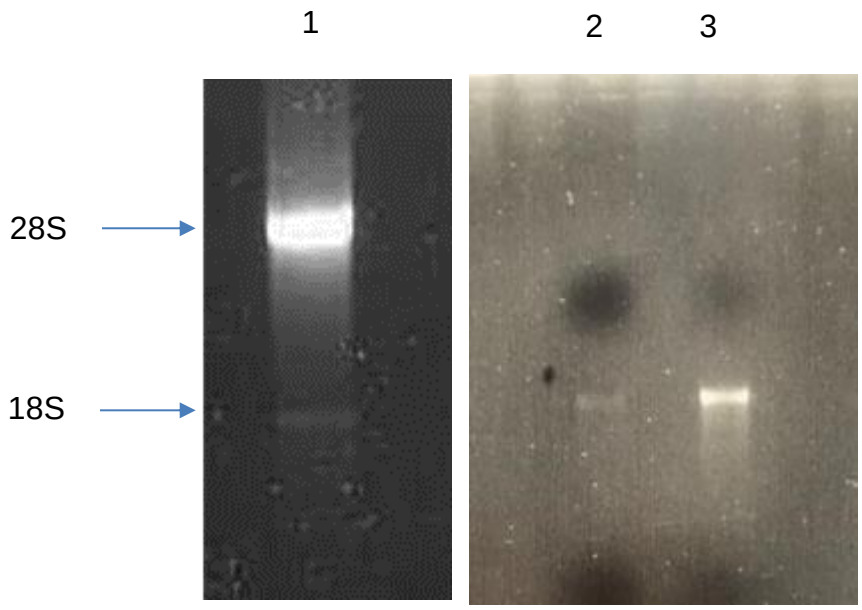


Figure 4.6: RNA comparison from 3 species. *Lane 1= Mammalian RNA showing 28s and 18s subunits, run by Kirsty Graham (former PhD student). Lane 2= PRM RNA, Lane 3= I. scapularis RNA; both previously extracted by Kirsty Graham and show the 1 band expected of an insect sample at 18s.*

The gel showed one band for both *I. scapularis* and for PRM, although PRM was a weaker band. This prompted a new RNA extraction from the PRM in order to get a strong signal on a gel which did not show three bands like the previous extraction (Figure 4.7).

Figure 4.7: 2nd PRM RNA extraction. *RNA was re-extracted from the PRM and run on a 1 % agarose gel in lane 1.*

The RNA was extracted from the PRM for a second time and run on an agarose gel. The result was a strong band on the gel which appeared to consist of two bands of a similar size. This could be consistent with the denaturing of the RNA and could show two fragments of the 28s that are a similar size. The band was a clear band with little smearing and had a 260/280 reading of 2.01 on the Nanodrop so was deemed of high purity. This was therefore used in subsequent downstream applications.

4.4.2 Amplification of Cytochrome b_5 and Cloning into Vector pET15b

Once RNA was deemed of high integrity, and had been converted to cDNA (see section 4.3.1), b_5 was amplified using the primers shown in Table 4.1. The results of this were run on a 1 % gel and can be viewed in Figure 4.8.

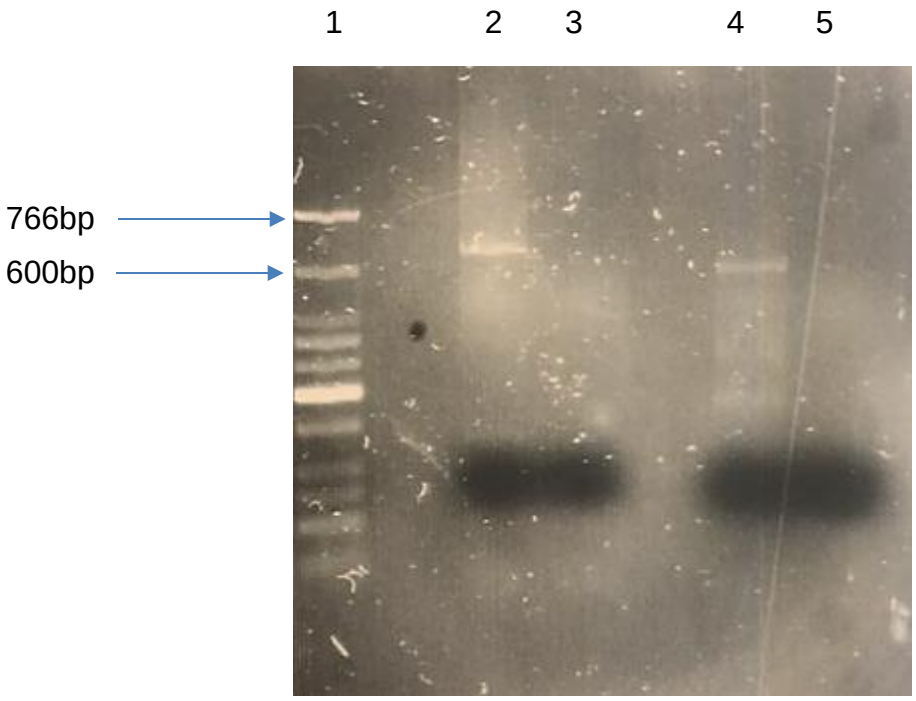


Figure 4.8: PCR of b_5 from cDNA. *Lane 1= Low molecular weight marker (NEB, Hertfordshire, UK), Lane 2= PCR product using primer set 1, Lane 3= PCR control with primer set 1, Lane 4= PCR product using primer set 5, Lane 5= PCR control with primer set 5.*

A low molecular weight ladder was used to analyse the size of the PCR product. The b_5 gene is 627 bp from the start of the GSP to the end. Both sets of primers appeared to produce a product of approximately the correct size but the smearing of the PCR product using primer set 1 led to primer set 5 being used to go forward. The PCR product from lane 4 was excised from the gel and cloned into pCR Blunt as described in section 4.3.3 and transformed into TOP10 cells (see section 4.3.3). Positive colonies were identified using kanamycin selective plates. pCR Blunt contains a lethal gene and ligation of the b_5 disrupts the expression of this gene, which allows growth of the colony and potentially only positive recombinants will grow on the plate.

A restriction digest with *EcoRI* (see section 4.3.4) was done on the colonies taken from the plate in order to identify positive colonies containing the correct size insert corresponding to the b_5 gene.

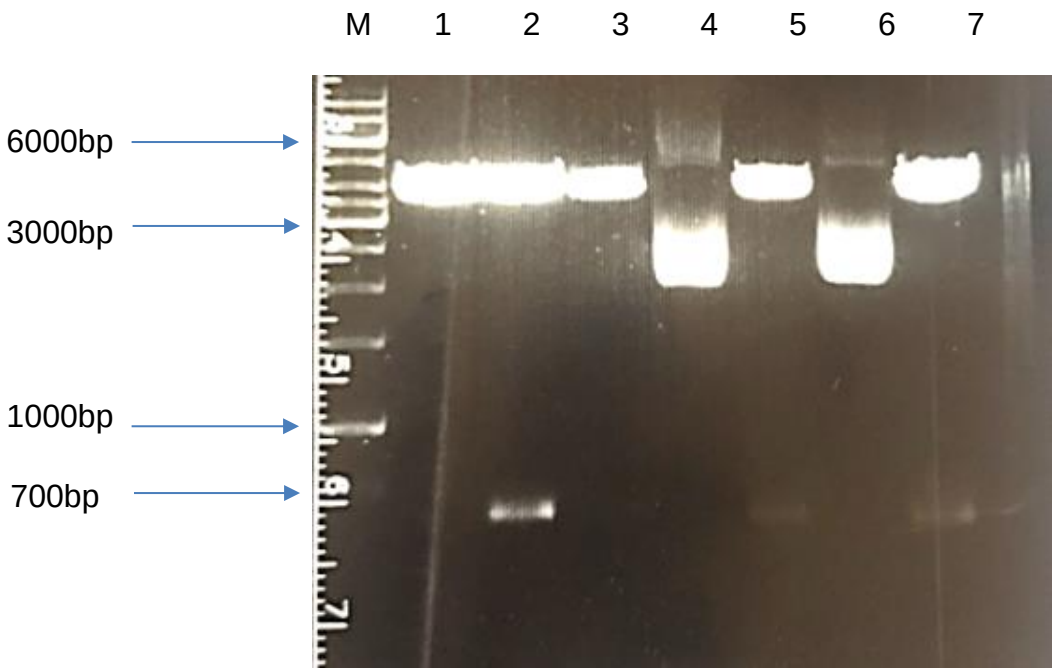


Figure 4.9: Results of EcoRI digest. *7 colonies of pCRBluntb_5 were digested with EcoRI and the results were run on a 1 % gel. Lane M= 1kb GeneRuler (Thermo Fisher, Paisley, UK), Lanes 1-7= colonies 1-7.*

The restriction digest (Figure 4.9) showed 3 colonies which seemed positive for the presence of the b_5 insert. The vector, pCR Blunt, is 3.5 kb and therefore should be one band higher on the molecular weight ladder than the 3000 bp line. The insert should be 627 bp long and therefore should be under the 700 bp line on the molecular weight ladder. Three colonies were a visual confirmation of this, and therefore colonies C2, C5 and C7 were sent for sequencing using M13 primers; M13 uni (21) TGT AAA ACG ACG GCC AGT and M13 rev (29) CAG

GAA ACA GCT ATG ACC. Sequence data was compared to the transcriptomic sequence data to confirm successful amplification.

Characterisation of the P450 accessory proteins requires them to be expressed and purified. To facilitate this, b_5 needed transferring into an expression vector which was suitable. pET15b allows expression of an N-teminally Histagged protein which can then be purified on a column using metal affinity chromatography. Primers were designed with the addition of the restriction sites *NdeI (5' CAT ATG 3')* and *XhoI* (5' CTC GAG 3') so the sequence could be ligated with pET15b (Table 4.2).

The first step in the process was the amplification of $PRMb_5$ to include the restriction sites required for cloning into pET15b. $PRMb_5C2$ was chosen to continue with and was amplified using PCR as described in section 4.3.5 (Figure 4.10).

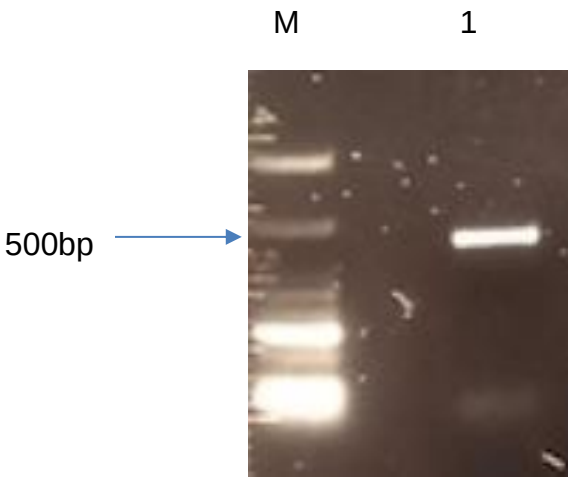


Figure 4.10: PCR of $PRMb_5C2$. *M= molecular weight ladder. L= PCR of $PRMb_5C2$.*

This PCR product, which now contained restriction sites for cloning into pET15b, was ligated with pCR Blunt and transformed into TOP10 cells. Overnight cultures

were made of ten colonies from the antibiotic selective plates which were then purified by miniprep and digested with *EcoRI. EcoRI* was chosen as there is a restriction site in the pCR Blunt vector at each side of the fragment insert which allows the fragment to be cut out of the vector and be visible on a 1 % agarose gel (Figure 4.11).

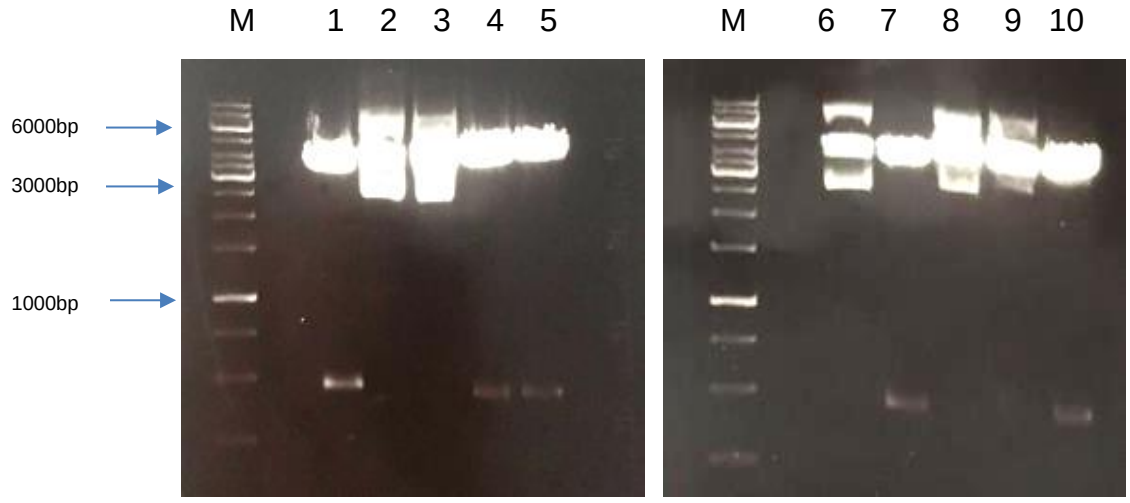


Figure 4.11: EcoRI digest of PRMb$_5$pCRblunt. *Gel is a 1 % agarose gel and the lanes 1-10 represent colonies 1-10. M= molecular weight marker, 1 kb GeneRuler (Thermo Fisher, Paisley, UK).*

The restriction digest showed 5 colonies which appeared to have a band at the correct migration for PRMb$_5$ as well as having the pCR Blunt vector at the correct migration of 3.5 kb. Colonies 1, 4 and 10 were sent to be sequenced using M13 primers and all three colonies came back with 100 % identity to the PRMb$_5$ gene.

With this confirmation, the fragment could now be ligated into pET15b. Both pET15b and PRMb$_5$ were digested with *NdeI,* as described in section 4.3.5, and can be viewed in Figure 4.12. Initial digest with *NdeI* was successful with pET15b and yielded one clear band. Digestion of PRMb$_5$pCRBlunt was only partial (Figure 4.12, lane 2) and so was further digested until only one band was present

on a 1 % agarose gel. These fragments were then put though a second digest using *XhoI* and the results of this can be seen in Figure 4.12, lanes 3-6. The bands were excised from the gel, pET15b was weaker than hoped but was successfully extracted from the gel, and the lower band from Figure 4.12, lanes 5 and 6, (b_5) was also extracted.

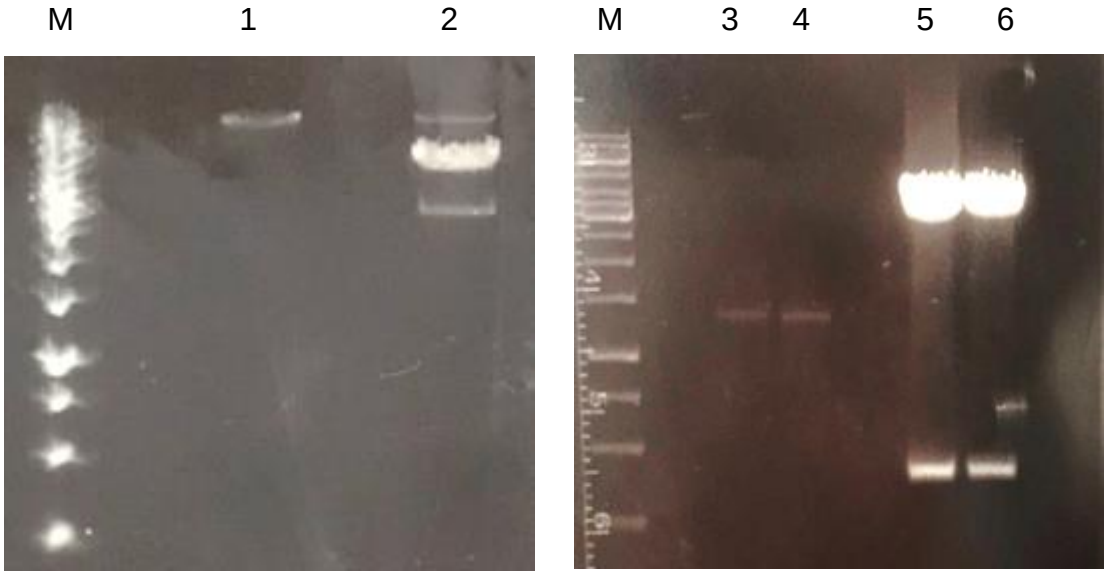


Figure 4.12: Restriction digest of PRMb$_5$pCRBlunt and pET15b. *M= Molecular weight markers. Lane 1= pET15b after digestion with NdeI. Lane 2= PRMb$_5$pCRBlunt after initial digest with NdeI. Lanes 3 and 4= digestion of pET15b after 2nd digest with XhoI. Lanes 5 and 6= digestion of PRMb$_5$pCRBlunt after 2nd digest with XhoI.*

With both bands extracted and having 'sticky ends', the two fragments could be ligated using T4 DNA ligase (see section 4.3.5) and transformed into TOP10 cells. The transformation was grown on ampicillin plates (50 µg/ml ampicillin) as pET15b contains an ampicillin resistance gene, meaning only colonies containing the pET15b construct will be viable on an ampicillin plate. 7 colonies were taken from the plates and grown overnight in LB containing 50 µg/ml ampicillin before

they were purified using a miniprep. A double digest using both *NdeI* and *XhoI* was performed to isolate colonies containing the b_5 gene and results were run on an agarose gel (Figure 4.13).

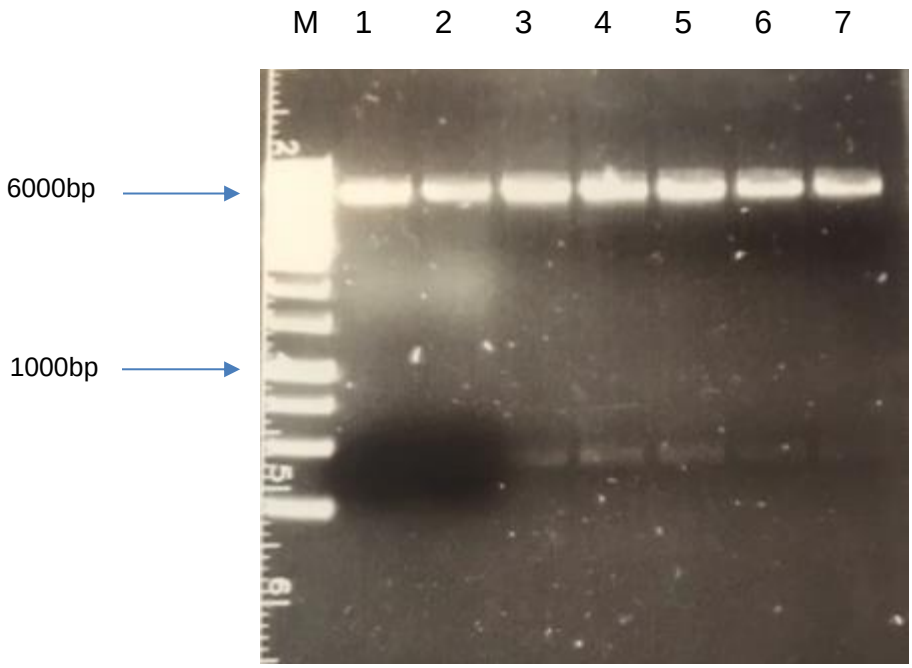


Figure 4.13: XhoI and NdeI digest of pET15bPRMb$_5$. *M= molecular weight ladder, 1 kb GeneRuler (Thermo Fisher, Paisley, UK), lanes 1-7= Colonies 1-7.*

The gel showed the presence of a band in approximately the correct weight for the b_5 gene in 5 of the colonies and the upper band was of approximately the correct weight for the pET15b vector (5.7 kb). Colonies C3 and C4 were sent for sequencing using T7 primers (see section 4.3.5).

This sequencing confirmed the presence of the b_5 gene in both sequences (Figure 4.14).

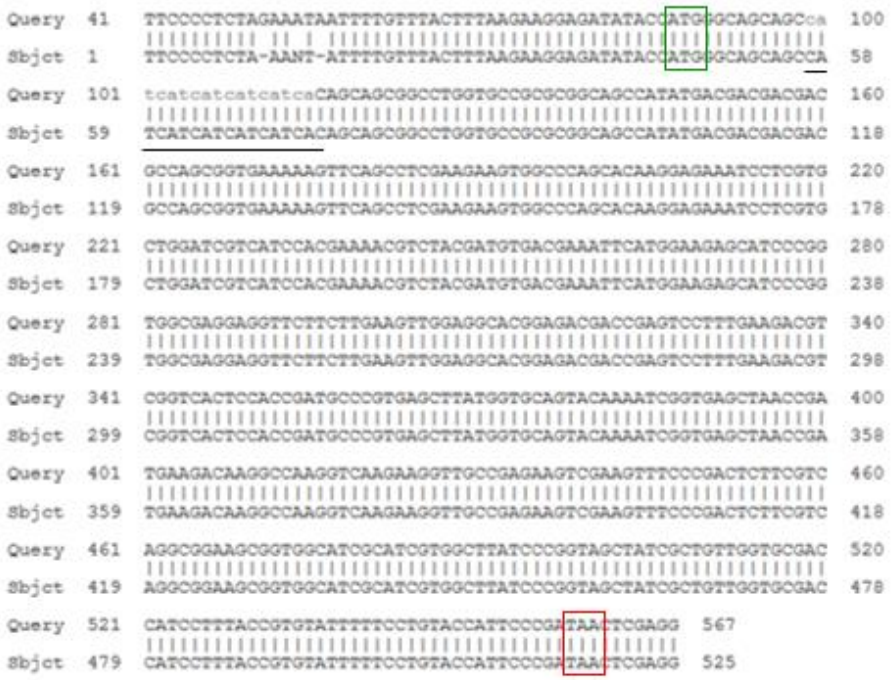


Figure 4.14: Sequencing results of PRMb$_5$pET15b. *The cloning of the Cytb5 fragment into pET15b. Query= in silico sequence to encode His-tagged PRM Cytb5. Subject= results of the sequencing for colony 3 returned from a sequencing company. The start and stop codons are highlighted by green and red boxes respectively. The six histidine tag is underlined. Alignment run in NCBI BLASTn.*

Sequencing confirmed the successful ligation of PRMb$_5$ into the pET15b vector, this has created a polyhistidine-tagged protein which can be purified using metal affinity purification.

