Identification of immunogenic gut-associated proteins from the poultry red mite, *Dermanyssus gallinae*

James Pritchard

Supervisors:
Professor Fiona Tomley
Professor Olivier Sparagano
Dr. Rachel Lawrence

Department of Pathology and Pathogen Biology
Royal Veterinary College
University of London

Thesis submitted for the degree of Doctor of Philosophy
September 2016

Declaration

I, **James Pritchard**, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Dermanyssus gallinae, the poultry red mite (PRM), is a haematophagous ectoparasite that infests layer hens, amongst other bird species, and causes significant welfare issues and economic losses to the poultry egg laying industry worldwide. Current control methods are not completely effective and widespread reports of genetic resistance to chemical acaricides have prompted research into alternative control strategies.

This project concerns the purification and identification of immunogenic proteins expressed on the surface of cells located within the digestive system of *Dermanyssus gallinae*. Hypothetically, such proteins could be excellent vaccine antigens because they are exposed to host blood after mites have fed. This means that while they are potential targets for neutralisation by antibodies in the blood meal, digestive proteins should not be subject to strong immune selection because the host immune system is not naturally exposed to these antigens during the act of feeding, only afterwards.

Homogenated poultry red mite mixture was fractionated by mechanical disruption followed by differential centrifugation to enrich specifically for proteins associated with cell membranes. Fractions were enriched for membrane–associated proteins by sedimentation of cell nuclei and mitochondria followed by treatment with Triton X-100 detergent to disrupt and release proteins from the phospholipid bilayer. Membrane proteins were increased from an initial 8% to a final 19% of a total of 1214 proteins identified by mass spectrometry and screening of a transcriptome database. Polyclonal antibodies against soluble and membrane proteins were used to probe Western blots and provided direct evidence that this methodology resulted in enrichment for both soluble and membrane-associated proteins in different fractions.

The use of a 'membrane-protein enriched' fraction for bio-panning resulted in the isolation of 62 phage from a total starting library of 1.37×10^8 phage variants (Tomlinson J, 2011) that each

bound specifically to the mite protein mixture. Each phage expresses a single chain variable fragment (scFv) molecule corresponding to the antigen binding site of a chicken immunoglobulin molecule. Immunohistochemical staining of paraffin-embedded and sectioned mites identified that 31 of the 62 phages expressed scFv variants that bound specifically to distinct PRM tissues. Nineteen scFv/phages bound to proteins expressed in the PRM midgut, caecae or hindgut and 10 of these were shown by sequencing to contain distinct hypervariable regions, indicating that they had different specificities and therefore are binding to different antigenic epitopes expressed within the gut tissue.

Acknowledgements

I would like to thank my supervisors Professor Fiona Tomley, Olivier Sparagano and Dr Rachel Lawrence for their guidance and encouragement during my PhD. I also want to express my deepest sadness for having to see this project through without Professor Declan McKeever being here to see it finished, he truly inspired passion in science and will is missed by all of us.

I also want to thank everyone in my group who have made working throughout the 4 years here at RVC a pleasure. My group have not just helped me with coffee and cake but have also given me invaluable academic and scientific advice. Matt, Tati, Virginia, Sarah, Ivan, Elaine, Kim, Daniel, Shazia and Charlotte, thank you!

Thanks also go to Dr. Damer Blake and Dr. Rob Noad, although they were not mentioned as supervisors, both played a very significant role in my PhD and I am indebted to both of them for giving up their time and sharing their experiences and knowledge. I also extend my deepest appreciation to all the people who aided with the project, notably including Dr David George (Newcastle), Dr. Alisdair Nisbet (Moredun), Dr. Kathryn Bartley (Moredun), Dr Dominic Kurian (Roslin), Miss Medina Gowers (RVC), Miss Elaine Shervill (RVC), Kathy Baker (Oaklands farm) and Richard Barton (Grassington Farm).

Finally, I would like to thank the British Egg Marketing Board and the Royal Veterinary College for allowing me to work on this project. Without the funding of the BEMB and the support structure of the RVC, none of this would have been possible, thank you.

Table of contents

List of Figures

List of Tables

Abbreviations

AI - Avian Influenza

APC – Antigen Presenting Cell

APMV - Avian Paramyxovirus

CD4/8 - Cluster of Differentiation 4/8

CO_2 – Carbon Dioxide

DEFRA - Department for Environment, Food & Rural Affairs

DNA – Deoxyribonucleic Acid

EST – Expressed Sequence Tag

EU – European Union

HACCP - Hazard Analysis and Critical Control Point

HPAI - Highly Pathogenic Asian Influenza

HRP – Horseradish Peroxidase

IgY/G/M – Immunoglobulin Y/G/M

LB – Luria Borth

MAC complex – Membrane Attack Complex

MHC - Major Histocompatibility Complex

NDV - Newcastle Disease Virus

PAMPs – Pathogen Associated Molecular Patterns

PCV - Porcine circovirus

PRM – Poultry Red Mite

PRR - Pattern Recognition Receptors

PTFE – Polytetrafluoroethylene

PVC – Packed Cell Volume

PVDF - Polyvinylidene fluoride

TcR – T cell Receptor

Th1/2 Cell – T helper cell 1/2

UK – United Kingdom

Species

Acaris siro - flour mite

Actinobacillus pleuropneumoniae - bacteria

Androlaelaps casalis – predatory species of mite

Ascaris - hookworm

Beauveria bassiana – fungi

Borrelia anserine - bacteria

Campylobacter jejuni - bacteria

Chlamydia spp. - bacteria

Candidatus cardinium – bacteria

Carcinops pumilio – histerid beetle

Clostridium chauvoei – bacteria

Coxiella burnetii - bacteria

Dendroctonus frontalis - southern pine beetle

Dermanyssus gallinae – poultry red mite

Dermatophagoides farina – dust mite

Diaphorina citri - Asian citrus psyllid

Dinothrombium sp – giant spider red velvet mite

Eimeria tenella – apicomplexan parasite

Escherichia coli - bacteria

Haematobia irritans exigua - buffalo fly

Haemonchus contortus – barber's pole worm

Hirsutella thompsonii - fungi

Hypoderma lineatum – warble fly

Hypoaspis aculeifer – predatory species of mite.

Hypoaspis miles - predatory species of mite.

Ixodes scapularis – deer tick

Leishmania major – kinetoplast parasite

Listeria monocytogenes - bacteria

Lucilia cuprina – sheep blowfly

Metarhizium anisopliae – fungi

Macrocheles muscaedomesticae – mite

Musca domestica – common house fly

Mycoplasma gallisepticum - bacteria

Nezara viridula - southern green stink bug

Ornithonyssus bursae – tropical fowl mite

Ornithonyssus sylviarum – northern fowl mite

Panonychus ulmi – European red mite

Pasteurella multocida - bacteria

Paecilomyces fumosoroseus – fungi

Phlebotomus papatasi – sand fly

Plasmodium falciparum – apicomplexan parasite

Psoroptes ovis – sheep scab mite

Rhipicephalus annulatus – blue cattle tick

Rhipicephalus microplus – cattle tick

Rickettsiella sp. – bacteria

Salmonella enteritidis - bacteria

Salmonella gallinarum - bacteria

Salmonella typhimurium- bacteria

Sarcoptes scabiei – itch mite

Scaphoideus titanus - American grapevine leafhopper

Schineria sp. – bacteria

Schistosoma spp. - trematode

Spiroplasma - bacteria

Staphylococcus spp. - bacteria

Stratiolaelaps scimitus – predatory species of mite

Streptomyces spp. - bacteria

Tetranychus urticae – red spider mite

Trichoderma album – fungi

Typanosoma brucei – kinetoplast endoparasite

Appendix – Regents

Blocking buffer (for IHC, ELISA and Western)

5g of Blotting grade blocker (Bio-rad)

100ml of x1 wash buffer (see below).

CAPS transfer buffer for western blot transfer

20x stock is 0.1% Tris/HCl

0.1% SDS

10% methanol

10Mm CAPS

pH9.5 – made up to 1x by 25ml stock + 475ml dH_2O.

Carnoy's fixative

60ml ethanol

30ml chloroform

10ml glacial acetic acid

Destaining solution for Comassie Blue stained gels.

250ml methanol

70ml acetic acid

made up to 1 litre with dH_2O.

Hypotonic buffer

10m NaCl (0.058g)

10mM Tris (0.121g)

1.5mM $MgCl_2$ (0.014g)

up to 100ml dH_20

Laemmli's buffer 2x (Sigma, UK)

4% SDS, 20% glycerol

10% 2-mercaptoethanol

0.004% bromphenol blue

0.125 M Tris HCl, pH 6.8

Mass spectrometry transfer buffer

8M Urea

50 mm Ammonium Bicarbonate

0.1% SDS

in dH_2O

MOPS buffer 20x (Invitrogen, UK)

50 mM MOPS

50 mM Tris Base

0.1% SDS

1 mM EDTA

pH 7.7, diluted in dH_2O to create 1x MOPS buffer.

PBS pH 7.4 (+protease inhibitors)

53.92g Disodium hydrogen orthophosphate (Na2HPO4)

3.12g Sodium dihydrogen orthophosphate (NaH2PO4)

17.0g Sodium chloride (NaCl)

Up to 1L with dH_2O

1 tablet SigmaFAST (Sigma #S8820) per 100ml buffer.

PEG6000

20% (w/v) Polyethylene glycol (10g)

2.5M NaCl (7.3g)

Up to 50ml dH_2O

RIPA buffer

150nm NaCl (0.435g)

1% Triton X-100 (500µl)

0.5% Sodium deoxychorlate (0.25g)

0.1% Sodium dodecyl sulphate

50mM Tris (0.3g)

pH 8.0, up to 50ml dH_20

Trypsin in TBSC (for eluting Phage)

40mg Trypsin

137mM NaCl

2mM $CaCl_2$

10mM Tris-HCl

up to 10ml with dH_20 pH8.0

Wash buffer (for IHC, ELISA and Western blots)