4.4.3 Expression of Cytochrome b$_5$ from *Dermanyssus gallinae, Ixodes scapularis* and *Rhipicephalus microplus*

Having successfully cloned PRMb$_5$ into pET15b, a transformation was required to transform the recombinant plasmid into BL21(DE3)pLysS cells using the protocol from section 4.3.3. The cells were chosen because pET15b has a T7 lac

promoter which allows transcription of the gene inserted, therefore the plasmid needed transferring into a cell line that contains a copy of T7 RNA polymerase gene (DE3), hence the choosing of BL21 DE3 cells which have such a genotype and contain a lacUV5 promoter. The T7 promotor and then lacUV5 promoter are both controlled by the lac repressor gene which causes the transcription of the T7 RNA polymerase gene in the host polymerase but halts the T7 lac promoter in the vector to stop transcription of the gene of interest by any RNA polymerase produced. pET15b vectors show a small level of expression but IPTG induces expression which allows the RNA polymerase to access the T7 promotor and cause transcription of the gene of interest.

BL21 DE3 cells can express high levels of protein without being subjected to proteolysis as they are lacking in OmpT and Lon proteases which could cause the proteolysis of the expressed b_5. The strain pLysS was chosen as a chloramphenicol resistant plasmid which codes for T7 lysozyme which naturally inhibits T7 RNA polymerase. Cells produce T7 lysozyme which supresses base expression of the T7 RNA polymerase before the cells are induced using IPTG. This allows the recombinant plasmid to remain stable whilst the cells are in the exponential growth phase, before being induced.

The $Cytb_5$ gene has been isolated from two veterinary important acari, *Rhipicephalus (Boophilus) microplus* (RM), *Ixodes scapularis* (IS) as well as from *Dermanyssus gallinae* (PRM). $Cytb_5$ was identified and isolated in these species by a former PhD student (Kirsty Graham) and the expression and purification of the genes allows comparisons with other arthropod species. Monitoring the changes in expression of b_5 when exposed to pesticides in different species in a baculovirus expression system will allow for more information on the potential role of b_5 in resistance mechanisms in arthropods and may allow for changes to

acaricide development. b_5 modifies the P450 complex and can donate the 2nd electron required in the P450 cycle, instead of the electron donation coming from POR. In doing so b_5 is around 10-100 times faster than POR and has been shown to stimulate, inhibit or have no effect on the activity of the P450 complex. The effect of b_5 has also been found to be dependant not only on specific P450 genes but also on the specific substrates of P450s, making knowledge of their intricate mechanism of great interest (Im and Waskell, 2011; Zhang et al., 2015). Downstream modelling of cytb_5 in these species will provide a molecular basis for the design of new acaricides or synergists targeting b_5 and thus provide a means of controlling populations of acari which have become resistant to the acaricides currently in use.

Transformations of PRM, IS and RM into BL21(DE3)pLysS were completed and grown on double antibiotic plates (50 µg/ml ampicillin and 34 µg/ml chloramphenicol) before being used to inoculate sterile TB (see section 4.3.6). Samples were taken of the unexpressed protein before IPTG and Aminolevulinic acid (ALA) were added to initiate protein expression. IPTG is a molecular mimic of allolactose, which functions by binding to the *lac* repressor and releasing the lac repressor from the lac operator which allows transcription of the gene inserted into the *lac* operon (Figure 4.15). ALA is an endogenous non-protein amino acid which is part of the porphyrin synthesis pathway which leads to heme production, this is added as the level in the cell can be a limiting factor.

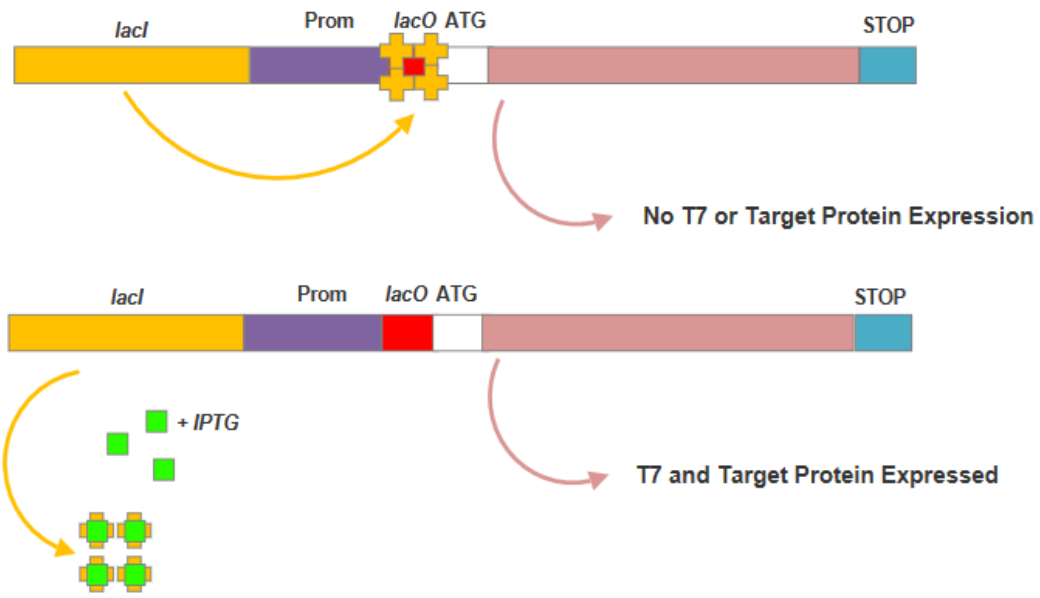


Figure 4.15: IPTG-inducible protein expression. *IPTG is a molecular mimic of allolactose and is represented by a green square. This binds to the lac repressor and releases the repressor from the lac operator in an allosteric manner, which allows the transcription of genes in the lac operon (lacO), such as* b_5.

Firstly, both C3 and C4 PRMb$_5$pET15b constructs were expressed as described in section 4.3.6 and one flask was left unexpressed as a control. The colonies were induced at 30 °C overnight and then spun down before the images were taken (Figure 4.16). The pink/red tone of the expressed samples suggests that the hemoprotein has correctly folded and has been significantly expressed. This first experiment was completed on a smaller scale to prove principle and then scaled up to produce large volumes of expressed protein.

1 2 3

Figure 4.16: Expression of cytochrome b_5 from PRM with the expression vector pET15b. *Image showing the pellet obtained from expressed b_5 and non-induced b_5. Tube 1= colony 3 without being induced by IPTG, tube 2= colony 3 after inducing with IPTG and tube 3= colony 4 after inducing with IPTG.*

Samples were taken of each colony before and after induction with IPTG and the results run on an SDS-PAGE gel (Figure 4.17), which confirmed the higher levels of protein expression in the samples induced with IPTG. Protein expression is seen to increase in the induced samples below the 25 kD mark, b_5 is expected to show up around the 17 kD mark and so the gel supports the observation of the presence of b_5 from the pink pellet.

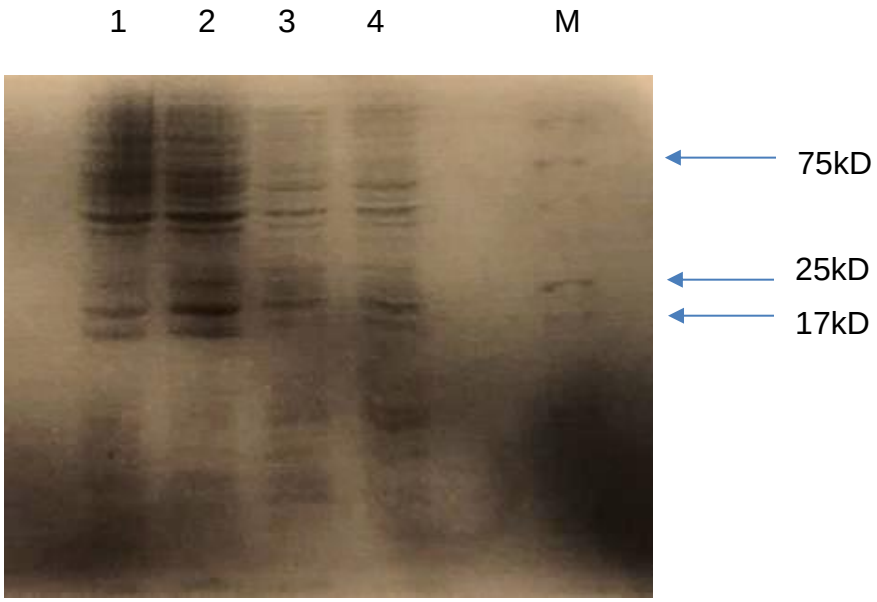
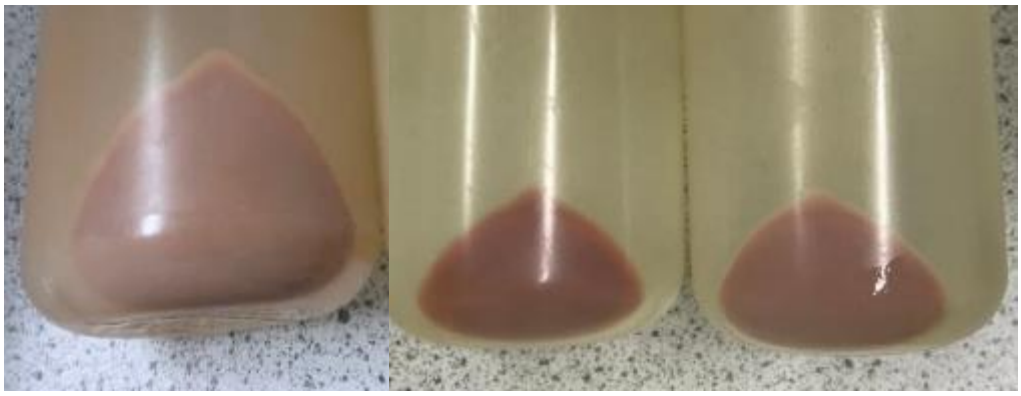


Figure 4.17: SDS-PAGE gel of induced and uninduced samples from PRM. *Lane 1= Colony 4 induced sample, Lane 2= Colony 3 induced sample, Lane 3= Colony 4 uninduced sample, Lane 4= Colony 3 uninduced sample, Lane M= weight marker.*

Cytochrome b_5 was then taken from RM and IS and expressed under the same conditions as PRM (see section 4.3.6). This was done in a larger culture for PRM (using colony 3) and produced three large pellets which indicated a successful expression of the protein. The results of the expression can be viewed in Figure 4.18.



1 2 3

Figure 4.18: Expression of cytochrome b_5 from D. gallinae, R. microplus and I. scapularis. *Tube 1= R. microplus, Tube 2= D. gallinae, Tube 3= I. scapularis. All three tubes were expressed using IPTG overnight at 30 ˚C.*

4.4.4 Purification of Cytochrome b_5 from *Dermanyssus gallinae, Ixodes scapularis* and *Rhipicephalus microplus*

Purification of the protein is essential to establish its role in the mechanisms of pesticide resistance and using biochemical characterisation to determine the effect of b_5 on P450 function. The value of this to PRM is great, as determining the cause of resistance mechanisms allows the ability for targeted product development, which in turn gives the farming community effective products and improves egg production. This would also be a significant advance to the IS and RM tick species, as they are both of veterinary importance due to their transmission of disease and both species are facing issues of resistance, similar to the PRM. Advancing the knowledge of one species of arthropod will assuredly advance the understanding of others and therefore having three species of veterinary importance at a purified stage and ready for biochemical analysis is of huge value to the field.

Purification of cytochrome b_5 was carried out using metal affinity chromatography on a 5 ml HisTrap column (GE, Buckinghamshire, UK) as described in section 4.3.6. IS b_5 was solubilised first and loaded on to the HisTrap column, immediately the binding of b_5 was visible as a red band and this aided the purification by allowing the accurate collection of the purified sample (Figure 4.19).

Figure 4.19: Loading of HisTrap column. *ISb_5 was first to be loaded on to the column and the red band was immediately visible, confirming binding.*

Samples were run on a 15 % SDS-PAGE gel and can be viewed in Figure 4.20. Protein expression is clearly increased in the induced sample in lane 2, (highlighted) compared to the uninduced sample in Lane 1. The first wash to come through the column shows very little protein, indicating the protein has

successfully bound to the column. The following washes show very little protein which ensures the protein was not washed off the column. The eluted and purified sample in Lane 8 shows a band of approximately the correct weight (15-17 kD). The two bands visible could be due to slight degradation of the protein sample, potentially due to the difficulty of keeping the sample chilled whilst purifying. Cytochrome b_5 is a polypeptide consisting of two distinct domains, a short hydrophobic C- terminal membrane binding domain and a large globular segment containing the heme domain. In other purifications of b_5 the loss of the C-terminus binding domain is seen, which could result in the different sized bands seen on the gel (Begum et al., 2000; Strittmatter et al., 1978; Sobrado et al., 2008).

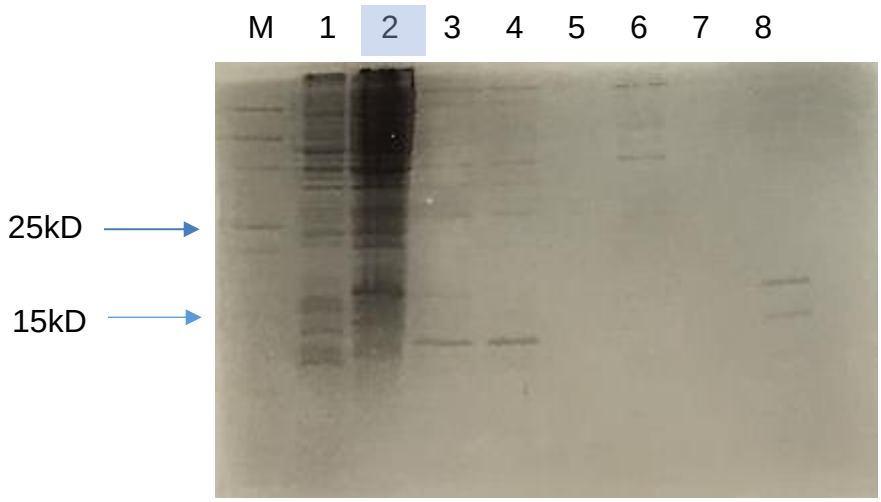


Figure 4.20: Purification of ISb$_5$. *M= molecular markers, Lane 1= protein before it was induced with IPTG, Lane 2= protein after it was induced (highlighted in blue), Lane 3= solubilised protein. Lane 4= flow through of solubilised protein through the column, Lane 5= flow through of the 15 mM imidazole wash, Lane 6= flow through of the 30 mM imidazole wash, Lane 7= flow through of the 50 mM imidazole wash, Lane 8= the eluted and salt exchanged sample.*

Following the purification of ISb$_5$, RMb$_5$ was purified using the same techniques (Figure 4.21). The prep of RMb$_5$ appeared the same in colour as the other species

protein preparations (Figure 4.18) but the SDS-PAGE revealed a more concentrated stock of protein than ISb5. A dense band was present at ~15 kD mark.

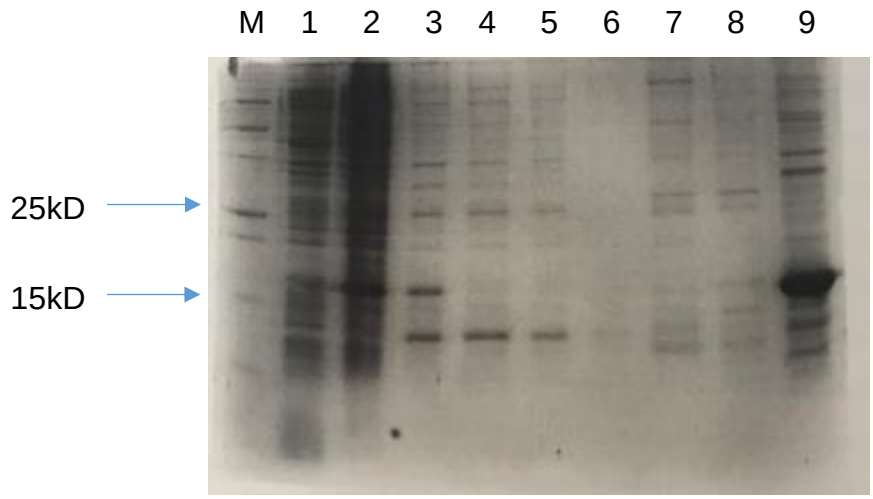


Figure 4.21: Purification of RMb5. *M= molecular markers, Lane 1= protein before it was induced with IPTG, Lane 2= protein after it was induced, Lane 3= solubilised protein. Lane 4= flow through of solubilised protein through the column, Lane 5= soluble fraction first wash, Lane 6= flow through of the 15 mM imidazole wash, Lane 7= flow through of the 30 mM imidazole wash, Lane 8= flow through of the 50 mM imidazole wash, Lane 9= the eluted and salt exchanged sample.*

Two samples were prepared from PRM, both soluble b_5 (the form in erythrocytes) and membrane bound b_5 (found localised to the endoplasmic reticulum and commonly found involved in pesticide metabolism). The membrane bound form is essential for *in vivo* studies of b_5 biochemistry and the soluble form could be used in the future for antibody purification. After the ultracentrifuge spin, as described in section 4.3.6, the supernatant was stored as the soluble fraction and the pellet was resuspended and stored in 1 X TSE buffer as the membrane bound

fraction. After the full purification, induced and uninduced samples as well as all washes and final purified samples were run on a gel as seen in Figure 4.22.

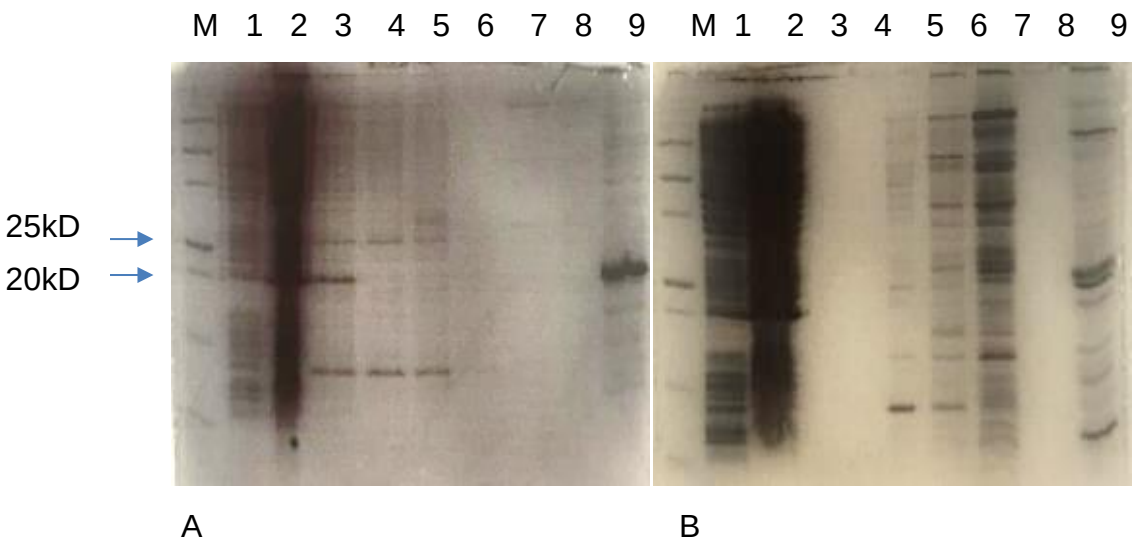


Figure 4.22: SDS-PAGE gel of both soluble and membrane bound b_5 in PRM. *Figure A= membrane bound protein, Figure B= Soluble protein. M= molecular markers, Lane 1= protein before it was induced with IPTG, Lane 2= protein after it was induced, Lane 3= solubilised protein. Lane 4= flow through of solubilised protein through the column, Lane 5= soluble fraction first wash, Lane 6= flow through of the 15 mM imidazole wash, Lane 7= flow through of the 30 mM imidazole wash, Lane 8= flow through of the 50 mM imidazole wash, Lane 9= the eluted and salt exchanged sample.*

Both PRM preps provided a strong purified band of protein at a similar molecular weight to one another. The bands appear heavier in molecular weight than that of IS and RM b_5, which is likely due to slight differences in protein weight in the different species, for example, IS b_5 is predicted to be 14.74 kDa, RM b_5 is predicted to be 15.1 kDa and PRM is predicted to be 15.51 kDa (Graham et al., 2016).

The benefit of having purified protein for all three of the pest species is invaluable. These proteins can be used in a host of downstream applications which have potential to make large breakthroughs in combatting resistance. The summary of this purification can be seen in Figure 4.23.

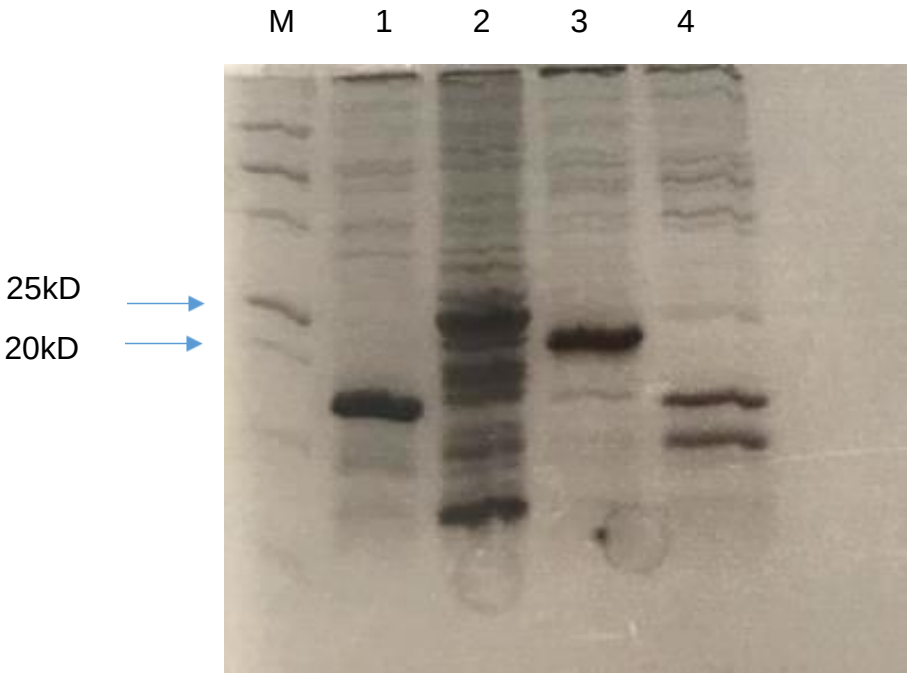


Figure 4.23: Summary of purification of cytochrome b_5. *M= molecular markers, Lane 1= R. microplus, Lane 2= D. gallinae soluble fraction, Lane 3= D. gallinae membrane bound fraction, Lane 4= I. scapularis.*

The PRM b_5 membrane fraction was confirmed using a Western blot with specific b_5 antibodies which were sent by a collaborator in Liverpool (Dr Mark Paine). The specificity of the antibody was first trialled using a dot blot (Figure 4.24), before being used in a Western blot.