1ml of Tween 20

Up to 1 litre with 1xPBS pH7.4

Chapter 1: Introduction

1.1 The Poultry Red Mite, *Dermanyssus gallinae*

The poultry red mite (PRM), *Dermanyssus gallinae*, is a haematophagous ectoparasite of both domesticated and commercially bred chickens, as well as other bird species (Brannstrom, Morrison et al. 2008). Though cases of PRM biting mammals is also reported (Gaaboub, Donia et al. 1982; Lucky, Sayers et al. 2001; Mignon and Losson 2008), it would appear a bird species is required as a definitive host for parasite survival and reproduction. Within the egg-laying sector, PRM causes a substantial economic loss due to lowered egg laying rates and chicken pathology. Existing chemical control techniques employed against PRM are often reported to be unreliable and ineffective, possibly due to the development of mite genetic resistance against active ingredients (Beugnet, Chauve et al. 1997; Harrington, George et al. 2011). There is a clear lack of other effective PRM control methods available to the poultry industry, necessitating research into alternative controls such as vaccine development. The following chapter is an introduction to the biology and economic importance of the poultry red mite and includes a review of work published to date on vaccine development and the requirements for such a vaccine to be successful.

1.1.1 Acari and 'general' mite characteristics

D. gallinae, the poultry red mite, forms part of the Acari subclass along with ~50,000 other described species of mites and ticks, although the true number of mite species is probably closer to one million (Walter and Proctor 1999). The Acari are a subclass of the phylum Arthropoda, which incorporates invertebrate classes including Insects, Arachnids (including Acari) and Crustaceans. Examples of medically and/or economically important Acari species

include the northern fowl mite (*Ornithonyssus sylviarum*), the itch mite (*Sarcoptes scabiei*), the dust mite (*Dermatophagoides farina*), the red spider mite (*Tetranychus urticae*), the deer tick (*Ixodes scapularis*), the cattle tick (*Rhipicephalus microplus*) and the poultry red mite (*Dermanyssus gallinae*). Mites appear to live in almost all environments on earth except in very cold conditions of <-25°C. Mites are predominantly known to science for their causative effect in asthma and allergies, as well as their parasitism of plants, bees and livestock. Some mites are detritivores, feeding on organic matter in soils. Others are parasitic and live on hosts including birds, fish, mammals and even other invertebrates such as bees (infested by *Varroa destructor*). Mite species are often small, about 1mm in length, although some species can reach sizes of 20mm, such as the giant red velvet mite, *Dinothrombium sp* (Cloudsley-Thompson 1962). Although many species are non-destructive to animal or plant life, some cause great devastation and economic loss such as the several hundred species of spider mite from the Tetranychidae family that feed on varieties of crop plants. Some predatory mite species can be used as a control against other mite species. For instance, species from the Phytoseiidae family are used as biological controls against mite species such as those of the Tetranychidae family (McMurtry and Scriven 1966).

1.1.2 The PRM life cycle and feeding

PRM has a metamorphic life cycle, developing through five developmental life stages, of which the latter three stages require a blood meal for growth and/or egg development (see Figure 1.1). The life cycle of PRM can be completed in 9 days in optimal environmental conditions, 20-30°C and 70% humidity, from egg to adult ((Nordenfors, Hoglund et al. 1999)Maurer & Baumgartner, 1992), although in poultry houses 14-16 days is more usual. It is predominantly only females of the protonymph, deutonymph and adult stages that feed on blood. Males have been observed to feed on both blood and detritus, although further research is required to elucidate whether blood is essential for male survival. Observations made by Wood (1917)

suggested males will not feed immediately straight after moulting but rather spend more time in the cracks and crevices, presumably to locate and fertilise females. Female adults typically lay clutches of 4-8 eggs with a maximum of 30 eggs total in a lifetime. Larvae have six legs (not eight as the other stages) and all stages spend the majority of their time away from the host, feeding intermittently for only short periods of up to 2 hours. Adults can live for up to 9 months without feeding (Kirkwood 1967), however typically they feed every third day where possible.