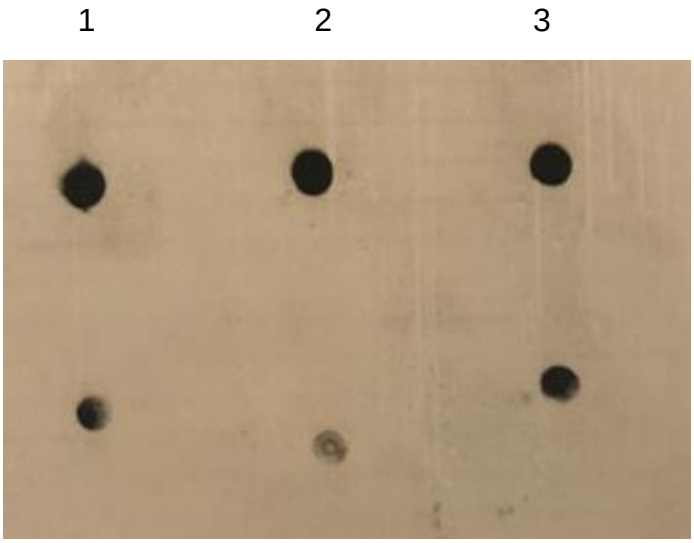


Figure 4.24: Dot blot using PRM membrane bound b_5. *Dot 1= 1:625 µl of antibody, Dot 2= 1:1250 µl of antibody, Dot 3= 1:2500 µl of antibody, Dot 4= 1:5000 µl of antibody, Dot 5= 1:7500 µl of antibody, Dot 6= 1:10,000 µl of antibody.*

50 ng of PRM membrane bound b_5 was added to each dot and decreasing concentrations of antibody were added to ensure the antibody had a high specificity for cytochrome b_5. Strong signals were picked up from all concentrations of antibody used, but in particular from the highest three concentrations, so a working concentration of 1:2500 was used going forwards in the project. Once it was ascertained that the b_5 could be easily detected using the antibody, a Western was completed using a range of b_5 amounts (Figure 4.25).

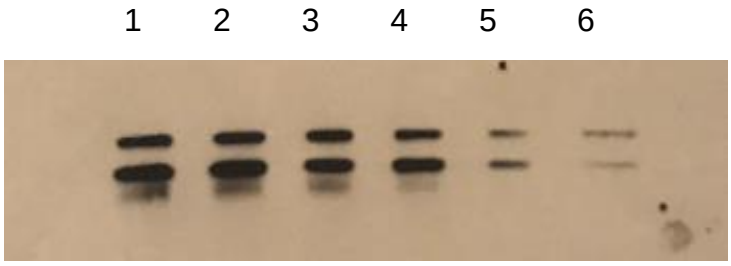


Figure 4.25: Western blot of membrane bound PRM b_5. *Lane 1= 100 ng b_5 loaded, Lane 2= 50 ng b_5 loaded, Lane 3= 40 ng b_5 loaded, Lane 4= 25 ng b_5 loaded, Lane 5= 10 ng b_5 loaded, Lane 6= 5 ng b_5 loaded,*

The Western showed a clear decrease in band density when the concentration of b_5 was lowered. The Western is sensitive for low concentrations of b_5 with 5 ng of protein being detected by the antibodies. This was essential for reliability of the Western results after expressing b_5 in the baculovirus system. The splitting of the b_5 into two bands on the Western was unexpected and could be due to the sensitivity of b_5 to the temperature variability. It could also represent an unidentified protein which may have been co-purified in the mosquito prep, which cross-reacts with the Anti-rabbit b_5 antibody (Sacco and Trepanier, 2010).

4.4.5 Generation of Baculovirus Expression System for Cytochrome b_5 and the Glutathione S-transferase from *Dermanyssus gallinae*

The success of the expression of b_5 in bacteria allowed the possibility of expressing the gene in a baculovirus expression system. Expressing proteins in a baculovirus system allows for functional assays to be carried out which allow the function of the b_5 gene to be monitored in terms of pesticide metabolism. Insect expression systems require the production of a recombinant baculovirus, which is time consuming, for use in Sf9 insect cells. The stages of the process

can be seen in Figure 4.26 and were carried out using adhering Sf9 cell culture grown in Serum Free media.

As well as taking b_5 through the process of the baculovirus expression system, a Glutathione S-transferase (GST) gene isolated from PRM was sent by a collaborator at the Moredun Institute, Kathryn Bartley. GSTs are known to facilitate the detoxification of drugs by catalysing the conjugation of reduced glutathione (GSH) to xenobiotic substrates, thereby playing a role in xenobiotic resistance (Bartley et al., 2015). Therefore, it was of interest to investigate the over expression of GST as well as the over expression of b_5 on the toxicity of three common pesticides at varying concentrations.

Figure 4.26 shows the first stage of creating the baculovirus expression system is to create a pFastBac recombinant bacmid, whereby the gene of interest is ligated into pFastBac before transforming into DH10 *E. coli* cells. pFastbac vector allows for the high level expression of the gene of interest in insect cells and contains a gentamycin resistance gene. This was first carried out with the b_5 gene from PRM, followed by the PRM GST.

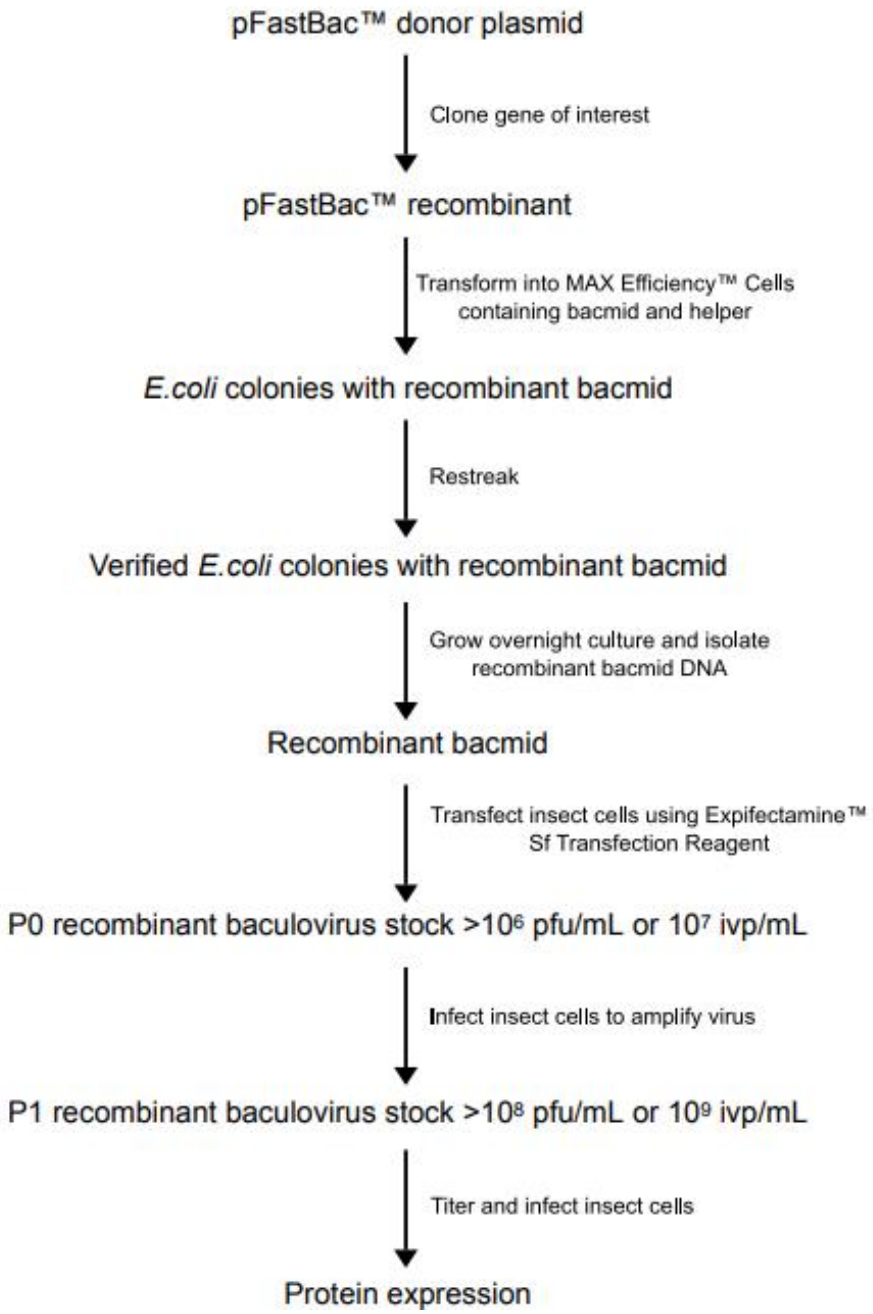


Figure 4.26: Overview of the work process of Bac-to-Bac Expression System. *Flowchart documenting the stages taken to produce a recombinant baculovirus and using the baculovirus to express the gene of interest in Sf9 cells. Flowchart taken from manufacturer's protocol.*

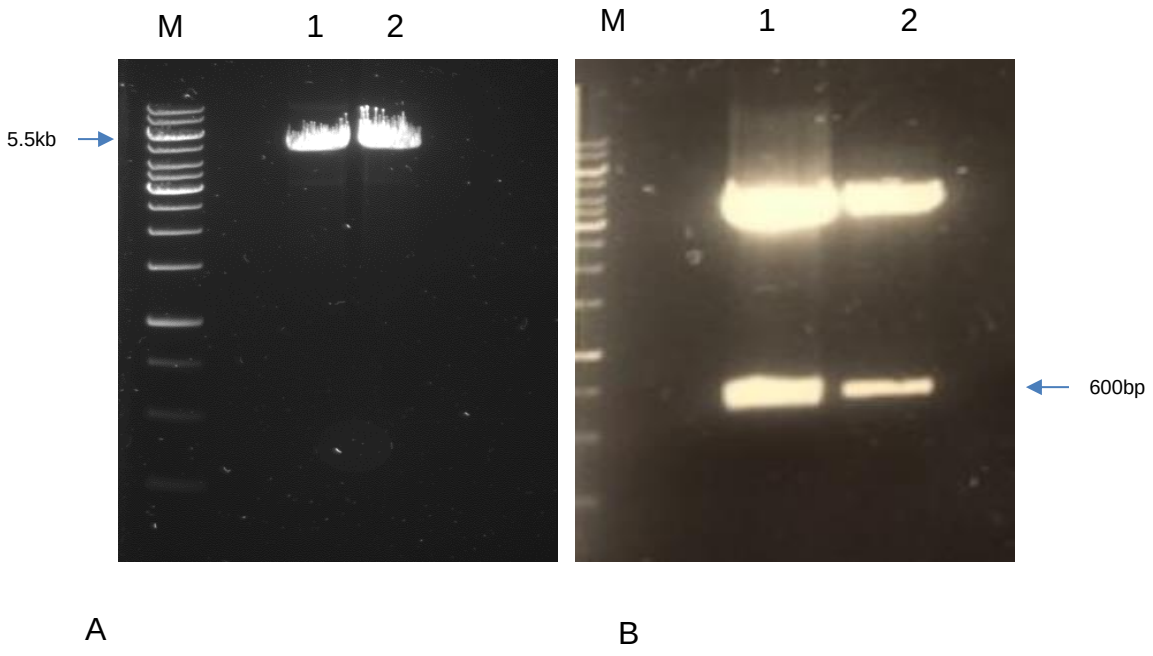


Figure 4.27: BamHI and XhoI digest of pFastBac and b_5. *M= molecular markers for all gels. A; Lane 1= pFastBac digest with BamHI followed by XhoI, Lane 2= pFastBac digest with XhoI followed by BamHI. B; Lane 1 and 2= PRMb_5pCRBlunt*

The first stage of the process was to miniprep and digest pFastBac and PRM cytochrome b_5 with both *BamHI* and *XhoI* in order to cut the vector in one place to allow for the insertion of the b_5 gene which can be viewed in Figure 4.27. Following this step, the 5.5 kb pFastBac band and ~600 bp b_5 band were excised from the gel and ligated using T4 DNA ligase before being cloned into TOP10 cells. These cells were grown overnight and checked for the presence of the b_5 gene using a restriction digest and PCR, with no success. This process was repeated, this time being digested with new *EcoRI,* in an attempt to negate any issues with ineffective enzymes (Figure 4.28).

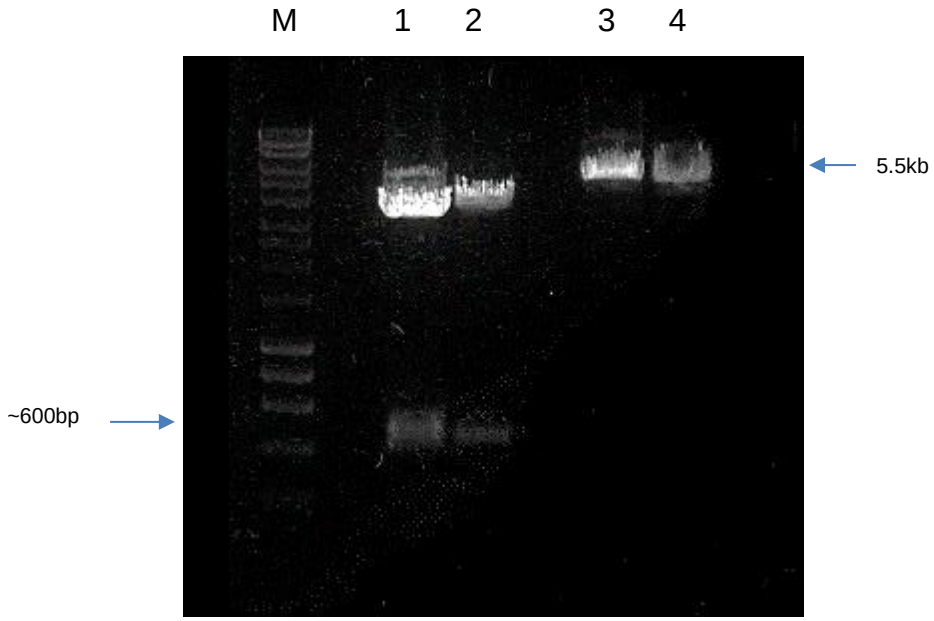


Figure 4.28: EcoRI digest of pFastBac and b_5. *M= molecular markers. Lanes 1 and 2= b_5 digested with EcoRI, Lanes 3 and 4= pFastbac digest with EcoRI.*

Both the presumed b_5 band and the pFastBac band were excised from the gel and ligated using T4 DNA ligase, before cloning into TOP10 Cells. These cells were grown overnight and checked for the presence of the b_5 gene using a restriction digest and PCR, with no success.

A third transformation of both pFastBac and PRM b_5 was completed and the miniprep of this transformation was used for a digest with new stocks of *BamHI* and *XhoI* (Figure 4.29). This digest was successful and bands were once again excised from the gel, ligated and cloned into TOP10 cells.

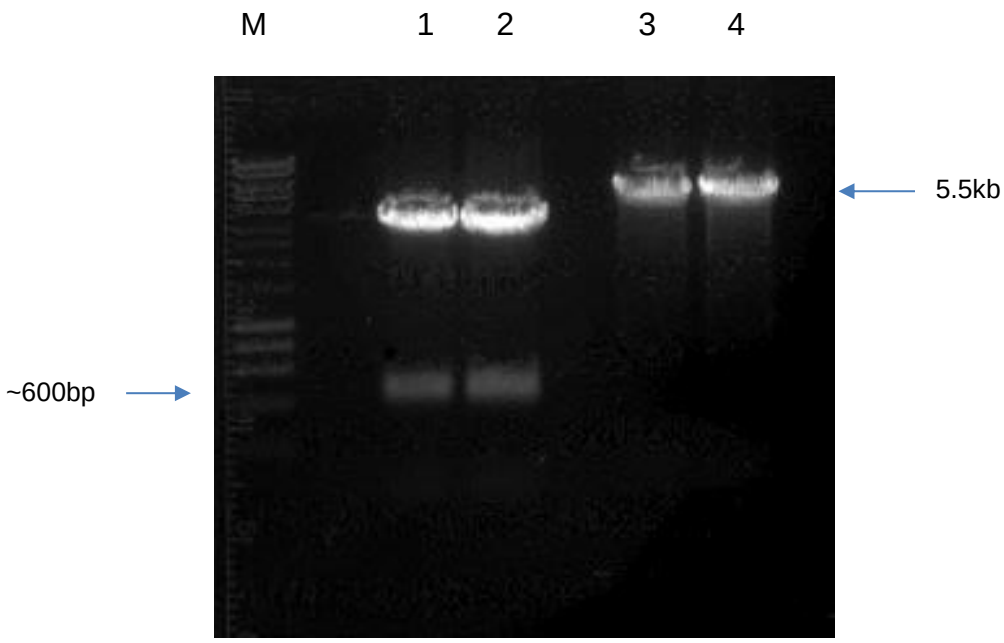


Figure 4.29: Repeat BamHI and XhoI digest of pFastBac and b_5. *M= Molecular weight markers. Lanes 1 and 2= b_5 digested with BamHI and XhoI, Lanes 3 and 4= pFastBac digested with BamHI and XhoI.*

A *BamHI* digest was used to screen the colonies for the presence of the b_5 fragment in the pFastBac vector. The results of this can be seen in Figure 4.30, with two colonies showing bands at the approximate correct molecular weight for the b_5 gene insert. The colonies were sent for sequencing which confirmed the full gene sequence for b_5 was present in the pFastBac vector of two colonies (Figure 4.30, lanes 6 and 8) and could be transformed into DH10 cells. The colonies from the transformation were screened using the PCR conditions from the manufacturer's protocol and colonies were found to be of the correct molecular weight according to the protocol (2300 bp plus fragment size; 2900 bp).

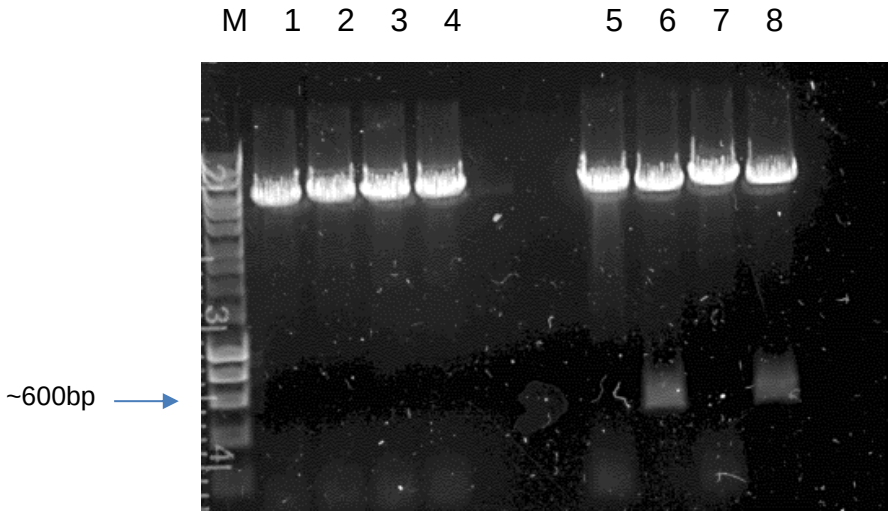


Figure 4.30: BamHI digest of pFastBacPRMb$_5$ colonies. *M= molecular weight markers. Lanes 1-4= colonies 1-4 from type 1 TOP10 Cells, Lanes 5-8= colonies 1-4 from type 2 TOP10 cells.*

With the b_5 successfully transformed into DH10 cells, the GST was next to be prepared. The first step was to design GST primers which would amplify the sequence and allow it to be ligated with pCRBlunt; Forward: 5'GCC ACC AGT TCG TGA TGG GC 3' and reverse: 5' GCC CAT CAC GAA CTG GTG GC 3'. Amplification with this set of primers produced a clear band at ~600 bp on the agarose gel, as seen in Figure 4.31, which allowed the excising of the GST fragment from the gel for ligation with pCRBlunt.

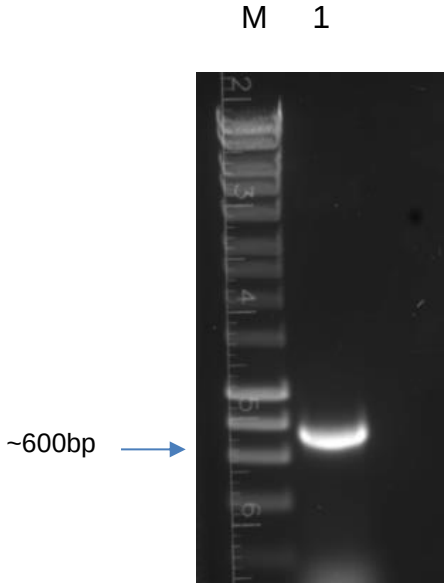


Figure 4.31: PCR amplification of GST. *M= Molecular weight markers, Lane 1= Amplification of GST.*

The ligation was transformed into TOP10 cells and digested with *EcoRI* to check for the presence of the GST fragment. Colonies appearing to contain the correct size fragment were confirmed with sequencing using M13 primers. With sequencing confirming the correct GST sequence had been amplified and ligated with pCRBlunt, it was then digested with *BamHI* and *KpnI* before being gel extracted, along with pFastBac, and the fragments ligated together using T4 DNA ligase (Figure 4.32).