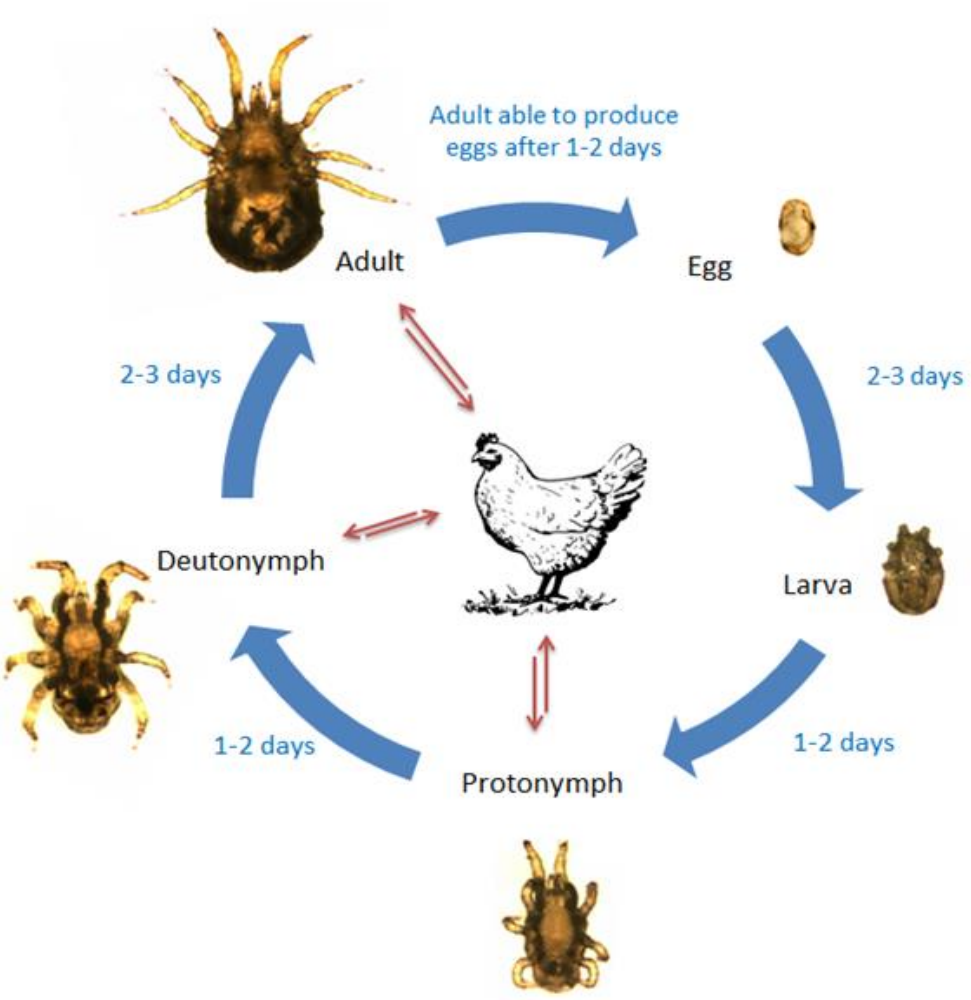


Figure 1.1: The life cycle of *Dermanyssus gallinae*. Going clockwise from top right - eggs develop into larvae then moult into protonymphs, deutonymphs and finally moult into adults. The whole life cycle commonly takes 7-14 days and fully-grown adult females are capable of oviposition after 9-16 days. Only protonymphs, deutonymphs and adults feed on blood (red arrows). All stages have eight legs, apart from larvae that have six legs. Legs should not be confused with the two palps present at the base of the mite head. Mite stages were photographed using an Olympus BM860 at x40.

1.1.3 Morphology & Anatomy 1: External morphology

PRM, along with other mite species, have an exoskeleton, 8 legs (6 in larval stages), palps and anteriorly extending feeding parts known as chelicerae. PRM larvae average 300-400µm in length and adults grow to 900-1000µm. Unlike some predatory mite species, PRM do not have eyes but sense their environment through palps and receptors at the extremities of their legs. Various types of receptor detect changes in micro vibrations, temperature and volatile chemicals such as pheromones or carbon dioxide (Cruz, Robles et al. 2005). The exoskeleton is made of a tough chitin polymer, surrounded by a waxy cuticle designed to retain water within the mite body. PRM dehydrate through respiration thus, their survival is optimised in humid conditions, suggesting that water retention is a fundamental necessity for PRM existence. Females can be differentiated from males microscopically by the identification of their ventral anal shield and the genitoventral shield. By contrast, male PRM have a larger, single fused holoventral shield.

1.1.4 Morphology & Anatomy 2: Internal morphology

Internally, PRM have an open circulatory system encased in a single body cavity known as the haemocoel. Organs are surrounded by haemolymph, that is analogous to blood in vertebrates and circulated by muscular movement. Haemolymph is blue when oxygenated due to copper molecules bound to soluble haemocyanin proteins that reversibly bind oxygen atoms during oxygen transportation. Haemolymph contains cells known as haemocytes, as well as nutrients such as proteins, sugars, inorganic salts and water. Unlike red blood cells, haemocytes are not involved in the transport of oxygen; but rather they play significant roles in the arthropod innate immune system involved with phagocytosis and encapsulation of pathogens (Lavine and Strand 2002). Arthropods do not have an adaptive immune system that jawed-vertebrates do, however incorporate a complex innate system including cellular and humoral immune responses (Kopáček, Hajdušek et al. 2010). This innate immunity is well conserved throughout

both vertebrates and invertebrates, with many arthropod immune molecules showing homology to vertebrate innate immune molecules (Zhu, Thangamani et al. 2005; Wang, Tan et al. 2006).

The mite digestive tract starts at at the gnathosoma (mouthparts), directing food posteriorly through an alimentary canal and into a midgut and six connecting caeca (Figure 1.2). In haematophagous Acari, including PRM, the midgut and caeca is where most blood digestion occurs (Hamilton, Nisbet et al. 2003; Sojka, Franta et al. 2013). Digested blood and waste products are collected from the haemolymph inside a pair of large malpighian tubules, similar to the function of kidneys in mammals. These tubules extend longitudinally and connect with the PRM hindgut and anus at the posterior.

Neural functions are coordinated via a centralised nervous mass known as the synganglion tissue (Figure 1.3). Eight major neural ganglia extend from this central mass, presumably each controlling a corresponding leg.