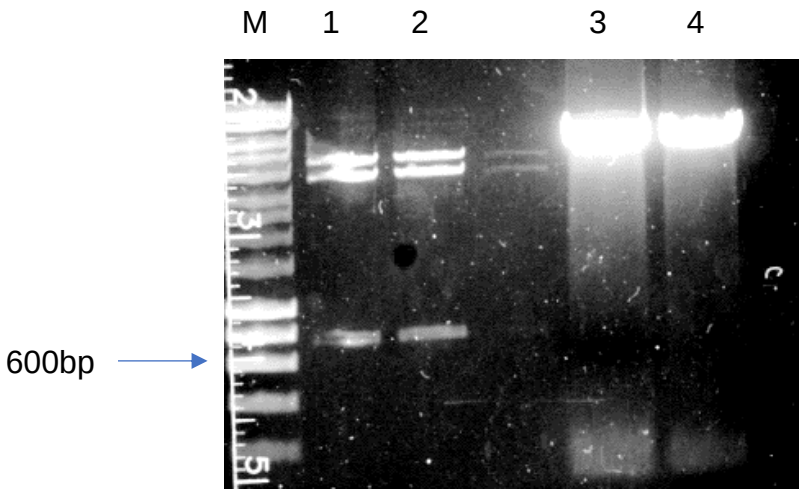


Figure 4.32: Agarose gel showing GST and pFastBac before gel extraction. *M= molecular markers, Lanes 1 and 2= GST samples digested from the pCRBlunt vector using BamHI and KpnI. The band of ~600 bp was excised from the gel, Lanes 3 and 4= pFastBac vector digested with BamHI and KpnI.*

The ligation was transformed into TOP10 cells and checked via PCR using the GSP for GST and Q5 high fidelity polymerase, the results can be seen in Figure 4.33 and show 24 of the 25 clones tested to have a fragment at the approximate correct size of the GST fragment. This was also checked using a restriction double digest with *KpnI* and *BamHI* with 9 of the positive PCR colonies (Figure 4.34).

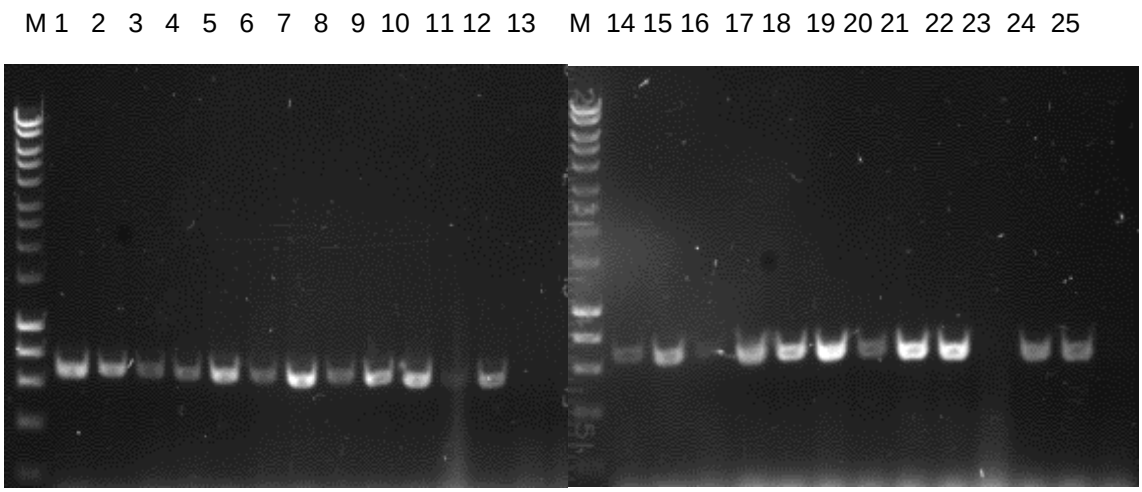


Figure 4.33: PCR of GST pFastBac construct using GST GSPs. *M= molecular weight ladder. Lanes 1-25= clones 1-15.*

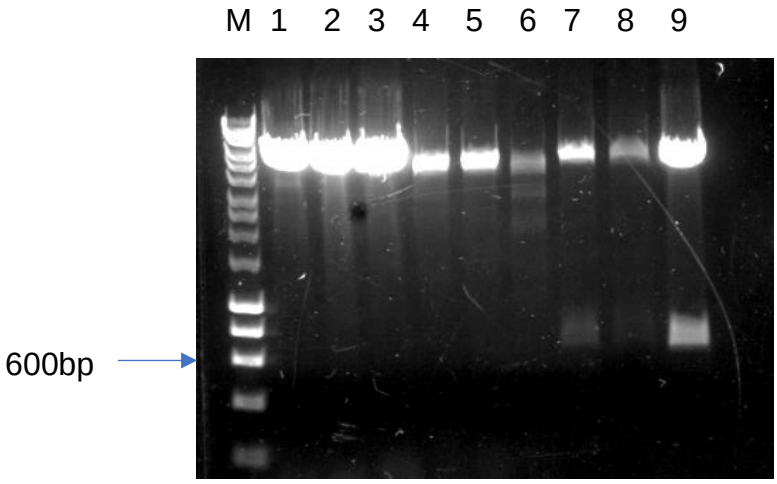


Figure 4.34: Restriction digest of GST pFastBac construct with KpnI and BamHI. *M= Molecular weight ladder.*

Two colonies that contained the correct size band were sent for sequencing (Figure 4.34, lanes 7 and 9) and were confirmed to contain the GST sequence, allowing them to be transformed into DH10 cells. GST pFastBac DH10 colonies were checked using the same method as the b_5 pFastBac DH10 colonies, using the PCR recommended by the manufacturer's protocol (Figure 4.35).

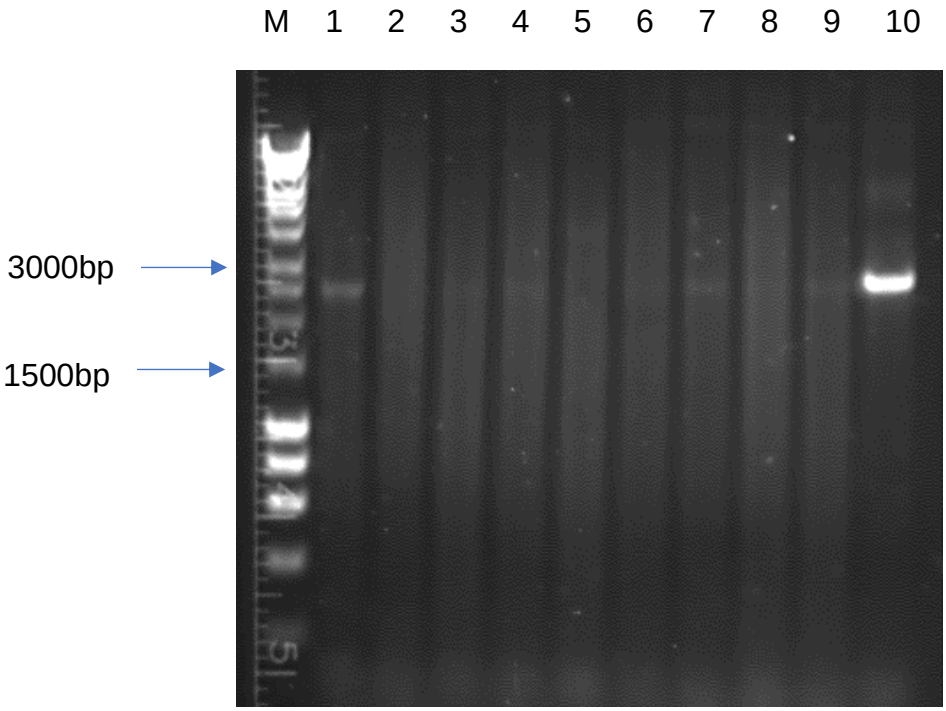


Figure 4.35: Agarose gel showing PCR of transformed GSTpFastBac colonies. *M= molecular markers, Lanes 1-10= colonies 1-10.*

Clones 1 and 10 were kept as successful transformations and used to proceed with the Sf9 cell based pesticide assay.

4.4.6 Pesticide Toxicity Assay Using Baculovirus Expression System

Sf9 insect cells were transfected with the recombinant baculoviral bacmids that had been produced in order to isolate and propagate recombinant baculoviral stocks to allow recombinant proteins expression. Cells were transfected with the baculovirus and the viral stock was amplified over time to give a high titre viral stock for both b_5 and GST expression as described in section 4.3.8. A sample of this stock has been stored at both 4 °C and -80 °C for future research.

The first aim was to ensure the cells were expressing the GST and b_5 genes as expected. The b_5 plate was assessed using a Western blot with b_5 specific

antibodies, which was run with control cells which had not been exposed to the b_5 baculovirus (Figure 4.36). The cells expressing GST were assessed using a GST assay, which is described in section 4.3.9, and was adapted from a recommended protocol (Bartley et al., 2015).

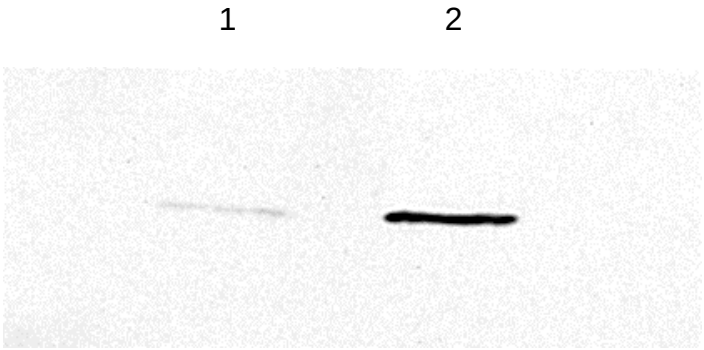


Figure 4.36: Western blot of cells expressing b_5. *Lane 1= Control cells not exposed to baculovirus, Lane 2= cells transfected with b_5 baculovirus. Loading control= Ponceau red.*

The Western blot (Figure 4.36) shows a strong band at approximately the 20 kD marker, which is consistent with the binding of the b_5 specific antibody to the protein from the cells transformed with the b_5 baculovirus. The faint band in the control well, from cells not transfected with the b_5 baculovirus, could be representing the baseline expression of b_5 in normally functioning cells.

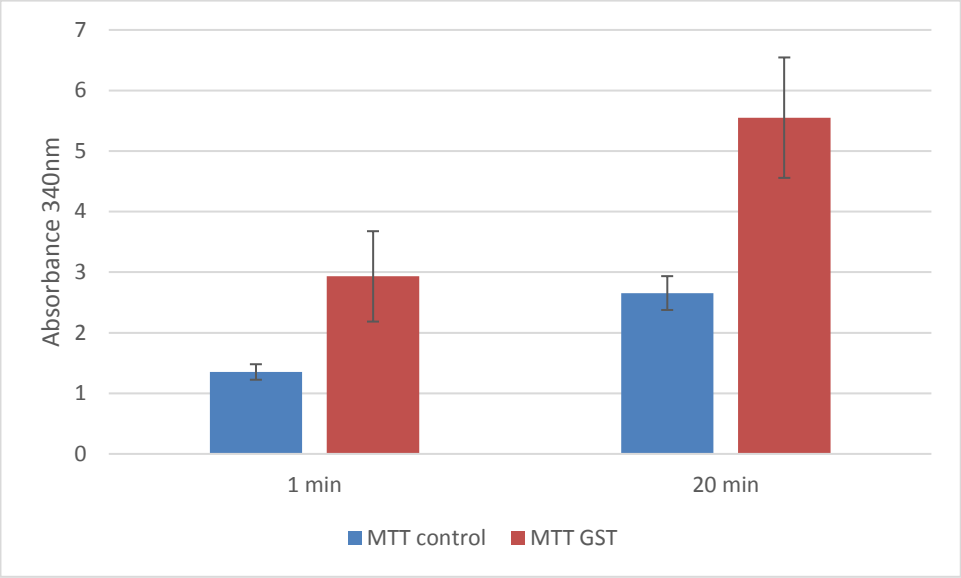


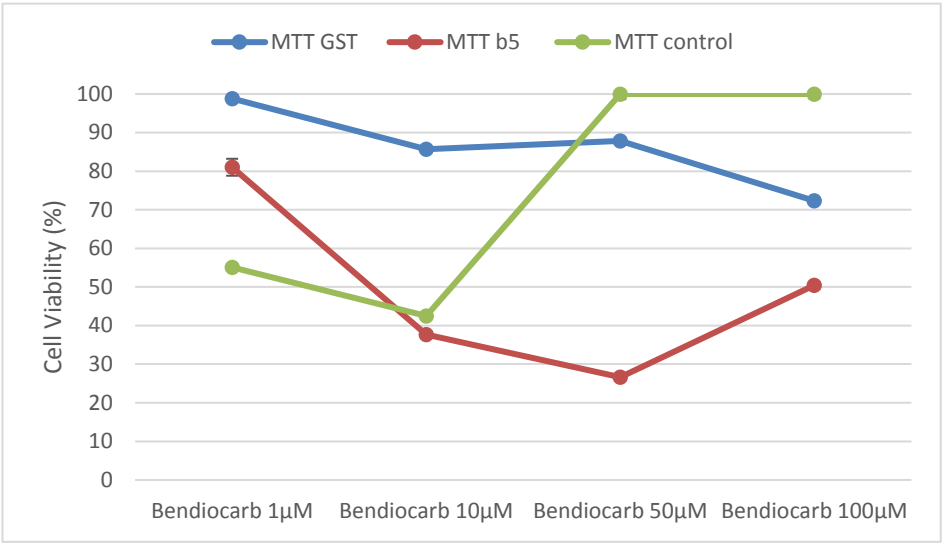
Figure 4.37: Graph of GST assay results. *GST assay was measured at 340 nm and the results were measured at 1 minute and 20 minutes for both the control plate (blue) and the cells expressing GST (orange). Standard deviation is shown for both results and n= 3.*

The ability of the GST to catalyse the conjugation of GSH to CDNB was assessed by measuring the absorbance at 340 nm at both an initial time point and after 20 minutes (Figure 4.37)(Lee et al., 2002; Bartley et al., 2015). The results of the assay show a clear increase in absorbance in the samples transfected with the GST baculovirus versus control at both 1 minute (P = 0.0225) and 20 minutes (P = 0.0083) and indicates the increased metabolic functioning of those cells.

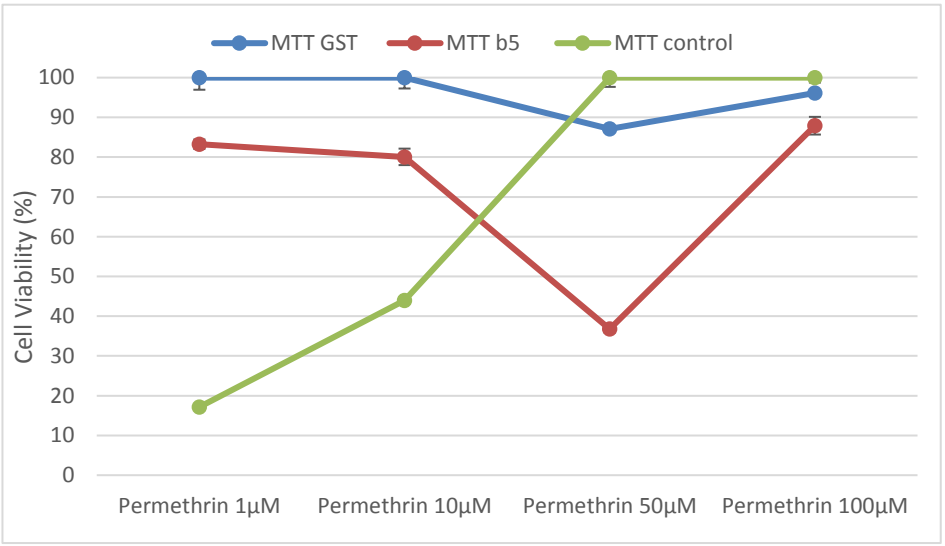
Once protein expression had been confirmed, the pesticide assay was carried out using bendiocarb, permethrin and deltamethrin. These active ingredients of common pesticides were chosen to give results for a range of different types of pesticides. Bendiocarb is part of the carbamate family which inhibits AChE, the role of which is to terminate nerve impulses (David et al., 2013; Antonio-Nkondjio et al., 2016). Permethrin and deltamethrin are part of the pyrethroid family which

is a major class of neurotoxic insecticides. They are synthetic analogues of acids found in a flower, *Chrysanthemum cinerafolis*, and the alcohol moiety of the pyrethrins has natural variations which leads to different classes of pyrethroids. Chrysanthemic acid produces Type I pyrethroids and pyrethric acid produces Type II pyrethroids (Davies et al., 2007). Permethrin is a Type I pyrethroid which induce repetitive firing in nerve axons that results in incoordination, hyperactivity and finally, paralysis (Gross and Bloomquist, 2018). Type II pyrethroids, such as deltamethrin, cause a convulsive phase which is caused by irreversible nerve axon depolarisation. Type II pyrethroids are often more effective pesticides as their effects last several seconds compared to milliseconds in type I pyrethroids (Davies et al., 2007).

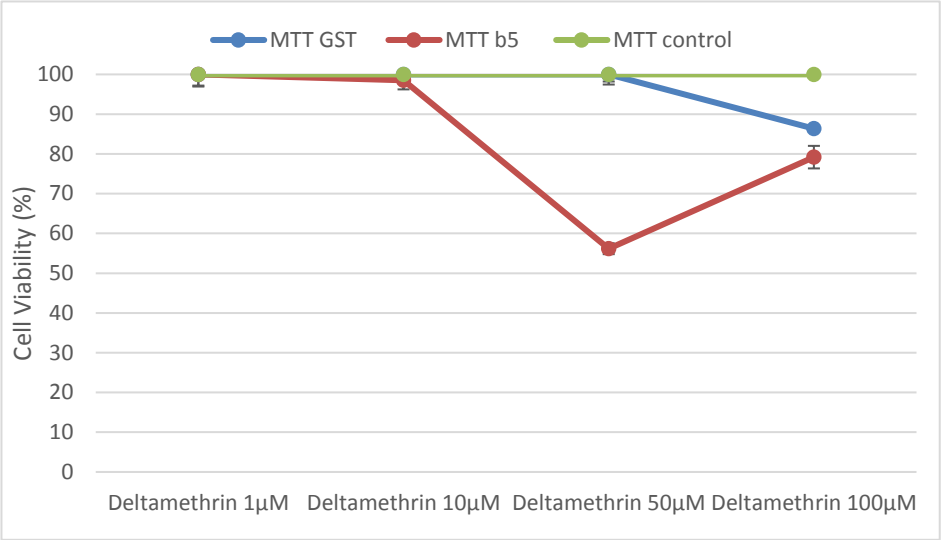
The pesticides were added to the plate as described in section 4.3.9 and an MTT assay was carried out in order to determine the number of viable cells in the well post treatment. The MTT assay functions by active cells reducing MTT to a strongly pigmented formazan product. The absorbance of the formazan can be measured on a plate reader and hence provides a measure of cell viability. Three plates were set up and treated with the range of concentrations, as described in section 4.3.9; a b_5 plate, a GST plate and a control plate.


MTT GST
MTT b5
MTT control
Cell Viability (%)
100
90
80
70
60
50
40
30
20
10
0
Bendiocarb 1µM
Bendiocarb 10µM
Bendiocarb 50µM
Bendiocarb 100µM

A


MTT GST
MTT b5
MTT control
Cell Viability (%)
100
90
80
70
60
50
40
30
20
10
0
Permethrin 1µM
Permethrin 10µM
Permethrin 50µM
Permethrin 100µM

B



C

Figure 4.38: Results of the pesticide toxicity testing. *The graphs show the viability of cells exposed to the pesticides as a percentage. Standard deviations are shown for all results. N= 3 for all results. Viability has been corrected for the control from each plate (b_5, GST and control) and the MTT control plate was not exposed to baculovirus. A= bendiocarb, B= permethrin and C= deltamethrin.*

The results of the toxicity testing can be viewed in Figure 4.38. Overall, the cells showed high viability levels after exposure to the pesticides for 48 hours. This could be due to the range of lower concentration levels used than other similar studies. Lower concentrations had to be used due to the difficulties with solubility of the pesticides in water. The actives are readily soluble in solvents, however levels of over 1 % solvents are not recommended to be used on Sf9 cells and hence 1 % was the maximum concentration able to be used (Boonsuepsakul et al., 2008; Duangkaew et al., 2011).

Cells exposed to b_5 baculovirus have been most effected by bendiocarb (Figure 4.37, graph A), with an LD50 of 10 µM compared to 50 µM in permethrin and

more than 100 µM in deltamethrin. Carbamates work via a different pathway to the pyrethroids, with no role for P450s in their metabolism, which is demonstrated by their increased effect on PRM (Oduola et al., 2012). Permethrin (Figure 4.38, graph B) shows increased cell viability in b_5 cells compared to the control plate for both 1 µM and 10 µM concentrations but deltamethrin (Figure 4.38, graph C) shows reduced cell viability in the b_5 cells. Deltamethrin is a type II pyrethroid whereas permethrin is type I. The increased cell viability in cells b_5 when exposed to permethrin could indicate the different pathways of detoxification and suggest b_5 plays a greater role in the detoxification of type I pyrethroids and could potentially inhibit the P450 complex when exposed to a type II substrate.

GST appears to have a positive affect at improving cell viability overall, with increasing survival rates from the control cells for both bendiocarb up to 50 µM and permethrin up to 50 µM and being equally effective at metabolising deltamethrin other than at the 100µM dose. These results show that greater research needs to be carried out in order to fully understand the role of GSTs, which preliminary data suggests, play a role in detoxifying pesticide.

4.5 Conclusions and Further Research Plan

As well as further investigation into the role of GSTs, greater work needs to be carried out in order to understand the role of cytochrome b_5. This project has developed a baculovirus system which allows for the monitoring of the effects of b_5 on xenobiotic metabolism. Going forward, this system can be used with increased doses of pesticides as well as upregulation of P450s once identified, for more in depth study of specific pesticides. As well as the study of b_5, once POR has been expressed and cloned into the baculovirus expression system it can be used in co-expression studies with cytochrome b_5 to allow for a deeper understanding of the role of accessory proteins in detoxification. It has been previously suggested that the role of b_5 in detoxification is dependent on the increased expression of P450s. The isolation of P450 complexes, made easier by this project, will be vital to assessing the role of b_5 in the detoxification of pesticides. Once P450s complexes have been identified, their use in co-expression studies with the GST baculovirus system could investigate multiple changes in expression as GSTs are expected to play a role in pesticide metabolism based on research in both PRM and similar species (Bartley et al., 2015; Tchigossou et al., 2018; Saldivar et al., 2008) .