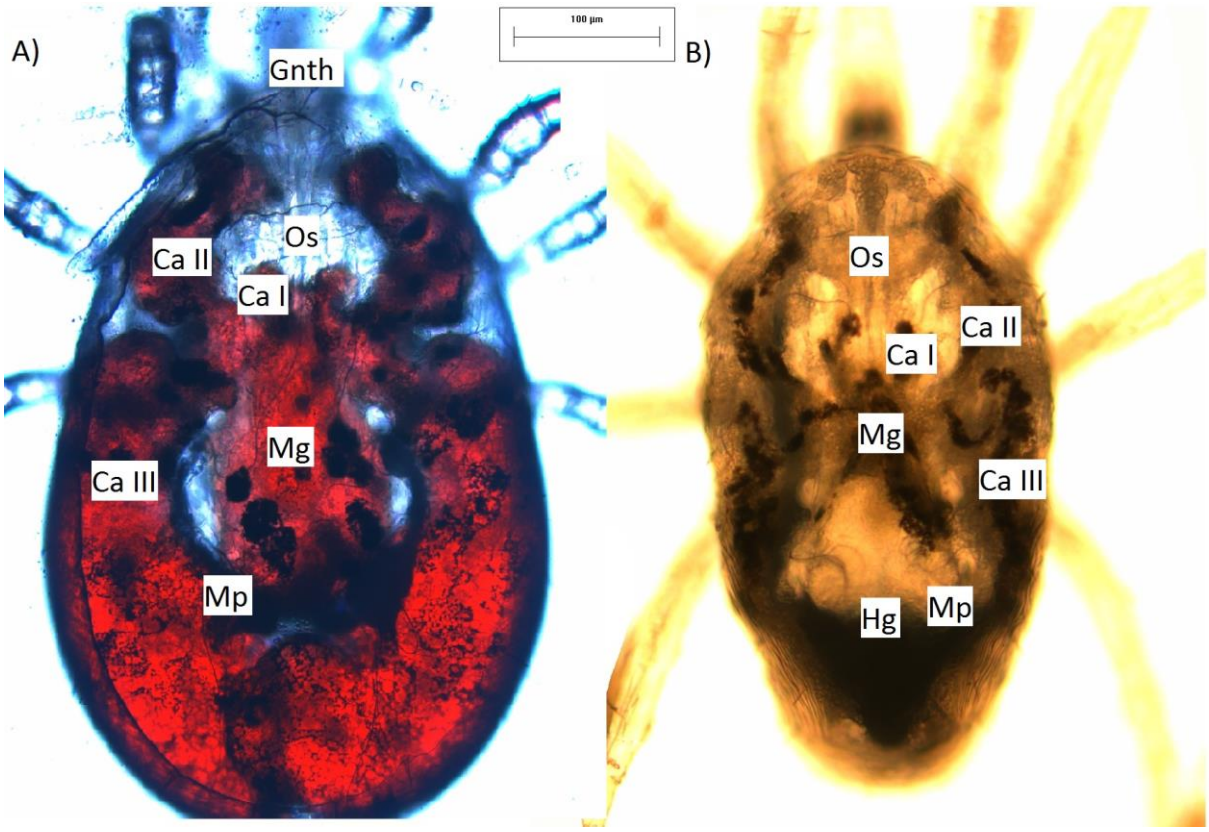


Figure 1.2: Comparison of the PRM digestive system in blood fed (A) and unfed (B) mites. Mites were observed at ×200 magnification from the dorsal side (Olympus BM860 microscope, axiom software). Both fed and unfed mites were photographed within one day of collection. The PRM digestive tract extends from the gnathosoma posteriorly through the oesophagus, midgut and caeca and ending in the hindgut. Most blood digestion occurs in the three caecal pairings (Ca I–III) and central midgut (Mg). Malpighian tubules elongate longitudinally along the idiosoma, connected to the anterior hindgut. These tubules are involved in nitrogenous waste collection and osmoregulation. Waste leaves through the posterior hindgut and through the anal opening (not shown). Note: mite body shape increases and gets rounder during feeding and the digestive tract completes >70% of the body cavity of the PRM when full (Figure 3A), compared to that of an unfed mite (Figure 3B). Bar = 100µm. Gnth – gnathosoma (mouthparts); Os – oesophagus; Ca I–III – caeca I–III; Mp – malpighian tubules; Hg – hindgut.