Chapter 5: Conclusions and Further Work

5.1 Survey Data and Toxicity Testing

The data generated in Chapter two of this study has been vast, with the first nationwide study of perceived resistance being carried out aimed at mapping acaricide resistance in PRM here in the UK or any European country. While the results are partly based on the perceived effectiveness of products by the egg-producing community, it is clear that this data has some guidance value in determining which products are potentially most effective on the whole, thus allowing the industry to take a more informed decision in their efforts to control PRM infestations and additionally facilitate efforts to limit the spread of resistance. For example, on average across the entire country, Milben Ex scored highest out of products tested with more than three responses, with an average perceived effectiveness of 7.74/10. However, Milben Ex is from the pyrethroid class of pesticide, and resistance to this class has already been noted in this study.

Toxicity testing shows that this is not enough to rely solely on the data from the survey. Resistance appears to be widespread across the UK with PRM tested from farms in close geographic proximity from one another having different mortality rates in PRM for the same product. This suggests individual farms need to be toxicity tested in order to fully determine the best practice to use on a per farm basis, and that a sweeping approach to mite control across the UK is not the best practice. It is likely that PRM have formed different genetic mutations that confer resistance and these appear different on an individual farm level.

5.2 Analysis of Cytochrome P450s

As well as resistance mapping, this project aimed to characterise the mechanisms responsible for inferring resistance and to investigate novel ways of

targeting such mechanisms. From the analysis of PRM transcriptome data, a database has been created of potential pesticide metabolising P450 sequences, the first of its kind in PRM. This database is a result of extensive gene comparison to known xenobiotic metabolising sequences from other species, which have resulted in 35 putative pesticide metabolising sequences in PRM. These sequences are of value to research into the resistance mechanisms within PRM and the future work in maintaining the efficacy of products already on the market.

Using Activity Based Probes to identify the pathways of pesticide metabolism is essential for this preservation of efficacy. This project has attempted to adapt and develop a protocol for microsomal preparation containing active P450 proteins, similar to that used for several insect species (Ismail et al., 2013). Different microsome preps have been made in order to determine the most effective method of producing a microsome prep containing active P450s for PRM. However, only samples containing inactive P450 were found and further work needs to be done to modify the protocol in order for those P450s to remain active in the sample.

Once this microsome preparation technique has been adapted such that active P450s are present in the sample, any peptides isolated using the ABPs can be compared to the sequence database generated and provide confirmation of gene function. There is then the ability to clone and isolate P450s in a targeted manner which function in the metabolism of specific acaricides. This production of a library of sequences with probable pesticide metabolising functions is novel in the PRM and allows a wealth of future work to be done which could allow greater understanding of pesticide metabolism in PRM.

5.3 Isolation of Cytochrome P450 Oxidoreductase

The importance of understanding the role of accessory proteins in the function of metabolising enzymes is vital to developing control methods for the pest species PRM.

The sequence that was isolated as potentially being PRM POR was found in two sections, the 3’ end and the 5’ end, and in order to find the full-length sequence, RACE (Rapid Amplification of cDNA Ends) ready cDNA was prepared using the GeneRacer Kit. PRM POR was expected to be around 2200 bp long in total, based on the length of the *R. microplus* POR sequence.

PCR of the sequence using gene specific primers as well as the RACE primers was attempted to be optimised over many different PCRs. One PCR provided a band for the 5’ RACE product which was the correct presumed size for the PRM POR gene, but this band could not be reproduced in order to make a larger prep of the PCR for the band to be excised from the gel. Attempts were made to optimise this PCR over several months however the band could not be reproduced on the gel. As well as extensive optimisation, the original RNA extraction from PRM was repeated in order to remake RACE ready cDNA. This new cDNA preparation was also used for PCR and was repeated several times with several optimisation steps.

Ultimately, POR was not amplified using the GeneRacer kit, and the band that was initially seen could never be reproduced. Similar issues occurred when scaling up PCRs in a project with *R. microplus,* that had originally produced a band using a smaller scale PCR, and may represent a difficultly when amplifying acari sequences.

There is great importance in isolating the POR gene in PRM as it allows for the production of a fully functioning cytochrome P450 model which could then be tested with both pesticides and synergists to enable to full picture of pesticide metabolism within PRM for the first time.

5.4 Expression of Cytochrome b_5

It was essential to isolate the PRM b_5 sequence using the transcriptomic data provided in order to characterise its function as part of the P450 complex. Using the cytochrome b_5 sequence from *R. microplus* as bait, a highly similar sequence was identified from the PRM transcriptomic database (Graham et al., 2016). This sequence was isolated and ligated into a pET15b vector for expression.

As well as the expression of cytochrome b_5 from PRM, the same procedure was used to ligate cytochrome b_5 from *R. microplus* and *I. scapularis* into pET15b expression vector. All three species could then be expressed and purified which has allowed for the storage of three novel purified cytochrome b_5 proteins. This will be of future value to researchers of metabolic resistance mechanisms in either of the three pest species as this protein has been shown to have a modifying effect on P450 activity (Gruenke et al., 1995).

PRM b_5 was not only expressed and purified, but used to create a recombinant baculovirus which was used for pesticide studies in the Sf9 cell line. This research was the first to show Sf9 cells expressing PRM b_5, with the expression confirmed using a Western blot with b_5 specific antibodies.

The effect of cytochrome b_5 on the ability of the Sf9 cells metabolism of pesticides was tested, and it was determined that increasing the expression of cytochrome b_5 alone did not increase the rate of metabolism of the pesticides. It is therefore suggested in the project that cytochrome b_5 does not play a role in resistant in

PRM, or that the resistance is co-dependent on the increased expression of accessory protein POR or the increased expression of the P450 itself. This is different from other species, where cytochrome b_5 has been found to directly affect the function of the P450 complex by either stimulation or inhibition of the complex (Finn et al., 2008; McLaughlin et al., 2010; Im and Waskell, 2011; Gruenke et al., 1995).

As well as creating a recombinant baculovirus expression system for the PRM cytochrome b_5 gene, the same system was created for a PRM GST gene which was provided by collaborators at the Moredun institute. Cells which had been transformed with the GST baculovirus had an increase in metabolic function when tested using a GST assay, however pesticide toxicity testing results were inconclusive and further work is required to full determine the effects of GST expression on pesticide metabolism.

5.5 Future Recommendations

From this project, it is known that all farms need to be tested for resistance levels on an individual farm basis. As current research into resistance mechanisms in PRM is lacking, current control method advice is of great use to the farming community, and the knowledge that each farm has entirely unique pesticide requirements is vital. This knowledge needs to be passed on to the farming community, by publications which make this data accessible.

Further to this knowledge, the lack of detoxifying mechanism data in the PRM is evident. Methods undertaken in this project have been extensive and involved various steps of optimisation. Now this initial step has been taken, future work on establishing a functioning P450 complex assay is closer, and can answer some important questions into the role of P450 and its accessory proteins in resistance

in PRM. This is of great value to future control of PRM. If work could be continued on this project, an imperative step would be to optimise the isolation of POR in order to gain both accessory proteins for use with the P450 complex and the recent publishing of the draft genome assembly of PRM may aid future efforts to this end (Burgess et al., 2018). Development of the microsome preparation assay to include active P450s would allow for the use of Activity Based Probes which could isolate specific pesticide metabolising sequences in PRM. This would allow for a fully functioning P450 complex when combined with cytochrome b_5 and POR, allowing for characterisation of pesticide metabolism in PRM for the first time, and would allow for best practice treatment recommendations for farmers.

Data generated from knowledge of PRM detoxification mechanisms, including knowledge of the role of the accessory proteins, would also be of great value to pesticide development, which could be developed in order to bypass the mechanism causing resistance and therefore allow treatments to be effective in control of PRM, reducing costs for farmers and improving farming productivity.

This data will allow future links between *D. gallinae* genetics and resistance/susceptibility to be established which could facilitate the development of rapid testing kits for resistance and the real-time tailoring of treatment programmes to ensure maximum efficacy. All of the work contributed in this project has provided new knowledge of an important, but under researched pest, which will be of use to the farming community whilst establishing methods and resources for continuation of this research in the future.

References

ABBAS, R. Z., COLWELL, D. D., IQBAL, Z. & KHAN, A. 2014. Acaricidal drug resistance in poultry red mite (Dermanyssus gallinae) and approaches to its management. *Worlds Poultry Science Journal,* 70**,** 113-124.

ABDIGOUDARZI, M., MIRAFZALI, M. S. & BELGHEISZADEH, H. 2014. Human Infestation with Dermanyssus gallinae (Acari: Dermanyssidae) in a Family Referred with Pruritus and Skin Lesions. *J Arthropod Borne Dis,* 8**,** 119-23.

ADRION, E. R., AUCOTT, J., LEMKE, K. W. & WEINER, J. P. 2015. Health care costs, utilization and patterns of care following Lyme disease. *PLoS One,* 10**,** e0116767.

ALDRIDGE, W. N. 1950. Some properties of specific cholinesterase with particular reference to the mechanism of inhibition by diethyl p-nitrophenyl thiophosphate (E 605) and analogues. *Biochemical Journal* 46**,** 451-460.

AMELIA-YAP, Z. H., SOFIAN-AZIRUN, M., CHEN, C. D., LAU, K. W., SUANA, I. W., SYAHPUTRA, E., RAZAK, A. & LOW, V. L. 2019. V1016G Point Mutation: The Key Mutation in the Voltage-Gated Sodium Channel (Vgsc) Gene of Pyrethroid-Resistant Aedes aegypti (Diptera: Culicidae) in Indonesia. *J Med Entomol,* 56**,** 953-958.

AMENYA, D. A., NAGURAN, R., LO, T. C., RANSON, H., SPILLINGS, B. L., WOOD, O. R., BROOKE, B. D., COETZEE, M. & KOEKEMOER, L. L. 2008. Over expression of a cytochrome P450 (CYP6P9) in a major African malaria vector, Anopheles Funestus, resistant to pyrethroids. *Insect Mol Biol,* 17**,** 19-25.

ANTONIO-NKONDJIO, C., POUPARDIN, R., TENE, B. F., KOPYA, E., COSTANTINI, C., AWONO-AMBENE, P. & WONDJI, C. S. 2016. Investigation of mechanisms of bendiocarb resistance in Anopheles gambiae populations from the city of Yaounde, Cameroon. *Malar J,* 15**,** 424.

ANTONIO-NKONDJIO, C., SONHAFOUO-CHIANA, N., NGADJEU, C. S., DOUMBE-BELISSE, P., TALIPOUO, A., DJAMOUKO-DJONKAM, L., KOPYA, E., BAMOU, R., AWONO-AMBENE, P. & WONDJI, C. S. 2017. Review of the evolution of insecticide resistance in main malaria vectors in Cameroon from 1990 to 2017. *Parasit Vectors,* 10**,** 472.

ARZEY, G. 1990. Mechanism of spread of Newcastle disease. *New South Wales, Agriculture and Fisheries* Sydney.

BAK, B., WILDE, J. & SIUDA, M. 2012. Characteristics of north-eastern population of Varroa destructor resistant to synthetic pyrethroids. *Medycyna Weterynaryjna,* 68**,** 603-606.

BARIMANI, A., YOUSSEFI, M. R. & TABARI, M. A. 2016. Traps containing carvacrol, a biological approach for the control of Dermanyssus gallinae. *Parasitology Research,* 115**,** 3493-3498.

BARLOW, J. 2018. *Enriched and colony cages* [Online]. PoultryKeeper.com.

BARNABA, C., GENTRY, K., SUMANGALA, N. & RAMAMOORTHY, A. 2017. The catalytic function of cytochrome P450 is entwined with its membrane-bound nature. *F1000Res,* 6**,** 662.

BARTLEY, K., WRIGHT, H. W., BULL, R. S., HUNTLEY, J. F. & NISBET, A. J. 2015. Characterisation of Dermanyssus gallinae glutathione S-transferases and their potential as acaricide detoxification proteins. *Parasit Vectors,* 8**,** 350.

BBC. 2002. *Battery hens 'to be banned'* [Online]. BBC News website BBC.

BEAK TRIMMING ACTION GROUP 2015. The Beak Trimming Action Group's Review. *In:* DEPARTMENT FOR ENVIRONMENT, F. R. A. (ed.). GOV.UK.

BEGUM, R. R., NEWBOLD, R. J. & WHITFORD, D. 2000. Purification of the membrane binding domain of cytochrome b5 by immobilised nickel chelate chromatography. *J Chromatogr B Biomed Sci Appl,* 737**,** 119-30.

BELLGARD, M. I., MOOLHUIJZEN, P. M., GUERRERO, F. D., SCHIBECI, D., RODRIGUEZ-VALLE, M., PETERSON, D. G., DOWD, S. E., BARRERO, R., HUNTER, A., MILLER, R. J. & LEW-TABOR, A. E. 2012. CattleTickBase: an integrated Internet-based bioinformatics resource for Rhipicephalus (Boophilus) microplus. *Int J Parasitol,* 42**,** 161-9.

BERNHARDT, R. 2006. Cytochromes P450 as versatile biocatalysts. *J Biotechnol,* 124**,** 128-45.

BEUGNET, F., CHAUVE, C., GAUTHEY, M. & BEERT, L. 1997. Resistance of the red poultry mite to pyrethroids in France. *Vet Rec,* 140**,** 577-9.

BHATT, S., WEISS, D. J., CAMERON, E., BISANZIO, D., MAPPIN, B., DALRYMPLE, U., BATTLE, K., MOYES, C. L., HENRY, A., ECKHOFF, P. A., WENGER, E. A., BRIET, O., PENNY, M. A., SMITH, T. A., BENNETT, A., YUKICH, J., EISELE, T. P., GRIFFIN, J. T., FERGUS, C. A., LYNCH, M., LINDGREN, F., COHEN, J. M., MURRAY, C. L. J., SMITH, D. L., HAY, S. I., CIBULSKIS, R. E. & GETHING, P. W. 2015. The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature,* 526**,** 207-211.

BOONSUEPSAKUL, S., LUEPROMCHAI, E. & RONGNOPARUT, P. 2008. Characterization of Anopheles minimus CYP6AA3 expressed in a recombinant baculovirus system. *Arch Insect Biochem Physiol,* 69**,** 13-21.

BRAUNEIS, M. D., ZOLLER, H., WILLIAMS, H., ZSCHIESCHE, E. & HECKEROTH, A. R. 2017. The acaricidal speed of kill of orally administered fluralaner against poultry red mites (Dermanyssus gallinae) on laying hens and its impact on mite reproduction. *Parasites & Vectors,* 10.

BROOKE, B. D. 2008. kdr: can a single mutation produce an entire insecticide resistance phenotype? *Transactions of the Royal Society of Tropical Medicine and Hygiene,* 102**,** 524-525.

BURGESS, S. T. G., BARTLEY, K., NUNN, F., WRIGHT, H. W., HUGHES, M., GEMMELL, M., HALDENBY, S., PATERSON, S., ROMBAUTS, S., TOMLEY, F. M., BLAKE, D. P., PRITCHARD, J., SCHICHT, S., STRUBE, C., OINES, O., VAN LEEUWEN, T., VAN DE PEER, Y. & NISBET, A. J. 2018. Draft Genome Assembly of the Poultry Red Mite, Dermanyssus gallinae. *Microbiol Resour Announc,* 7.

BUSS, L., CARTER, E. & GILLETT-KAUFMAN, J. 2014. *Featured Creatures: Dermanyssus gallinae* [Online]. Univeristy of Florida: Entomology and Nematology: Univeristy of Florida.

CAFIERO, M., CAMARDA, A., CIRCELLA, E., RAELE, D., GIANGASPERO, A. & SPARAGANO, O. 2008. Dermanyssus gallinae prevalence in laying hen units in the Apulia region, Italy. *British society for Parasitology.* Newcastle, UK.

CAFIERO, M. A., GALANTE, D., CAMARDA, A., GIANGASPERO, A. & SPARAGANO, O. 2011. Why dermanyssosis should be listed as an occupational hazard. *Occup Environ Med,* 68**,** 628.

CAFIERO, M. A., VIVIANO, E., LOMUTO, M., RAELE, D. A., GALANTE, D. & CASTELLI, E. 2018. Dermatitis due to Mesostigmatic mites (Dermanyssus gallinae, Ornithonyssus [O.] bacoti, O. bursa, O. sylviarum) in residential settings. *J Dtsch Dermatol Ges,* 16**,** 904-906.

CARINO, F., KOENER, J., PLAPP, F. & FEYEREISEN, R. 1992. Expression of the Cytochrome P450 gene CYP6A1 in the Housefly, Musca domestica. *In:* MULLIN C, S. J. (ed.) *Molecular mechanisms of Insecticide Resistance* American chemical society

CARINO, F. A., KOENER, J. F., PLAPP, F. W., JR. & FEYEREISEN, R. 1994. Constitutive overexpression of the cytochrome P450 gene CYP6A1 in a house fly strain with metabolic resistance to insecticides. *Insect Biochem Mol Biol,* 24**,** 411-8.

CENCEK, T. 2003. Prevalence of Dermanyssus gallinae in poultry farms in silesia region in Poland. *Bulletin of the Veterinary Institute in Pulawy,* 47**,** 465-469.

CHAMBERLAIN, R. W. & SIKES, R. K. 1955. Laboratory investigations on the role of bird mites in the transmission of eastern and western equine encephalitis. *Am J Trop Med Hyg,* 4**,** 106-18.

CHAMBERLAIN, R. W., SIKES, R. K. & SUDIA, W. D. 1957. Attempted laboratory infection of bird mites with the virus of St. Louis encephalitis. *Am J Trop Med Hyg,* 6**,** 1047-53.

CHANDOR-PROUST, A., BIBBY, J., REGENT-KLOECKNER, M., ROUX, J., GUITTARD-CRILAT, E., POUPARDIN, R., RIAZ, M. A., PAINE, M., DAUPHIN-VILLEMANT, C., REYNAUD, S. & DAVID, J. P. 2013. The central role of mosquito cytochrome P450 CYP6Zs in insecticide detoxification revealed by functional expression and structural modelling. *Biochem J,* 455**,** 75-85.

CHANG, K. S., KIM, H. C., KLEIN, T. A. & JU, Y. R. 2017. Insecticide resistance and cytochrome-P450 activation in unfed and blood-fed laboratory and field populations of Culex pipiens pallens. *J Pest Sci (2004),* 90**,** 759-771.

CHAUVE, C. 1998. The poultry red mite Dermanyssus gallinae (De Geer, 1778): current situation and future prospects for control. *Vet Parasitol,* 79**,** 239-45.

CHEN, X. & ZHANG, Y. 2015. Identification and characterization of NADPH-dependent cytochrome P450 reductase gene and cytochrome b(5) gene from Plutella xylostella: possible involvement in resistance to beta-cypermethrin. *Gene,* 558**,** 208-14.

CHIRICO, J., ERIKSSON, H., FOSSUM, O. & JANSSON, D. 2003. The poultry red mite, Dermanyssus gallinae, a potential vector of Erysipelothrix rhusiopathiae causing erysipelas in hens. *Med Vet Entomol,* 17**,** 232-4.

CHURCHER, T. S., LISSENDEN, N., GRIFFIN, J. T., WORRALL, E. & RANSON, H. 2016. The impact of pyrethroid resistance on the efficacy and effectiveness of bednets for malaria control in Africa. *Elife,* 5.

CIOLCA, A., TANASE, I. & MAY, I. 1968. Role of the poultry red mite, Dermanyssus gallinae, in the transmission of spirochaetosis. *Arch Vet Pol,* 5.

CIRCELLA, E., PUGLIESE, N., TODISCO, G., CAFIERO, M. A., SPARAGANO, O. A. & CAMARDA, A. 2011. Chlamydia psittaci infection in canaries heavily infested by Dermanyssus gallinae. *Exp Appl Acarol,* 55**,** 329-38.

CLAUDIANOS, C., RANSON, H., JOHNSON, R. M., BISWAS, S., SCHULER, M. A., BERENBAUM, M. R., FEYEREISEN, R. & OAKESHOTT, J. G. 2006. A deficit of detoxification enzymes: pesticide sensitivity and environmental response in the honeybee. *Insect Molecular Biology,* 15**,** 615-636.

CLEMENTS, J., SCHOVILLE, S., PETERSON, N., HUSETH, A. S., LAN, Q. & GROVES, R. L. 2017. RNA interference of three up-regulated transcripts associated with insecticide resistance in an imidacloprid resistant population of Leptinotarsa decemlineata. *Pestic Biochem Physiol,* 135**,** 35-40.

COLE, S. P. & DEELEY, R. G. 2006. Transport of glutathione and glutathione conjugates by MRP1. *Trends Pharmacol Sci,* 27**,** 438-46.

CORDOVA, P., GONZALEZ, A. M., NELSON, D. R., GUTIERREZ, M. S., BAEZA, M., CIFUENTES, V. & ALCAINO, J. 2017. Characterization of the cytochrome P450 monooxygenase genes (P450ome) from the carotenogenic yeast Xanthophyllomyces dendrorhous. *BMC Genomics,* 18**,** 540.

COSOROABA, I. 2001. Massive Dermanyssus gallinae invasion in battery-husbandry raised fowls. *Revue De Medecine Veterinaire,* 152**,** 89-96.

COSSIO-BAYUGAR, R., MARTINEZ-IBANEZ, F., AGUILAR-DIAZ, H. & MIRANDA-MIRANDA, E. 2018. Pyrethroid Acaricide Resistance Is Proportional to P-450 Cytochrome Oxidase Expression in the Cattle Tick Rhipicephalus microplus. *Biomed Res Int,* 2018**,** 8292465.

DABERT, M., WITALINSKI, W., KAZMIERSKI, A., OLSZANOWSKI, Z. & DABERT, J. 2010. Molecular phylogeny of acariform mites (Acari, Arachnida): strong conflict between phylogenetic signal and long-branch attraction artifacts. *Mol Phylogenet Evol,* 56**,** 222-41.