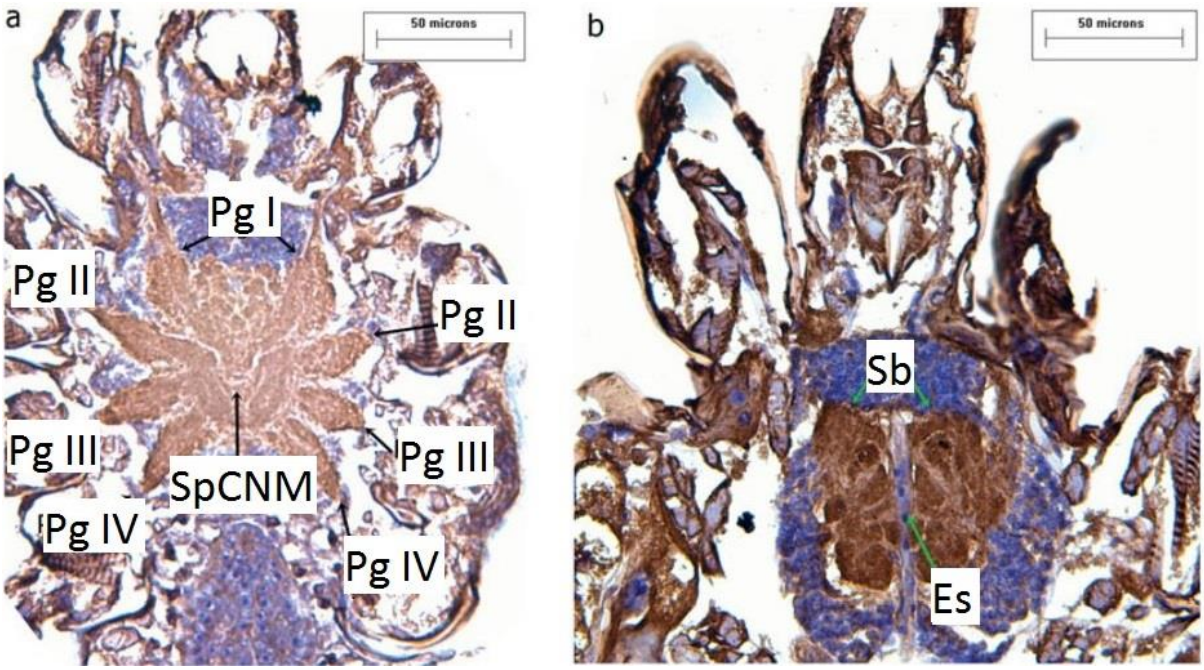


Figure 1.3: The synganglion tissue (brain) of the PRM. Longitudinal sections of 10-µm thickness observed at ×200 magnification. Sections were embedded in wax, hydrated and stained with 1:100 anti-Cathepsin D chicken IgY (kindly donated by Dr Alistair Nisbet) then stained with 1:1,000 goat anti-rabbit IgG HRP and counter-stained with haematoxylin. The PRM synganglion tissue, as in all Acari, is centrally divided by the oesophagus into two connected masses – the supraoesophageal mass (a) and the suboesophageal mass (b). Figure 1.3a shows the supra-oesophageal central nervous mass connected to eight pedal ganglia, each extending distally to the corresponding leg. Figure 1.3b shows the sub-oesophageal mass, comparatively more rounded and divided by the oesophagus extending longitudinally down though the centre. Bar = 50 µm. Pg I–IV – pedal ganglion 1–4; SpCNM – supra-oesophageal central nervous mass; Sb – sub-oesophageal mass; Es – oesophagus.

1.2 Epidemiology and pathology

1.2.1 Global distribution of the poultry industry and PRM

Poultry production around the world totals an estimated 65 million birds globally each year, producing 1.1 trillion eggs and 90 million tonnes of meat (http://www.faostat.fao.org/). Bird numbers have tripled in the past 20 years and chicken egg and meat production is expanding in areas where human populations, urbanisation and industrialisation are also increasing rapidly (Pi, Rou et al. 2014; Vaswani 2015). Between 60-70% of food consumption in Asia and >50% in Africa is now from urbanised environments (Reardon, Tschirley et al. 2014). The poultry red mite appears to have a worldwide distribution including Europe, the USA, China and Japan (Chauve 1998; Mullen and O'Connor 2009; Sparagano, Pavlicevic et al. 2009; Wang, Wang et al. 2010). Infestation rates are variable between seasons and farming systems used, however 58% of poultry units in England (Fiddes, Le Gresley et al. 2005), 64% of farms in China (Wang, Wang et al. 2010) and 33-67% of farms in Sweden (Höglund, Nordenfors et al. 1995) were reported to be infested by PRM. PRM is seen as the predominant mite species within poultry houses in most countries, though it is reportedly less numerous than the Northern fowl mite*, Ornithonyssus sylviarum*, within the United States (Wales, Carrique Mas et al. 2010). The importance of PRM in currently emerging markets such as India and Africa is not well documented, though PRM presence has been reported in several African countries (Mungube, Bauni et al. 2008; Sahibi, Sparagano et al. 2008).

1.2.2 Habitat and ecological limitations

PRM spend the majority of their time 'off host', living in dark cracks and crevices in walls and nest boxes (Chauve 1998). PRM thrive in high humidity (70-90%) and warm temperatures (~25°C), however they are unable to survive outside temperatures of -25°C to 45°C and humidities below 20% (Nordenfors, Hoglund et al. 1999). This preference for dark, moist places makes acaricidal spraying against PRM time consuming to spray every creviced area. Even after

spraying, there are reports of PRM repopulating hen houses quickly, starting from hidden mites. As explained by Höglund, Nordenfors et al. (1995), mite infestations are further exacerbated in 'low-density' free-range farming systems compared to 'high-density' caged farming systems. Conventional metal cage systems offer fewer refugia for hiding PRM, including reduction in bird perches/laying boxes and removal of wooden material that presents small cracks ideal for mite habitation. Such cages were heavily used in the European Union until their removal in 2013 due to changes in animal welfare laws (Appleby 2003; Sparagano, Pavlicevic et al. 2009). Bigger, more open free range and barn systems offer increased refugia for PRM habitation and many authors have reported increases of PRM in such free-range systems (Höglund, Nordenfors et al. 1995; Guy, Khajavi et al. 2004; Fiddes, Le Gresley et al. 2005). For the immediate future therefore, it would appear developments in farming infrastructure within the European Union will contribute to increasing PRM numbers in Europe.