DANIELS, T. 2015. *The life of a battery hen* [Online]. PoultryKeeper.com.

DAVID, J. P., ISMAIL, H. M., CHANDOR-PROUST, A. & PAINE, M. J. 2013. Role of cytochrome P450s in insecticide resistance: impact on the control of mosquito-borne diseases and use of insecticides on Earth. *Philos Trans R Soc Lond B Biol Sci,* 368**,** 20120429.

DAVID, J. P., STRODE, C., VONTAS, J., NIKOU, D., VAUGHAN, A., PIGNATELLI, P. M., LOUIS, C., HEMINGWAY, J. & RANSON, H. 2005. The Anopheles gambiae detoxification chip: a highly specific microarray to study metabolic-based insecticide resistance in malaria vectors. *Proc Natl Acad Sci U S A,* 102**,** 4080-4.

DAVIDSON, G. 1956. Insecticide resistance in Anopheles gambiae giles. *Nature,* 178**,** 705-6.

DAVIES, T. G., FIELD, L. M., USHERWOOD, P. N. & WILLIAMSON, M. S. 2007. DDT, pyrethrins, pyrethroids and insect sodium channels. *IUBMB Life,* 59**,** 151-62.

DE SOUSA, M. D. G. & FILHO, F. B. 2018. Gamasoidosis (bird mite dermatitis): dermanyssus gallinae in a young boy. *Pan Afr Med J,* 30**,** 144.

DENG, J., CARBONE, I. & DEAN, R. A. 2007. The evolutionary history of cytochrome P450 genes in four filamentous Ascomycetes. *BMC Evol Biol,* 7**,** 30.

DHOORIA, M. S. 2016. Morphology and Anatomy of Acari. *Fundamentals of Applied Acarology.* Singapore: Springer Singapore.

DI PALMA, A., GIANGASPERO, A., CAFIERO, M. A. & GERMINARA, G. S. 2012. A gallery of the key characters to ease identification of Dermanyssus gallinae (Acari: Gamasida: Dermanyssidae) and allow differentiation from Ornithonyssus sylviarum (Acari: Gamasida: Macronyssidae). *Parasit Vectors,* 5**,** 104.

DJOGBENOU, L., DABIRE, R., DIABATE, A., KENGNE, P., AKOGBETO, M., HOUGARD, J. M. & CHANDRE, F. 2008. Identification and geographic distribution of the ACE-1R mutation in the malaria vector Anopheles gambiae in south-western Burkina Faso, West Africa. *Am J Trop Med Hyg,* 78**,** 298-302.

DONG, K., DU, Y., RINKEVICH, F., NOMURA, Y., XU, P., WANG, L., SILVER, K. & ZHOROV, B. S. 2014. Molecular biology of insect sodium channels and pyrethroid resistance. *Insect Biochem Mol Biol,* 50**,** 1-17.

DONNELLY, M. J., CORBEL, V., WEETMAN, D., WILDING, C. S., WILLIAMSON, M. S. & BLACK, W. C. 2009. Does kdr genotype predict insecticide-resistance phenotype in mosquitoes? *Trends in Parasitology,* 25**,** 213-219.

DORLING, P. D. 2007. *The Myth of The North* [Online]. University of Sheffield.

DU, Y., NOMURA, Y., SATAR, G., HU, Z., NAUEN, R., HE, S. Y., ZHOROV, B. S. & DONG, K. 2013. Molecular evidence for dual pyrethroid-receptor sites on a mosquito sodium channel. *Proc Natl Acad Sci U S A,* 110**,** 11785-90.

DUANGKAEW, P., KAEWPA, D. & RONGNOPARUT, P. 2011. Protective efficacy of Anopheles minimus CYP6P7 and CYP6AA3 against cytotoxicity of pyrethroid

insecticides in Spodoptera frugiperda (Sf9) insect cells. *Trop Biomed,* 28**,** 293-301.

DUMIC, I. & SEVERNINI, E. 2018. "Ticking Bomb": The Impact of Climate Change on the Incidence of Lyme Disease. *Can J Infect Dis Med Microbiol,* 2018**,** 5719081.

DURDEN, L. A., LINTHICUM, K. J. & MONATH, T. P. 1993. Laboratory transmission of eastern equine encephalomyelitis virus to chickens by chicken mites (Acari: Dermanyssidae). *J Med Entomol,* 30**,** 281-5.

DURDEN, L. A., LINTHICUM, K. J. & TURELL, M. J. 1992. Mechanical transmission of Venezuelan equine encephalomyelitis virus by hematophagous mites (Acari). *J Med Entomol,* 29**,** 118-21.

ELADL, A. H., HAMED, H. R. & EL-SHAFEI, R. A. 2018. Prevalence of mites and their impact on laying hen (Gallus gallus domesticus) farm facilities in Egypt, with an analysis of deltamethrin residues in eggs and tissue. *Avian Pathol,* 47**,** 161-171.

ELZEN, P. J., EISCHEN, F. A., BAXTER, J. B., PETTIS, J., ELZEN, G. W. & WILSON, W. T. 1998. Fluvalinate resistance in Varroa jacobsoni from several geographic locations. *American Bee Journal,* 138**,** 674-676.

ENTREKIN, D. L. & OLIVER, J. H., JR. 1982. Aggregation of the chicken mite, Dermanyssus gallinae (acari: Dermanyssidae). *J Med Entomol,* 19**,** 671-8.

ETZERODT, T., WETTERHORN, K., DIONISIO, G. & RAYMENT, I. 2017. Functional characterization of a soluble NADPH-cytochrome P450 reductase from Fusarium graminearum. *Protein Expr Purif,* 138**,** 69-75.

FANG, Y., SHI, W. Q., WU, J. T., LI, Y. Y., XUE, J. B. & ZHANG, Y. 2019. Resistance to pyrethroid and organophosphate insecticides, and the geographical distribution and polymorphisms of target-site mutations in voltage-gated sodium channel and acetylcholinesterase 1 genes in Anopheles sinensis populations in Shanghai, China. *Parasites & Vectors,* 12.

FAOSTAT. 2016. *FAOSTAT- Food and Agriculture Organization of the United Nations* [Online].

FELIX, R. C., MULLER, P., RIBEIRO, V., RANSON, H. & SILVEIRA, H. 2010. Plasmodium infection alters Anopheles gambiae detoxification gene expression. *BMC Genomics,* 11**,** 312.

FEYEREISEN, R. 2006. Evolution of insect P450. *Biochem Soc Trans,* 34**,** 1252-5.

FEYEREISEN, R. 2011. Arthropod CYPomes illustrate the tempo and mode in P450 evolution. *Biochim Biophys Acta,* 1814**,** 19-28.

FIDDES, M. D., LE GRESLEY, S., PARSONS, D. G., EPE, C., COLES, G. C. & STAFFORD, K. A. 2005. Prevalence of the poultry red mite (Dermanyssus gallinae) in England. *Veterinary Record,* 157**,** 233-235.

FINN, R. D., MCLAUGHLIN, L. A., RONSEAUX, S., ROSEWELL, I., HOUSTON, J. B., HENDERSON, C. J. & WOLF, C. R. 2008. Defining the in Vivo Role for cytochrome b5 in cytochrome P450 function through the conditional hepatic deletion of microsomal cytochrome b5. *J Biol Chem,* 283**,** 31385-93.

FLOCHLAY, A. S., THOMAS, E. & SPARAGANO, O. 2017. Poultry red mite (Dermanyssus gallinae) infestation: a broad impact parasitological disease that still remains a significant challenge for the egg-laying industry in Europe. *Parasites & Vectors,* 10.

FOSSOG TENE, B., POUPARDIN, R., COSTANTINI, C., AWONO-AMBENE, P., WONDJI, C. S., RANSON, H. & ANTONIO-NKONDJIO, C. 2013. Resistance to DDT in an urban setting: common mechanisms implicated in both M and S forms of Anopheles gambiae in the city of Yaounde Cameroon. *PLoS One,* 8**,** e61408.

GASSEL, M., WOLF, C., NOACK, S., WILLIAMS, H. & ILG, T. 2014. The novel isoxazoline ectoparasiticide fluralaner: selective inhibition of arthropod gamma-aminobutyric acid- and L-glutamate-gated chloride channels and insecticidal/acaricidal activity. *Insect Biochem Mol Biol,* 45**,** 111-24.

GAVRILOVIC, P., KECMAN, V. & JOVANOVIC, M. 2015. Diagnosis of skin lesions caused by Dermanyssus gallinae in five patients. *Int J Dermatol,* 54**,** 207-10.

GENCHI, C., HUBER, H. & TRALDI, G. 1984. The efficacy of flumethrin (Bayticol Bayer) for the control of chicken mite Dermanyssus gallinae. *Archivio Veterinario Italiano* 35**,** 125-128.

GEORGE, D. R., CALLAGHAN, K., GUY, J. H. & SPARAGANO, O. A. E. 2008a. Lack of prolonged activity of lavender essential oils as acaricides against the poultry red mite (Dermanyssus gallinae) under laboratory conditions. *Research in Veterinary Science,* 85**,** 540-542.

GEORGE, D. R., FINN, R. D., GRAHAM, K. M., MUL, M. F., MAURER, V., MORO, C. V. & SPARAGANO, O. A. E. 2015. Should the poultry red mite Dermanyssus gallinae be of wider concern for veterinary and medical science? *Parasites & Vectors,* 8.

GEORGE, D. R., OLATUNJI, G., GUY, J. H. & SPARAGANO, O. A. E. 2010a. Effect of plant essential oils as acaricides against the poultry red mite, Dermanyssus gallinae, with special focus on exposure time. *Veterinary Parasitology,* 169**,** 222-225.

GEORGE, D. R., SHIEL, R. S., APPLEBY, W. G., KNOX, A. & GUY, J. H. 2010b. In vitro and in vivo acaricidal activity and residual toxicity of spinosad to the poultry red mite, Dermanyssus gallinae. *Vet Parasitol,* 173**,** 307-16.

GEORGE, D. R., SMITH, T. J., SPARAGANO, O. A. E. & GUY, J. H. 2008b. The influence of 'time since last blood meal' on the toxicity of essential oils to the poultry red mite (Dermanyssus gallinae). *Veterinary Parasitology,* 155**,** 333-335.

GEORGE, D. R., SPARAGANO, O. A., PORT, G., OKELLO, E., SHIEL, R. S. & GUY, J. H. 2009. Repellence of plant essential oils to Dermanyssus gallinae and toxicity to the non-target invertebrate Tenebrio molitor. *Vet Parasitol,* 162**,** 129-34.

GHARBI, M., SAKLY, N. & DARGHOUTH, M. A. 2013. Prevalence of Dermanyssus gallinae (Mesostigmata: Dermanyssidae) in industrial poultry farms in North-East Tunisia. *Parasite,* 20**,** 41.

GIBSON, G. & SKETT, P. 2001. *Introduction to Drug Metabolism,* Chelenham UK, Nelson Thornes.

GIDDINGS, J., GAGNE, J. & SHARP, J. 2016. Synergistic effect of piperonyl butoxide on acute toxicity of pyrethrins to Hyalella azteca. *Environ Toxicol Chem,* 35**,** 2111-6.

GILBERT, L. I. 2004. Halloween genes encode P450 enzymes that mediate steroid hormone biosynthesis in Drosophila melanogaster. *Molecular and Cellular Endocrinology,* 215**,** 1-10.

GILLESPIE, J. J., JOHNSTON, J. S., CANNONE, J. J. & GUTELL, R. R. 2006. Characteristics of the nuclear (18S, 5.8S, 28S and 5S) and mitochondrial (12S and 16S) rRNA genes of Apis mellifera (Insecta: Hymenoptera): structure, organization, and retrotransposable elements. *Insect Mol Biol,* 15**,** 657-86.

GINSBURG, J. M. 1937. Studies in Reducing Volume of Oil Necessary to Kill Mosquito Larvae By Incorporating Pyrethrum. *Journal of Economic Entomology* 30**,** 328-332.

GLEAVE, K., LISSENDEN, N., RICHARDSON, M., CHOI, L. & RANSON, H. 2018. Piperonyl butoxide (PBO) combined with pyrethroids in insecticide-treated nets to prevent malaria in Africa. *Cochrane Database Syst Rev,* 11**,** CD012776.

GONZALEZ-CABRERA, J., DAVIES, T. G. E., FIELD, L. M., KENNEDY, P. J. & WILLIAMSON, M. S. 2013. An Amino Acid Substitution (L925V) Associated with Resistance to Pyrethroids in Varroa destructor. *Plos One,* 8.

GONZALEZ-CABRERA, J., RODRIGUEZ-VARGAS, S., DAVIES, T. G., FIELD, L. M., SCHMEHL, D., ELLIS, J. D., KRIEGER, K. & WILLIAMSON, M. S. 2016. Novel Mutations in the Voltage-Gated Sodium Channel of Pyrethroid-Resistant Varroa destructor Populations from the Southeastern USA. *PLoS One,* 11**,** e0155332.

GRACIA-SALINAS, M. J., FERRER-DUFOL, M., LATORRE-CASTRO, E., MONERO-MANERA, C., CASTILLO-HERNANDEZ, J. A., LUCIENTES-CURD, J. & PERIBANEZ-LOPEZ, M. A. 2006. Detection of fluvalinate resistance in Varroa destructor in Spanish apiaries. *Journal of Apicultural Research,* 45**,** 101-105.

GRAHAM, K. M., SPARAGANO, O. A. & FINN, R. D. 2016. Isolation of the monooxygenase complex from Rhipicephalus (Boophilus) microplus - clues to understanding acaricide resistance. *Ticks Tick Borne Dis,* 7**,** 614-23.

GREBENYUK, R., CHIROV, P. & KADYSHEVA, A. 1972. The role of wild animals and blood-sucking arthropods in the epizootiology of infection with Listeria.

GROSS, A. D. & BLOOMQUIST, J. R. 2018. Characterizing Permethrin and Etofenprox Resistance in Two Common Laboratory Strains of Anopheles gambiae (Diptera: Culicidae). *Insects,* 9.

GROSSO, C. G., BLARIZA, M. J., MOUGABURE-CUETO, G., PICOLLO, M. I. & GARCIA, B. A. 2016. Identification of three cytochrome P450 genes in the Chagas' disease vector Triatoma infestans: Expression analysis in deltamethrin susceptible and resistant populations. *Infect Genet Evol,* 44**,** 459-470.

GRUENKE, L. D., KONOPKA, K., CADIEU, M. & WASKELL, L. 1995. The stoichiometry of the cytochrome P-450-catalyzed metabolism of methoxyflurane and benzphetamine in the presence and absence of cytochrome b5. *J Biol Chem,* 270**,** 24707-18.

GRUIANU, A., MAGDAS, C., DURLEA, F., IONITA, M. & MITREA, I. L. 2018. Dermanyssus gallinae infestation in poultry farms, in Romania: results of a pilot survey. *Journal of Biotechnology,* 280**,** S39-S40.

GUENGERICH, F. P. 2006. Cytochrome P450s and other enzymes in drug metabolism and toxicity. *AAPS J,* 8**,** E101-11.

GUERRERO, F. D., LOVIS, L. & MARTINS, J. R. 2012. Acaricide resistance mechanisms in Rhipicephalus (Boophilus) microplus. *Rev Bras Parasitol Vet,* 21**,** 1-6.

GUO, Y., WU, H., ZHANG, X., MA, E., GUO, Y., ZHU, K. Y. & ZHANG, J. 2016. RNA interference of cytochrome P450 CYP6F subfamily genes affects susceptibility to different insecticides in Locusta migratoria. *Pest Manag Sci,* 72**,** 2154-2165.

GUY, J. H., KHAJAVI, M., HLALEL, M. M. & SPARAGANO, O. 2004. Red mite (Dermanyssus gallinae) prevalence in laying units in Northern England. *Br Poult Sci,* 45 Suppl 1**,** S15-6.

HADADZADEH, H., MAJID, T. & MOHAMMAD, R. 2001. Evaluation of the effect of deltamethrin on Dermanysus gallinae in an egg layer house in Qom province in Iran. *Scientific-Research Iranian Veterinary Journal,* 4**,** 29-35.

HAMIDI, A., SHERIFI, K., MUJI, S., BEHLULI, B., LATIFI, F., ROBAJ, A., POSTOLI, R., HESS, C., HESS, M. & SPARAGANO, O. 2011. Dermanyssus gallinae in layer farms in Kosovo: A high risk for Salmonella prevalence. *Parasites & Vectors,* 4.

HEMINGWAY, J., BEATY, B. J., ROWLAND, M., SCOTT, T. W. & SHARP, B. L. 2006. The innovative vector control consortium: improved control of mosquito-borne diseases. *Trends in Parasitology,* 22**,** 308-312.

HEMINGWAY, J., HAWKES, N. J., MCCARROLL, L. & RANSON, H. 2004. The molecular basis of insecticide resistance in mosquitoes. *Insect Biochemistry and Molecular Biology,* 34**,** 653-665.

HEMINGWAY, J. & RANSON, H. 2000. Insecticide resistance in insect vectors of human disease. *Annu Rev Entomol,* 45**,** 371-91.

HENDERSON, C. & TILTON, W. 1955. Tests with Acaricides against the Brown Wheat Mite. *Journal of Economic Entomology,* 48**,** 157-161.

HODGSON, E. & PHILPOT, R. 1974. Interactions of methylenedioxyphenyl (1,3-benzodioxole) compounds with enzymes and their effects on mammals. *Drug Metabolism Reviews,* 3**,** 231-301.

HOFFMANN, G. 1987. Vogelmilben als L¨astlinge, Krankheitserzeuger und Vektoren bei Mensch und

Nutztier. *Dtsch. Tier¨arztl. Wschr.,* 95**,** 7-10.

HOGLUND, J., NORDENFORS, H. & UGGLA, A. 1995. Prevalence of the Poultry Red Mite, Dermanyssus-Gallinae, in Different Types of Production Systems for Egg Layers in Sweden. *Poultry Science,* 74**,** 1793-1798.

HOJLAND, D. H., JENSEN, K. M. & KRISTENSEN, M. 2014. Expression of xenobiotic metabolizing cytochrome P450 genes in a spinosad-resistant Musca domestica L. strain. *PLoS One,* 9**,** e103689.

HPRA. 2009. *ByeMite 500 mg/ml concentrate for spraying emulsion for laying hens* [Online]. The Health Products Regulatory Authority (HPRA) website.

HPRA. 2016. *ByeMite 500mg/ml Summary of Product Characteristics* [Online]. Health Products Regulatory Authority Website.

HRYCAY E & BANDIERA, S. 2015. *Monooxygenase, Peroxidase and Peroxygenase Properties and Mechanisms of Cytochrome P450*, Springer.

HUANG, Y., JIANG, H. B., SHEN, G. M., DOU, W. & WANG, J. J. 2012. Molecular characterizations of two cytochrome P450 genes encoding CYP6A41 and CYP6EK1 from the oriental fruit fly, Bactrocera dorsalis (Diptera: Tephritidae). *Arch Insect Biochem Physiol,* 79**,** 31-46.

HUBER, K., ZENNER, L. & BICOUT, D. J. 2011. Modelling population dynamics and response to management options in the poultry red mite Dermanyssus gallinae (Acari: Dermanyssidae). *Veterinary Parasitology,* 176**,** 65-73.

HUBERT, J., NESVORNA, M., KAMLER, M., KOPECKY, J., TYL, J., TITERA, D. & STARA, J. 2014. Point mutations in the sodium channel gene conferring tau-fluvalinate resistance in Varroa destructor. *Pest Manag Sci,* 70**,** 889-94.

HUI BON HOA, G., DI PRIMO, C., DONDAINE, I., SLIGAR, S. G., GUNSALUS, I. C. & DOUZOU, P. 1989. Conformational changes of cytochromes P-450cam and P-450lin induced by high pressure. *Biochemistry,* 28**,** 651-6.

IBM, C. 2013. IBM SPSS Statistics for Windows. 22.0 ed. Armonk, NY.

IM, S. C. & WASKELL, L. 2011. The interaction of microsomal cytochrome P450 2B4 with its redox partners, cytochrome P450 reductase and cytochrome b(5). *Arch Biochem Biophys,* 507**,** 144-53.

IMAI, Y. & SATO, R. 1967. Conversion of P-450 to P-420 by neutral salts and some other reagents. *Eur J Biochem,* 1**,** 419-26.

ISHAK, I. H., KAMGANG, B., IBRAHIM, S. S., RIVERON, J. M., IRVING, H. & WONDJI, C. S. 2017. Pyrethroid Resistance in Malaysian Populations of Dengue Vector Aedes aegypti Is Mediated by CYP9 Family of Cytochrome P450 Genes. *PLoS Negl Trop Dis,* 11**,** e0005302.

ISMAIL, H. M., BARTON, V., PHANCHANA, M., CHAROENSUTTHIVARAKUL, S., WONG, M. H., HEMINGWAY, J., BIAGINI, G. A., O'NEILL, P. M. & WARD, S. A. 2016. Artemisinin activity-based probes identify multiple molecular targets within the asexual stage of the malaria parasites Plasmodium falciparum 3D7. *Proc Natl Acad Sci U S A,* 113**,** 2080-5.

ISMAIL, H. M., O'NEILL, P. M., HONG, D. W., FINN, R. D., HENDERSON, C. J., WRIGHT, A. T., CRAVATT, B. F., HEMINGWAY, J. & PAINE, M. J. I. 2013. Pyrethroid activity-based probes for profiling cytochrome P450 activities associated with insecticide interactions. *Proceedings of the National Academy of Sciences of the United States of America,* 110**,** 19766-19771.

ISTVAN, E. S., MALLARI, J. P., COREY, V. C., DHARIA, N. V., MARSHALL, G. R., WINZELER, E. A. & GOLDBERG, D. E. 2017. Esterase mutation is a mechanism of resistance to antimalarial compounds. *Nat Commun,* 8**,** 14240.

JANADAREE BANDARA, K. M. U. & PARAKRAMA KARUNARATNE, S. H. P. 2017. Mechanisms of acaricide resistance in the cattle tick Rhipicephalus (Boophilus) microplus in Sri Lanka. *Pestic Biochem Physiol,* 139**,** 68-72.

JONSSON, N. N., CUTULLE, C., CORLEY, S. W. & SEDDON, J. M. 2010. Identification of a mutation in the para-sodium channel gene of the cattle tick Rhipicephalus microplus associated with resistance to flumethrin but not to cypermethrin. *Int J Parasitol,* 40**,** 1659-64.

KILPINEN, O. 2001. Activation of the poultry red mite, Dermanyssus gallinae (Acari: Dermanyssidae), by increasing temperatures. *Exp Appl Acarol,* 25**,** 859-67.

KILPINEN, O. 2005. How to obtain a bloodmeal without being eaten by a host: the case of poultry red mite, Dermanyssus gallinae. *Physiological Entomology,* 30**,** 232-240.

KILPINEN, O. & MULLENS, B. A. 2004. Effect of food deprivation on response of the mite, Dermanyssus gailinae, to heat. *Medical and Veterinary Entomology,* 18**,** 368-371.

KILPINEN, O., ROEPSTORFF, A., PERMIN, A., NORGAARD-NIELSEN, G., LAWSON, L. G. & SIMONSEN, H. B. 2005. Influence of Dermanyssus gallinae and Ascaridia galli infections on behaviour and health of laying hens (Gallus gallus domesticus). *British Poultry Science,* 46**,** 26-34.

KIM, S. I., NA, Y. E., YI, J. H., KIM, B. S. & AHN, Y. J. 2007. Contact and fumigant toxicity of oriental medicinal plant extracts against Dermanyssus gallinae (Acari: Dermanyssidae). *Vet Parasitol,* 145**,** 377-82.

KIM, S. I., YI, J. H., TAK, J. H. & AHN, Y. J. 2004. Acaricidal activity of plant essential oils against Dermanyssus gallinae (Acari : Dermanyssidae). *Veterinary Parasitology,* 120**,** 297-304.

KNIGHT-JONES, T. J. D., GIBBENS, J., WOOLDRIDGE, M. & STARK, K. D. C. 2011. Assessment of Farm-Level Biosecurity Measures after an Outbreak of Avian Influenza in the United Kingdom. *Transboundary and Emerging Diseases,* 58**,** 69-75.

KOENRAADT, C. J. & DICKE, M. 2010. The role of volatiles in aggregation and host-seeking of the haematophagous poultry red mite Dermanyssus gallinae (Acari: Dermanyssidae). *Exp Appl Acarol,* 50**,** 191-9.

KONYALI, C., ERDEM, H. & SAVAŞ, T. 2018. *Prevalence of Dermanyssus gallinae and Relation with Henhouse Conditions of Backyards in Çanakkale Province in Turkey*.

KOWALSKI, A. & SOKOL, R. 2009. Influence of Dermanyssus gallinae (poultry red mite) invasion on the plasma levels of corticosterone, catecholamines and proteins in layer hens. *Pol J Vet Sci,* 12**,** 231-5.

KRANTZ, G. & WALTER, D. 2009. *A Manual of Acarology*, Texas Tech University Press.

KWON, D. H., SEONG, G. M., KANG, T. J. & LEE, S. H. 2010. Multiple resistance mechanisms to abamectin in the two-spotted spider mite. *Journal of Asia-Pacific Entomology,* 13**,** 229-232.

LAKE, I. R. 2017. Food-borne disease and climate change in the United Kingdom. *Environ Health,* 16**,** 117.

LAURSEN, T., JENSEN, K. & MOLLER, B. L. 2011. Conformational changes of the NADPH-dependent cytochrome P450 reductase in the course of electron transfer to cytochromes P450. *Biochim Biophys Acta,* 1814**,** 132-8.

LEE, A. J., HUNTLEY, J., VAN DEN BROEK, A., COATES, D. & ISAAC, R. E. 2002. Expression and characterisation of a Psoroptes ovis glutathione S-transferase. *Vet Parasitol,* 105**,** 49-63.

LEGER, E., VOURC'H, G., VIAL, L., CHEVILLON, C. & MCCOY, K. D. 2013. Changing distributions of ticks: causes and consequences. *Exp Appl Acarol,* 59**,** 219-44.

LEHANE, M. 2005. The Blood-Sucking Insect Groups. *The Biology of Blood-Sucking in Insects.* Cambridge University Press.

LIU, D., ZHOU, X., LI, M., ZHU, S. & QIU, X. 2014. Characterization of NADPH-cytochrome P450 reductase gene from the cotton bollworm, Helicoverpa armigera. *Gene,* 545**,** 262-70.

LIU, S., LIANG, Q. M., ZHOU, W. W., JIANG, Y. D., ZHU, Q. Z., YU, H., ZHANG, C. X., GURR, G. M. & ZHU, Z. R. 2015. RNA interference of NADPH-cytochrome P450 reductase of the rice brown planthopper, Nilaparvata lugens, increases susceptibility to insecticides. *Pest Manag Sci,* 71**,** 32-9.

LUNDH, J., WIKTELIUS, D. & CHIRICO, J. 2005. Azadirachtin-impregnated traps for the control of Dermanyssus gallinae. *Veterinary Parasitology,* 130**,** 337-342.

MA, Z., LI, J., ZHANG, Y., SHAN, C. & GAO, X. 2017. Inheritance mode and mechanisms of resistance to imidacloprid in the house fly Musca domestica (Diptera:Muscidae) from China. *PLoS One,* 12**,** e0189343.

MARANGI, M., CAFIERO, M. A., CAPELLI, G., CAMARDA, A., SPARAGANO, O. A. E. & GIANGASPERO, A. 2009. Evaluation of the poultry red mite, Dermanyssus gallinae (Acari: Dermanyssidae) susceptibility to some acaricides in field populations from Italy. *Experimental and Applied Acarology,* 48**,** 11-18.

MARANGI, M., MORELLI, V., PATI, S., CAMARDA, A., CAFIERO, M. A. & GIANGASPERO, A. 2012. Acaricide residues in laying hens naturally infested by red mite Dermanyssus gallinae. *PLoS One,* 7**,** e31795.

MARTINIS, S. A., BLANKE, S. R., HAGER, L. P., SLIGAR, S. G., HOA, G. H., RUX, J. J. & DAWSON, J. H. 1996. Probing the heme iron coordination structure of pressure-induced cytochrome P420cam. *Biochemistry,* 35**,** 14530-6.

MAURER, V. & BAUMGARTNER, J. 1992. Temperature influence on life table statistics of the chicken mite Dermanyssus gallinae (Acari: Dermanyssidae). *Exp Appl Acarol,* 15**,** 27-40.

MAURER, V., PERLER, E. & HECKENDORN, F. 2009. In vitro efficacies of oils, silicas and plant preparations against the poultry red mite Dermanyssus gallinae. *Exp Appl Acarol,* 48**,** 31-41.

MCLAUGHLIN, L. A., NIAZI, U., BIBBY, J., DAVID, J. P., VONTAS, J., HEMINGWAY, J., RANSON, H., SUTCLIFFE, M. J. & PAINE, M. J. 2008. Characterization of inhibitors and substrates of Anopheles gambiae CYP6Z2. *Insect Mol Biol,* 17**,** 125-35.

MCLAUGHLIN, L. A., RONSEAUX, S., FINN, R. D., HENDERSON, C. J. & ROLAND WOLF, C. 2010. Deletion of microsomal cytochrome b5 profoundly affects hepatic and extrahepatic drug metabolism. *Mol Pharmacol,* 78**,** 269-78.

MCLEAN, K. J., SABRI, M., MARSHALL, K. R., LAWSON, R. J., LEWIS, D. G., CLIFT, D., BALDING, P. R., DUNFORD, A. J., WARMAN, A. J., MCVEY, J. P., QUINN, A. M., SUTCLIFFE, M. J., SCRUTTON, N. S. & MUNRO, A. W. 2005. Biodiversity of cytochrome P450 redox systems. *Biochem Soc Trans,* 33**,** 796-801.

MCLEOD, R. & KRISTJANSON, P. 1999. Final report of joint esys/ILRI/ACIAR Tick Cost project – Economic impact of ticks and tick-borne diseases to livestock in Africa, Asia and Australia. Nairobi: International Livestock Research Institute.

MILLER, R. J., DAVEY, R. B. & GEORGE, J. E. 2007. First report of permethrin-resistant Boophilus microplus (Acari: Ixodidae) collected within the United States. *J Med Entomol,* 44**,** 308-15.

MO, W., JIAN-XIA, T., JU-LIN, L., MEI-HUA, Z., JING, C., SUI, X., YUAN-YUAN, C., MENG-MENG, Y., GUO-DING, Z., HUA-YUN, Z. & JUN, C. 2018. [Study on expression characteristics of cytochrome P450 genes in Anopheles sinensis]. *Zhongguo Xue Xi Chong Bing Fang Zhi Za Zhi,* 30**,** 149-154.

MORGAN, J. A., CORLEY, S. W., JACKSON, L. A., LEW-TABOR, A. E., MOOLHUIJZEN, P. M. & JONSSON, N. N. 2009. Identification of a mutation in the para-sodium channel gene of the cattle tick Rhipicephalus (Boophilus) microplus associated with resistance to synthetic pyrethroid acaricides. *Int J Parasitol,* 39**,** 775-9.

MORO, C. V., FRAVALO, P., AMELOT, M., CHAUVE, C., SALVAT, G. & ZENNER, L. Experimental studies on the potential role of the poultry red mite, Dermanyssus gallinae, as a vector of Salmonella serotype Enteritidis. 2010 Dordrecht. Springer Netherlands, 521-525.

MRC PROTEIN PHOSPHORYLATION AND UBIQUITYLATION UNIT, V. L. 2016. Activity-based profiling of ubiquitin conjugation. University of Dundee

MUELLER, G. C. & MILLER, J. A. 1948. The metabolism of 4-dimethylaminoazobenzene by rat liver homogenates. *J Biol Chem,* 176**,** 535-44.

MUL, M. 2013. *Fact sheet Poultry Red Mite in Europe*.

MUL, M., VAN NIEKERK, T., CHIRICO, J., MAURER, V., KILPINEN, O., SPARAGANO, O., THIND, B., ZOONS, J., MOORE, D., BELL, B., GJEVRE, A. G. & CHAUVE, C. 2009. Control methods for Dermanyssus gallinae in systems for laying hens: results of an international seminar. *Worlds Poultry Science Journal,* 65**,** 589-599.

MUL, M. F., VAN NIEKERK, T. G. C. M., REUVEKAMP, B. F. J. & VAN EMOUS, R. A. 2010. Dermanyssus gallinae in Dutch poultry farms: Results of a questionnaire on severity, control treatments, cleaning, and biosecurity. *Trends in Acarology***,** 513-516.

MUL, M. F., VAN RIEL, J. W., ROY, L., ZOONS, J., ANDRE, G., GEORGE, D. R., MEERBURG, B. G., DICKE, M., VAN MOURIK, S. & GROOT KOERKAMP, P. W. G. 2017. Development of a model forecasting Dermanyssus gallinae's population dynamics for advancing Integrated Pest Management in laying hen facilities. *Vet Parasitol,* 245**,** 128-140.

MULLER, P., DONNELLY, M. J. & RANSON, H. 2007. Transcription profiling of a recently colonised pyrethroid resistant Anopheles gambiae strain from Ghana. *BMC Genomics,* 8**,** 36.

MUNGUBE, E. O., BAUNI, S. M., TENHAGEN, B. A., WAMAE, L. W., NZIOKA, S. M., MUHAMMED, L. & NGINYI, J. M. 2008. Prevalence of parasites of the local scavenging chickens in a selected semi-arid zone of Eastern Kenya. *Tropical Animal Health and Production,* 40**,** 101-109.

MUNOZ-PINEIRO, M. & ROBOUGH, P. 2018. Fipronil In Eggs European Commission Science Hub

MUNRO, A. W., GIRVAN, H. M. & MCLEAN, K. J. 2007. Cytochrome P450--redox partner fusion enzymes. *Biochim Biophys Acta,* 1770**,** 345-59.

NAKAMAE, H., FUJISAKI, K., KISHI, S., YASHIRO, M., OSHIRO, S. & FURUTA, K. 1997. The new parasitic ecology of chicken mites Dermanyssus gallinae, parasitizing and propagating on chickens even in the daytime. *J. Poult. Sci. ,* 34**,** 110-116.

NAUEN, R. & STUMPF, N. 2002. Fluorometric microplate assay to measure glutathione S-transferase activity in insects and mites using monochlorobimane. *Anal Biochem,* 303**,** 194-8.

NECHITA, I. S., POIREL, M. T., COZMA, V. & ZENNER, L. 2015. The repellent and persistent toxic effects of essential oils against the poultry red mite, Dermanyssus gallinae. *Veterinary Parasitology,* 214**,** 348-352.

NORDENFORS, H. & CHIRICO, J. 2001. Evaluation of a sampling trap for Dermanyssus gallinae (Acari: Dermanyssidae). *J Econ Entomol,* 94**,** 1617-21.

NORDENFORS, H. & HOGLUND, J. 2000. Long term dynamics of Dermanyssus gallinae in relation to mite control measures in aviary systems for layers. *British Poultry Science,* 41**,** 533-540.

NORDENFORS, H., HOGLUND, J., TAUSON, R. & CHIRICO, J. 2001. Effect of permethrin impregnated plastic strips on Dermanyssus gallinae in loose-housing systems for laying hens. *Veterinary Parasitology,* 102**,** 121-131.

NORDENFORS, H., HOGLUND, J. & UGGLA, A. 1999. Effects of temperature and humidity on oviposition, molting, and longevity of Dermanyssus gallinae (Acari: Dermanyssidae). *J Med Entomol,* 36**,** 68-72.

NYANGIWE, N., HORAK, I. G., VAN DER MESCHT, L. & MATTHEE, S. 2017. Range expansion of the economically important Asiatic blue tick, Rhipicephalus microplus, in South Africa. *J S Afr Vet Assoc,* 88**,** e1-e7.

O'REILLY, A. O., WILLIAMSON, M. S., GONZALEZ-CABRERA, J., TURBERG, A., FIELD, L. M., WALLACE, B. A. & DAVIES, T. G. 2014. Predictive 3D modelling of the interactions of pyrethroids with the voltage-gated sodium channels of ticks and mites. *Pest Manag Sci,* 70**,** 369-77.

ODUOLA, A. O., IDOWU, E. T., OYEBOLA, M. K., ADEOGUN, A. O., OLOJEDE, J. B., OTUBANJO, O. A. & AWOLOLA, T. S. 2012. Evidence of carbamate resistance in urban populations of Anopheles gambiae s.s. mosquitoes resistant to DDT and deltamethrin insecticides in Lagos, South-Western Nigeria. *Parasit Vectors,* 5**,** 116.

OMURA, T. & SATO, R. 1964a. The Carbon Monoxide-Binding Pigment of Liver Microsomes. I. Evidence for Its Hemoprotein Nature. *J Biol Chem,* 239**,** 2370-8.

OMURA, T. & SATO, R. 1964b. The Carbon Monoxide-Binding Pigment of Liver Microsomes. Ii. Solubilization, Purification, and Properties. *J Biol Chem,* 239**,** 2379-85.

PASAY, C., ARLIAN, L., MORGAN, M., GUNNING, R., ROSSITER, L., HOLT, D., WALTON, S., BECKHAM, S. & MCCARTHY, J. 2009. The Effect of Insecticide Synergists on the Response of Scabies Mites to Pyrethroid Acaricides. *Plos Neglected Tropical Diseases,* 3.

PAVLIDI, N., KAMPOURAKI, A., TSELIOU, V., WYBOUW, N., DERMAUW, W., RODITAKIS, E., NAUEN, R., VAN LEEUWEN, T. & VONTAS, J. 2018. Molecular characterization of pyrethroid resistance in the olive fruit fly Bactrocera oleae. *Pestic Biochem Physiol,* 148**,** 1-7.

PENILLA, R. P., RODRIGUEZ, A. D., HEMINGWAY, J., TORRES, J. L., ARREDONDO-JIMENEZ, J. I. & RODRIGUEZ, M. H. 1998. Resistance management strategies in malaria

vector mosquito control. Baseline data for a large-scale field trial against Anopheles albimanus in Mexico. *Med Vet Entomol,* 12**,** 217-33.

PEREIRA, B. B., LIMONGI, J. E., CAMPOS, E. O., JR., LUIZ, D. P. & KERR, W. E. 2014. Effects of piperonyl butoxide on the toxicity of the organophosphate temephos and the role of esterases in the insecticide resistance of Aedes aegypti. *Rev Soc Bras Med Trop,* 47**,** 579-82.

PERMIN, A. & HANSEN, J. 1998. Epidemiology, diagnosis and control of poultry parasites. *In:* FAO (ed.) *Animal Health Manual.* Rome.

PETROV, D. 1975. [Study of Dermanyssus gallinae as a carrier of Pasteurella multocida]. *Vet Med Nauki,* 12**,** 32-6.

PHILIPPOU, D., BORZATTA, V., CAPPARELLA, E., MORONI, L., FIELD, L. & MOORES, G. 2013. The interactions between piperonyl butoxide and E4, a resistance-associated esterase from the peach-potato aphid, Myzus persicae Sulzer (Hemiptera: Aphididae). *Pest Manag Sci,* 69**,** 499-506.

POGACNIK, L. & FRANKO, M. 1999. Determination of organophosphate and carbamate pesticides in spiked samples of tap water and fruit juices by a biosensor with photothermal detection. *Biosens Bioelectron,* 14**,** 569-78.

PRITCHARD, J., KUSTER, T., SPARAGANO, O. & TOMLEY, F. 2015. *Understanding the biology & control of the poultry red mite Dermanyssus gallinae : a review*.

PUGLIESE, N., CIRCELLA, E., MARINO, M., DE VIRGILIO, C., COCCIOLO, G., LOZITO, P., CAFIERO, M. A. & CAMARDA, A. 2018. Circulation dynamics of Salmonella enterica subsp. enterica ser. Gallinarum biovar Gallinarum in a poultry farm infested by Dermanyssus gallinae. *Med Vet Entomol*.

RANASINGHE, C. & HOBBS, A. A. 1999. Induction of cytochrome P450 CYP6B7 and cytochrome b5 mRNAs from Helicoverpa armigera (Hubner) by pyrethroid insecticides in organ culture. *Insect Mol Biol,* 8**,** 443-7.

RANSON, H. & LISSENDEN, N. 2016. Insecticide Resistance in African Anopheles Mosquitoes: A Worsening Situation that Needs Urgent Action to Maintain Malaria Control. *Trends Parasitol,* 32**,** 187-196.

RINKEVICH, F. D., DU, Y. Z. & DONG, K. 2013. Diversity and convergence of sodium channel mutations involved in resistance to pyrethroids. *Pesticide Biochemistry and Physiology,* 106**,** 93-100.

ROSEN, S., YERUHAM, I. & BRAVERMAN, Y. 2002. Dermatitis in humans associated with the mites Pyemotes tritici, Dermanyssus gallinae, Ornithonyssus bacoti and Androlaelaps casalis in Israel. *Medical and Veterinary Entomology,* 16**,** 442-444.

ROSENKRANZ, P., AUMEIER, P. & ZIEGELMANN, B. 2010. Biology and control of Varroa destructor. *Journal of Invertebrate Pathology,* 103**,** S96-S119.

ROY, L. & CHAUVE, C. M. 2007. Historical review of the genus Dermanyssus Duges, 1834 (Acari: Mesostigmata: Dermanyssidae). *Parasite,* 14**,** 87-100.

ROY, L., DOWLING, A. P., CHAUVE, C. M., LESNA, I., SABELIS, M. W. & BURONFOSSE, T. 2009. Molecular phylogenetic assessment of host range in five Dermanyssus species. *Exp Appl Acarol,* 48**,** 115-42.

RSC. 2019. *ChemSpider database* [Online]. Royal Society of Chemistry.

RUMSEY, J. 2018. United Kingdom Egg Statistics *In:* AFFAIRS, D. F. E. F. A. R. (ed.). Government website

SACCO, J. C. & TREPANIER, L. A. 2010. Cytochrome b5 and NADH cytochrome b5 reductase: genotype-phenotype correlations for hydroxylamine reduction. *Pharmacogenet Genomics,* 20**,** 26-37.

SAINGAMSOOK, J., SAEUNG, A., YANOLA, J., LUMJUAN, N., WALTON, C. & SOMBOON, P. 2017. A multiplex PCR for detection of knockdown resistance mutations, V1016G and F1534C, in pyrethroid-resistant Aedes aegypti. *Parasit Vectors,* 10**,** 465.

SALDIVAR, L., GUERRERO, F. D., MILLER, R. J., BENDELE, K. G., GONDRO, C. & BRAYTON, K. A. 2008. Microarray analysis of acaricide-inducible gene expression in the southern cattle tick, Rhipicephalus (Boophilus) microplus. *Insect Mol Biol,* 17**,** 597-606.

SANTOS, A. C., ROBERTS, J. A., COOK, A. J., SIMONS, R., SHEEHAN, R., LANE, C., ADAK, G. K., CLIFTON-HADLEY, F. A. & RODRIGUES, L. C. 2011. Salmonella Typhimurium and Salmonella Enteritidis in England: costs to patients, their families, and primary and community health services of the NHS. *Epidemiol Infect,* 139**,** 742-53.

SASAKI, M., AKAHIRA, A., OSHIMAN, K., TSUCHIDO, T. & MATSUMURA, Y. 2005. Purification of cytochrome P450 and ferredoxin, involved in bisphenol A degradation, from Sphingomonas sp. strain AO1. *Appl Environ Microbiol,* 71**,** 8024-30.

SCHENKMAN, J. B. & JANSSON, I. 2003. The many roles of cytochrome b5. *Pharmacol Ther,* 97**,** 139-52.

SCOTT, M. L. & MCALLISTER, J. C. 2012. Comparison of biochemical and molecular tests for detecting insecticide resistance due to insensitive acetylcholinesterase in Culex quinquefasciatus. *J Am Mosq Control Assoc,* 28**,** 323-6.

SENGUL, M., KACAR, N., KARACA, M., ONER, S. Z. & ERGIN, C. 2017. [A case with scalp pruritus caused by Dermanysus gallinae (order: mesostigmata)]. *Mikrobiyol Bul,* 51**,** 293-298.

SERVICE, M. 2012. Medical Entomology for Students. Cambridge: Cambridge.

SHEEHAN, D., MEADE, G., FOLEY, V. M. & DOWD, C. A. 2001. Structure, function and evolution of glutathione transferases: implications for classification of non-mammalian members of an ancient enzyme superfamily. *Biochem J,* 360**,** 1-16.