1.2.3 Transmission and population dynamics

PRM primarily parasitize commercial layer hens, though are also reported to infest >30 species of wild bird populations (Kristofík, Masan et al. 1996; Brannstrom, Morrison et al. 2008). Genetic analysis of PRM populations in Brazil indicated multiple introduction events of PRM to Brazil over several years, suggesting migrating wild bird populations may have played a role in transmission (Roy and Buronfosse 2011). Genetic variation between PRM of domesticated birds and wild birds in Europe however suggested that European wild bird populations are of low importance as PRM natural reservoirs (Brannstrom, Morrison et al. 2008). PRM transmission between laying farms in Europe is thus thought to be predominantly through synanthropic factors such as the exchange of contaminated material or infested birds between facilities (Oines and Brannstrom 2011).

PRM have been reported to infest cats, dogs, rodents, horses and humans (Brockis 1980) though it is unknown if PRM can sustain a full life cycle through non-avian hosts. Pathology in humans is most common in poultry farm workers, usually as pruritus and dermatitis of the hands and arms (Rosen, Yeruham et al. 2002; Cafiero, Galante et al. 2011). PRM infestations of humans have been reported in public places due to contact with parasitized bird species, however infestations have never been reported without a definitive avian host population nearby (Auger, Nantel et al. 1979; Lucky, Sayers et al. 2001; Cafiero, Camarda et al. 2008).

1.2.4 Bird pathology and economic effect

Pathology due to PRM in parasitized birds is variable depending on infestation rates. Continual biting from mites causes behavioural symptoms such as increased self-preening, lack of sleep and increased uptake of water or food (Kilpinen, Roepstorff et al. 2005). In extreme conditions, behavioural traits can include amplified bird aggression and even cannibalism, both promoted by increased bird stress. Physiologically, severe mite infestations of 500,000 mites per bird can lead to a decline in health through anaemia. Blood loss reduces bird growth and weight, reduces egg laying and can cause mortality (Chauve, 1998; Kilpinen, et al., 2005). Egg quality is also affected as eggs can be damaged or unsuitable for commercial sale due to shell thinning and spots of blood on the egg (Chauve 1998; Cosoroaba 2001; De Luna, Arkle et al. 2008; Valiente Moro, Thioulouse et al. 2009). It is been estimated that PRM may have an economic impact of over €130,000,000 p.a. within the E.U. (Van Emous 2005), including €3,000,000 p.a. within the U.K. (Sparagano, Pavlicevic et al. 2009) due to product loss and cost of prevention and treatment.

1.2.5 Mite vectorial capacity

The capacity of Acari species to act as vectors of pathogens dangerous to animals and humans appears to be largely understudied and underreported. This is reflected in specific studies of PRM vectorial capacity that often report incomplete findings or require follow up reports.

Pathogens such as *Salmonella gallinarum*, *Listeria monocytogenes* and Newcastle Disease Virus (NDV) have been isolated from PRM (see Table 1.1), however pathogen ability to replicate within PRM has not been demonstrated. Spirochetes are not capable of surviving in blood meals greater than 48 hours, though can be transmitted between chickens via mite feeding (Ciolca, Tanase et al. 1968). PRM-mediated transmission between hens has also been shown directly for *Coxiella burnetii*, fowl poxvirus and eastern equine encephalitis virus (Chamberlain & Sikes, 1955; Shirinov et al., 1972; De Luna et al., 2008; Valiente Moro et al., 2009). *Salmonella gallinae* has been isolated from PRM and transmission of *Salmonella enteritidis* between mite and chicken has been proven. *Salmonella* spp. are important pathogenic agents of food borne diseases worldwide and more research is required to define the absolute vectorial competence of PRM (Valiente Moro, Chauve et al. 2007). As defined by Moro, Chauve et al. (2005), the role of PRM as a vector of such a wide spread and important pathogen should not be underestimated.

Pathogen		Transmission potential	Reference
Bacteria	*Salmonella gallinarum*	Isolated from mites	(Zeman, Stika et al. 1981)
	Salmonella enteritidis	Transmission demonstrated	(Valiente Moro, De Luna et al. 2009)
	Pasteurella multocida	Transmission demonstrated	(Petrov 1974)
	Chlamydia spp.	Isolated from mites	(Circella, Pugliese et al. 2011)
	Borrelia anserina	Unknown	(Hoffmann 1988)
	Erysipelothrix rhusiopathiae	Isolated from mites	(Chirico, Eriksson et al. 2003)
	Listeria monocytogenes	Isolated from mites	(Grebenyuk, Chirov et al. 1972)
	Coxiella burnetii	Transmission demonstrated	(Zemskaya and Pchelkina 1967)
	Escherichia coli	Isolated from mites	(Valiente Moro, De Luna et al. 2009)
	Staphylococcus spp.	Isolated from mites	(Valiente Moro, De Luna et al. 2009)
	Streptomyces spp.	Isolated from mites	(Valiente Moro, De Luna et al. 2009)
	Spirochetes	Transmission demonstrated	(Ciolca, Tanase et al. 1968)
Viruses	Viruses Avian leucosis	Unknown	(Hoffmann 1988)
	Newcastle disease	Isolated from mites	(Arzey 1990)
	Fowl poxvirus	Transmission demonstrated	(Shirinov, Ibragimova et al. 1972)
	St. Louis encephalitis	Transmission not demonstrated	(Chamberlain, Sikes et al. 1957)
	Tick-borne encephalitis	Transmission not demonstrated	(Wegner 1975)
	Eastern equine encephalitis	Transmission demonstrated	(Durden, Linthicum et al. 1993)
	Western equine encephalitis	Transmission demonstrated	(Chamberlain and Sikes 1955)
	Venezuelan equine encephalitis	Transmission demonstrated	(Durden, Linthicum et al. 1992)