SHIRINOV, F., IBRAGIMOVA, A. & MISIROV, Z. 1972. The dissemination of the fowl-pox by the mite Dermanyssus gallinae. *Veterinarya,* 4**,** 48-49.

SIEKEVITZ, P. 1963. Protoplasm: endoplasmic reticulum and microsomes and their properties. *Annu Rev Physiol,* 25**,** 15-40.

SILVA, A. P. B., SANTOS, J. M. M. & MARTINS, A. J. 2014. Mutations in the voltage-gated sodium channel gene of anophelines and their association with resistance to pyrethroids - a review. *Parasites & Vectors,* 7.

SILVER, K. S., DU, Y., NOMURA, Y., OLIVEIRA, E. E., SALGADO, V. L., ZHOROV, B. S. & DONG, K. 2014. Voltage-Gated Sodium Channels as Insecticide Targets. *Adv In Insect Phys,* 46**,** 389-433.

SMIT, R. & POSTMA, M. J. 2015. Lyme borreliosis: reviewing potential vaccines, clinical aspects and health economics. *Expert Rev Vaccines,* 14**,** 1549-61.

SMITH, L. B., KASAI, S. & SCOTT, J. G. 2016. Pyrethroid resistance in Aedes aegypti and Aedes albopictus: Important mosquito vectors of human diseases. *Pestic Biochem Physiol,* 133**,** 1-12.

SMITH, L. B., TYAGI, R., KASAI, S. & SCOTT, J. G. 2018. CYP-mediated permethrin resistance in Aedes aegypti and evidence for trans-regulation. *PLoS Negl Trop Dis,* 12**,** e0006933.

SOBRADO, P., GOREN, M. A., JAMES, D., AMUNDSON, C. K. & FOX, B. G. 2008. A Protein Structure Initiative approach to expression, purification, and in situ delivery of human cytochrome b5 to membrane vesicles. *Protein Expr Purif,* 58**,** 229-41.

SODERLUND, D. M. 2012. Molecular mechanisms of pyrethroid insecticide neurotoxicity: recent advances. *Arch Toxicol,* 86**,** 165-81.

SODERLUND, D. M. & KNIPPLE, D. C. 2003. The molecular biology of knockdown resistance to pyrethroid insecticides. *Insect Biochemistry and Molecular Biology,* 33**,** 563-577.

SPARAGANO, O., PAVLICEVIC, A., MURANO, T., CAMARDA, A., SAHIBI, H., KILPINEN, O., MUL, M., VAN EMOUS, R., LE BOUQUIN, S., HOEL, K. & CAFIERO, M. A. 2009. Prevalence and key figures for the poultry red mite Dermanyssus gallinae infections in poultry farm systems. *Exp Appl Acarol,* 48**,** 3-10.

SPARAGANO, O. A., GEORGE, D. R., HARRINGTON, D. W. & GIANGASPERO, A. 2014. Significance and control of the poultry red mite, Dermanyssus gallinae. *Annu Rev Entomol,* 59**,** 447-66.

STANEK, G., FINGERLE, V., HUNFELD, K. P., JAULHAC, B., KAISER, R., KRAUSE, A., KRISTOFERITSCH, W., O'CONNELL, S., ORNSTEIN, K., STRLE, F. & GRAY, J. 2011. Lyme borreliosis: clinical case definitions for diagnosis and management in Europe. *Clin Microbiol Infect,* 17**,** 69-79.

STONE, N. E., OLAFSON, P. U., DAVEY, R. B., BUCKMEIER, G., BODINE, D., SIDAK-LOFTIS, L. C., GILES, J. R., DUHAIME, R., MILLER, R. J., MOSQUEDA, J., SCOLES, G. A., WAGNER, D. M. & BUSCH, J. D. 2014. Multiple mutations in the para-sodium channel gene are associated with pyrethroid resistance in Rhipicephalus microplus from the United States and Mexico. *Parasit Vectors,* 7**,** 456.

STRITTMATTER, P., FLEMING, P., CONNORS, M. & CORCORAN, D. 1978. Purification of cytochrome b5. *Methods Enzymol,* 52**,** 97-101.

SUN, X., GONG, Y., ALI, S. & HOU, M. 2018. Mechanisms of resistance to thiamethoxam and dinotefuran compared to imidacloprid in the brown planthopper: Roles of cytochrome P450 monooxygenase and a P450 gene CYP6ER1. *Pestic Biochem Physiol,* 150**,** 17-26.

SZTAL, T., CHUNG, H., BERGER, S., CURRIE, P. D., BATTERHAM, P. & DABORN, P. J. 2012. A Cytochrome P450 Conserved in Insects Is Involved in Cuticle Formation. *Plos One,* 7.

TANG, T., ZHAO, C., FENG, X., LIU, X. & QIU, L. 2012. Knockdown of several components of cytochrome P450 enzyme systems by RNA interference enhances the susceptibility of Helicoverpa armigera to fenvalerate. *Pest Manag Sci,* 68**,** 1501-11.

TCHIGOSSOU, G., DJOUAKA, R., AKOTON, R., RIVERON, J. M., IRVING, H., ATOYEBI, S., MOUTAIROU, K., YESSOUFOU, A. & WONDJI, C. S. 2018. Molecular basis of permethrin and DDT resistance in an Anopheles funestus population from Benin. *Parasit Vectors,* 11**,** 602.

THIND, B. B. & FORD, H. L. 2007. Assessment of susceptibility of the poultry red mite Dermanyssus gallinae (Acari : Dermanyssidae) to some acaricides using an adapted filter paper based bioassay. *Veterinary Parasitology,* 144**,** 344-348.

THOMAS, E., ZOLLER, H., LIEBISCH, G., ALVES, L. F. A., VETTORATO, L., CHIUMMO, R. M. & SIGOGNAULT-FLOCHLAY, A. 2018. In vitro activity of fluralaner and commonly used acaricides against Dermanyssus gallinae isolates from Europe and Brazil. *Parasit Vectors,* 11**,** 361.

TOH, S. Q., GLANFIELD, A., GOBERT, G. N. & JONES, M. K. 2010. Heme and blood-feeding parasites: friends or foes? *Parasit Vectors,* 3**,** 108.

TSOU, C. Y., MATSUNAGA, S. & OKADA, S. 2018. Molecular cloning and functional characterization of NADPH-dependent cytochrome P450 reductase from the green microalga Botryococcus braunii, B race. *J Biosci Bioeng,* 125**,** 30-37.

ULLMANN, A. J., LIMA, C. M., GUERRERO, F. D., PIESMAN, J. & BLACK, W. C. T. 2005. Genome size and organization in the blacklegged tick, Ixodes scapularis and the Southern cattle tick, Boophilus microplus. *Insect Mol Biol,* 14**,** 217-22.

UNION, N. F. 2014. *Farming in the South West* [Online].

VALIENTE MORO, C., DE LUNA, C. J., TOD, A., GUY, J. H., SPARAGANO, O. A. & ZENNER, L. 2009. The poultry red mite (Dermanyssus gallinae): a potential vector of pathogenic agents. *Exp Appl Acarol,* 48**,** 93-104.

VAN EMOUS, R. 2005. Wage war against the red mite. *Poultry International,* 44**,** 26-33.

VAN EMOUS, R. 2017. *Verwachtte schade bloedluis 21 miljoen euro* [Online]. Pluimveeweb.

VAN ROSSUM, A. J., JEFFERIES, J. R., RIJSEWIJK, F. A., LACOURSE, E. J., TEESDALE-SPITTLE, P., BARRETT, J., TAIT, A. & BROPHY, P. M. 2004. Binding of hematin by a new class of glutathione transferase from the blood-feeding parasitic nematode Haemonchus contortus. *Infect Immun,* 72**,** 2780-90.

VULULE, J. M., BEACH, R. F., ATIELI, F. K., MCALLISTER, J. C., BROGDON, W. G., ROBERTS, J. M., MWANGI, R. W. & HAWLEY, W. A. 1999. Elevated oxidase and esterase levels associated with permethrin tolerance in Anopheles gambiae from Kenyan villages using permethrin-impregnated nets. *Med Vet Entomol,* 13**,** 239-44.

WAAP, H., NUNES, T., MUL, M. F., GOMES, J. & BARTLEY, K. 2019. Survey on the prevalence of Dermanyssus gallinae in commercial laying farms in Portugal. *Avian Pathol***,** 1-8.

WALSH, T. K., JOUSSEN, N., TIAN, K., MCGAUGHRAN, A., ANDERSON, C. J., QIU, X., AHN, S. J., BIRD, L., PAVLIDI, N., VONTAS, J., RYU, J., RASOOL, A., BARONY MACEDO, I., TAY, W. T., ZHANG, Y., WHITEHOUSE, M. E. A., SILVIE, P. J., DOWNES, S., NEMEC, L. & HECKEL, D. G. 2018. Multiple recombination events between two cytochrome P450 loci contribute to global pyrethroid resistance in Helicoverpa armigera. *PLoS One,* 13**,** e0197760.

WALTER, D. & PROCTOR, H. 1999. Mites: Ecology, Evolution and Behaviour New York: CABI Publishing

WAN, H., LIU, Y., LI, M., ZHU, S., LI, X., PITTENDRIGH, B. R. & QIU, X. 2014. Nrf2/Maf-binding-site-containing functional Cyp6a2 allele is associated with DDT resistance in Drosophila melanogaster. *Pest Manag Sci,* 70**,** 1048-58.

WANG, F. F., WANG, M., XU, F. R., LIANG, D. M. & PAN, B. L. 2010. Survey of prevalence and control of ectoparasites in caged poultry in China. *Vet Rec,* 167**,** 934-7.

WANG, M., ROBERTS, D. L., PASCHKE, R., SHEA, T. M., MASTERS, B. S. & KIM, J. J. 1997. Three-dimensional structure of NADPH-cytochrome P450 reductase: prototype for FMN- and FAD-containing enzymes. *Proc Natl Acad Sci U S A,* 94**,** 8411-6.

WEGNER, Z. 1976. Laboratory study on some parasitic hematophagous arthropods as possible subsidiary links of the biocenosis of tick-borne encephalitis. *Bull Inst Marit Trop Med Gdynia,* 27**,** 75-85.

WEILL, M., MALCOLM, C., CHANDRE, F., MOGENSEN, K., BERTHOMIEU, A., MARQUINE, M. & RAYMOND, M. 2004. The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors. *Insect Mol Biol,* 13**,** 1-7.

WERCK-REICHHART, D. & FEYEREISEN, R. 2000. Cytochromes P450: a success story. *Genome Biol,* 1**,** REVIEWS3003.

WILLIAMSON, M. S., MARTINEZTORRES, D., HICK, C. A. & DEVONSHIRE, A. L. 1996. Identification of mutations in the housefly para-type sodium channel gene associated with knockdown resistance (kdr) to pyrethroid insecticides. *Molecular and General Genetics,* 252**,** 51-60.

WINDHORST, H. 2017. Dynamics and patterns of the EU egg industry. *International Egg Commission* Monte Carlo.

WINNEBECK, E. C., MILLAR, C. D. & WARMAN, G. R. 2010. Why does insect RNA look degraded? *J Insect Sci,* 10**,** 159.

WOJCIK, A. R., GRYGON-FRANCKIEWICZ, B., ZBIKOWSKA, E. & WASIELEWSKI, L. 2000. [Invasion of Dermanyssus gallinae (De Geer, 1778) in poultry farms in the Torun region]. *Wiad Parazytol,* 46**,** 511-5.

WOLSTENHOLME, A. J. & ROGERS, A. T. 2005. Glutamate-gated chloride channels and the mode of action of the avermectin/milbemycin anthelmintics. *Parasitology,* 131 Suppl**,** S85-95.

WRIGHT, A. T. & CRAVATT, B. F. 2007. Chemical proteomic probes for profiling cytochrome p450 activities and drug interactions in vivo. *Chem Biol,* 14**,** 1043-51.

YAKHCHALI, M., RASOULI, S. & ALBORZI, E. 2013. Prevalence and body distribution of the poultry red mite in layer farms from Markazi province of Iran. *Iranian Journal of Veterinary Research,* 14**,** 72-74.

YANOLA, J., SOMBOON, P., WALTON, C., NACHAIWIENG, W. & PRAPANTHADARA, L. A. 2010. A novel F1552/C1552 point mutation in the Aedes aegypti voltage-gated sodium channel gene associated with permethrin resistance. *Pesticide Biochemistry and Physiology,* 96**,** 127-131.

YU, C. & GUNSALUS, I. C. 1974. Cytochrome P-450cam. II. Interconversion with P-420. *J Biol Chem,* 249**,** 102-6.

YU, S. J., ROBINSON, F. A. & NATION, J. L. 1984. Detoxication Capacity in the Honey Bee, Apis-Mellifera L. *Pesticide Biochemistry and Physiology,* 22**,** 360-368.

ZEMAN, P. 1987. Encounter the poultry red mite resistance to acaricides in Czechoslovak poultry-farming. *Folia Parasitol (Praha),* 34**,** 369-73.

ZEMAN, P. 1988. Surface skin lipids of birds--a proper host kairomone and feeding inducer in the poultry red mite, Dermanyssus gallinae. *Exp Appl Acarol,* 5**,** 163-73.

ZEMAN, P., STIKA, V., SKALKA, B., BARTIK, M., DUSBABEK, F. & LAVICKOVA, M. 1982. Potential role of Dermanyssus gallinae De Geer, 1778 in the circulation of the agent of pullurosis-typhus in hens. *Folia Parasitol (Praha),* 29**,** 371-4.

ZEMSKAYA, A. & PCHELKINA, A. 1967. Gamasoid mites and Q fever. *Problemy Parazitologii***,** 258-259.

ZENNER, L., BON, G., CHAUVE, C., NEMOZ, C. & LUBAC, S. 2009. Monitoring of Dermanyssus gallinae in free-range poultry farms. *Experimental and Applied Acarology,* 48**,** 157-166.

ZHANG, H., HAMDANE, D., IM, S. C. & WASKELL, L. 2008. Cytochrome b5 inhibits electron transfer from NADPH-cytochrome P450 reductase to ferric cytochrome P450 2B4. *J Biol Chem,* 283**,** 5217-25.

ZHANG, H., MYSHKIN, E. & WASKELL, L. 2005. Role of cytochrome b5 in catalysis by cytochrome P450 2B4. *Biochem Biophys Res Commun,* 338**,** 499-506.

ZHANG, H., ZHAO, M. T., LIU, Y. R., ZHOU, Z. S. & GUO, J. Y. 2018. Identification of cytochrome P450 monooxygenase genes and their expression in response to high temperature in the alligatorweed flea beetle Agasicles hygrophila (Coleoptera: Chrysomelidae). *Scientific Reports,* 8.

ZHANG, L., LU, Y., XIANG, M., SHANG, Q. L. & GAO, X. W. 2016. The retardant effect of 2-Tridecanone, mediated by Cytochrome P450, on the Development of Cotton bollworm, Helicoverpa armigera. *Bmc Genomics,* 17.

ZHANG, M., LE CLAIR, S. V., HUANG, R., AHUJA, S., IM, S. C., WASKELL, L. & RAMAMOORTHY, A. 2015. Insights into the role of substrates on the interaction between cytochrome b5 and cytochrome P450 2B4 by NMR. *Sci Rep,* 5**,** 8392.

ZIAPOUR, S. P., KHEIRI, S., FAZELI-DINAN, M., SAHRAEI-ROSTAMI, F., MOHAMMADPOUR, R. A., AARABI, M., NIKOOKAR, S. H., SARAFRAZI, M., ASGARIAN, F., ENAYATI, A. & HEMINGWAY, J. 2017. Pyrethroid resistance in Iranian field populations of Rhipicephalus (Boophilus) annulatus. *Pestic Biochem Physiol,* 136**,** 70-79.

Appendices

A. Terrific broth

250 g modified Terrific Broth and 43 ml glycerol (both Sigma-Aldrich, Gillingham, UK) was dissolved in 5 L of distilled water and autoclaved.

B. SDS-PAGE

Running Buffer:

0.2 M Tris

0.2 M Glycine

10 % SDS

2x Sample Buffer:

100 mM Tris

4 % SDS

20 % Glycerol

Bromophenol blue

Coomassie Blue Stain:

45 % Methanol

45 % H2O

10 % Glacial Acetic Acid

Coomassie blue 252

Destain:

45 % Methanol

45 % H2O

10 % Glacial Acetic Acid

Components of SDS-PAGE gel (15 %):

Separating Gel	**Stacking Gel**
40 % acrylamide 3.75 ml	40 % acrylamide 1 ml
1.5 M Tris-HCL pH 8.8 2.5 ml	0.6 M Tris-HCL pH 6.8 2.5 ml
Distilled water 3.75 ml	Distilled water 6.5 ml
10 % APS 100 µl	10 % APS 200 µl
TEMED 6 µl	TEMED 20 µl

C. Survey

Poultry Red Mite (PRM) Questionnaire:

1) **Farm name:** .....

2) **County:** .....

3) **Nearest town:** .....

4) **Email Address:** .....

5) **Please provide a brief description of your production system(s)** (flock size, type/dimension/number of buildings, lighting used, feeding regime, other animals reared on the farm etc.):

6) **Product information** (please fill in the table for as many products as you currently use for PRM control. If more space is needed then please add on to an extra page as required):

Name of product used:	Product type (dust, spray, etc.):	Manufacturer (if known):	Frequency of use:	Timing of use (i.e. on empty or populated units):	Method of use (spot treatment, complete coverage, etc.):	Perceived effective-ness (scale of 0-10):

7) **Do you use any preventative methods other than the products mentioned?** (If yes please state)

.....

8) **Any other comments?**

Thank you for your time

D. PRM trap instructions

Mite Trap Usage

Ensure the black corrugated plastic sheet is in the slot inside the blue trap.

Find areas inside the unit to attach the traps. Best areas are places where mites have been observed before (mainly in dark cracks/crevices near the birds).

Use the cable ties through the holes in the blue traps to secure the traps to the housing.

Leave the traps in place for 7-14 days.

Cut the ties and remove the traps from the sheds. Quickly place traps into the plastic tub provided (ensure corrugated card is still inside trap).

Place tub inside plastic bag and then post back in envelope provided.

E. Product usage instructions

Milben Ex (Schopf): 100 ml to 5 litre water (sufficient for up to 150 m^2) at normal infestation.

Interkokask (Hysolv): Ready to use.

Ficam (Bayer): 15g of powder in 5 L of water.

Perbio Choc (Digrain): Ready to use.

CBM8 (Hysolv): 200 ml in 10 L of water.

Fendona (BASF): 5 ml in 1 L of water.

F. Ethical approval

Professor Kathleen McCourt, CBE FRCN

Executive Dean

This matter is being dealt with by:

Dr Richard N. Ranson
Applied Sciences Ethics Lead
Faculty of Health & Life Sciences
Northumberland Building
Newcastle upon Tyne
NE1 8ST

Date: 26/11/2014

Project Ref: BMS13JA2014

Period of Coverage: 3 years from date above unless the study has been significantly changed or completed.

Dear Joanne Atkinson,

Faculty of Health and Life Sciences Research –Biomedical Ethics Review.

Title:

Mapping, characterising and targeting acaricide resistance in the poultry red mite, *Dermanyssus gallinae*.

Following independent peer review of the above proposal the review process has been completed. I am therefore pleased to inform you that Departmental (and thus) Faculty approval has been granted for this proposal- subject to compliance with the University policies on ethics and consent and any other policies applicable to your individual research.

NB. If your research involves working with children and/or vulnerable adults you should also have recent Disclosure & Barring Service (DBS) and occupational health clearance.

The University's Policies and Procedures are available from the following web link: http://www.northumbria.ac.uk/researchandconsultancy/sa/ethgov/policies/?view=Standard

All researchers must give notice of the following:

- Any significant changes to the study design;
- Any incidents which have an adverse effect on participants, researchers or study outcomes;
- Any suspension or abandonment of the study;

Please keep this letter with your application as proof of ethical clearance and for any future auditing requirements.

Yours sincerely

Dr Richard N. Ranson

Applied Science and Biomedical Ethics Faculty Representative.

G. Conference contributions and workshops

05.03.2015 AgriFood Charities Partnership (AFCP) student forum Poster
'Identification of Pyrethroid Metabolising P450s in Poultry Red Mite using Bioinformatics'
Joanne Atkinson, David George, Olivier Sparagano, Robert Finn

20.05.2015 Northumbria Research Conference Poster
'Identification of Pyrethroid Metabolising P450s in Poultry Red Mite using Bioinformatics'
Joanne Atkinson, David George, Olivier Sparagano, Robert Finn

24.06.2015 Experimental Poultry Centre Poultry Red Mite Workshop

05.04.2016 WPSA Conference Chester Oral presentation and Poster (poster prize awarded)
'A preliminary assessment of the current state of resistance/susceptibility to the active compounds used in Acaricides for Poultry Red Mite, *Dermanyssus gallinae*, in the UK'
Joanne Atkinson, David George, Olivier Sparagano, Robert Finn

27.04.2016 Coremi COST action meeting Invited Speaker
'A preliminary assessment of the current state of resistance to active compounds used in Acaricides for Poultry Red Mite, *Dermanyssus gallinae*, in the UK'
Joanne Atkinson, David George, Olivier Sparagano, Robert Finn

01.08.2016 International conference of parasitology Poster
'A preliminary assessment of the current state of resistance/susceptibility to the active compounds used in Acaricides for Poultry Red Mite, *Dermanyssus gallinae*, in the United Kingdom'
Joanne Atkinson, David George, Olivier Sparagano, Robert Finn

08.09.2016 STVM-AITVM joint conference Berlin Poster
'A comparison between the cytochrome b_5 gene in *Dermanyssus gallinae*, *Rhipicephalus microplus* and *Ixodes scapularis* and their host species – potential for a new acaricide target'

Joanne Atkinson, Kirsty Graham, David George, Olivier Sparagano, Robert Finn

05.04.2017 AFCP student forum Oral presentation and Poster
‘A preliminary assessment of the current state of resistance/susceptibility to the Acaricides used for Poultry Red Mite, *Dermanyssus gallinae*, in the UK’
Joanne Atkinson, David George, Olivier Sparagano, Robert Finn

H. PRM transcriptomic data (127 sequences)

>isotig29458|cytochrome P450, putative [Ixodes scapularis]
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