Table 1.1: Bacterial and viral pathogens associated with *D. gallinae* vectorial capacity (adapted from Valiente Moro, De Luna et al. (2009) and Sparagano, George et al. (2014)).

1.3 Controlling poultry red mite

Current control of PRM principally includes the use of various classes of acaricides through spraying and/or dust application. Recorded cases of acaricide resistance in mites (Beugnet, Chauve et al. 1997; Fiddes, Le Gresley et al. 2005) as well as cases of acaricidal residues found in commercially sold eggs and meat (Hamscher, Prieß et al. 2003; Marangi, Morelli et al. 2012) demonstrates there is a clear requirement for alternative control techniques. As with other parasitic diseases, the search for reliable alternative control methods is a slow process (Chauve 1998; Willadsen 2006; Vaughan and Kappe 2012). Recent concern in the food industry has focused on the control of *Salmonella* spp. (Malorny, Bhunia et al. 2011) in place of ectoparasites, such as PRM. Salmonella is now however controlled to almost negligible levels in the poultry industry in many, but not all countries (Berge, Briese et al. 2009). Below is a brief outline of how PRM is currently controlled and an overview of the possible alternative controls that may be integrated into future control programmes.

1.3.1 Integrated control strategies and farm management

Control of PRM should integrate multiple control strategies, primarily focusing on good farming management and properly scheduled acaricidal spraying. Previously, such an integrated strategy had been adopted by agricultural policy makers and successfully implemented against crop-infesting mite species (Elliott and Dent 1995; Harrington, George et al. 2011). The European red mite, *Panonychus ulmi*, a major pest of tree fruit, was introduced into North America in the early 1900s and quickly spread (Lienk 1980). Initially, predatory species were used but were seen to have low efficacy, however introduction of sulphur-based fungicides along with predator significantly reduced *P. umli* populations (Flint and Van den Bosch 2012).

Integrated control strategies for PRM may benefit from using a Hazard Analysis and Critical Control Point (HACCP) review as designed by Mul and Koenraadt (2009). This review was

developed for help in identifying and assessing potential risks associated with PRM introduction and spread. High risks associated with the introduction of PRM include workers not changing clothes between farms, removal of cadavers, PRM transport by food/manure belts, PRM transport by moved crates/containers and movement of eggs between sheds. Activities that reduce these risks can be integrated with regular cleaning of equipment using acaricidal sprays/dusts as well as careful inspection of eggs/poultry. PRM can survive without a blood meal for >9 months, much than the typical 2-4 weeks rest period between removal of an aging flock (38-40 weeks old) and introduction of a new flock. Consequently, essential care should be taken before new flocks arrive. Time should be taken to remove litter, spray housing facilities and wash workers' clothes.

1.3.2 Acaricidal sprays

Spraying of acaricidal chemicals is the primary control method for PRM both in industry and in backyard flocks. In recent years over 30 potential compounds have been registered for the control of PRM (Chauve 1998), though only a few are currently licensed within the E.U. (See table 1.2). These products include organophosphates, carbamates, pyrethroids, amitraz and endectocides. Inefficiencies in monitoring and reports of anti-acaricidal resistance in mites have led to use of unlicensed or even banned substances in some areas (Maurer, Perler et al. 2009; Marangi, Morelli et al. 2012).

Country	Approved for use (year of approval)	Not specifically approved, but still widely used	Banned in the European Union since 2007
United Kingdom	Phoxim (2010), abermectin (2012), various pyrethroids (n/a)	Bendiocarb	Fenitrothion, carbaryl, dichlorvos, propoxur
Italy	Phoxim (2010)	Amitraz, permethrin, carbaryl	
France	Phoxim (2010)	-	
The Netherlands	Cyfluthrin (1997), phoxim (2010)	Amitraz, various pyrethroids	
Belgium	Phoxim (2010)	Various carbamates, various pyrethroids, various organophosphates	
Denmark	Phoxim (2010)	Propoxur, dichlorvos	
Germany	Phoxim (2010)	-	
Poland	-	Trichlorfon, dichlorvos	
Greece	Phoxim (2010)	Amitraz, carbaryl, various pyrethroids	
Sweden	Phoxim (2010)	Metrifonate, propoxur, various pyrethroids	

Table 1.2 (adapted from Sparagano, George et al. (2014)): Licensed and banned substances used for control of PRM within the European Union. Phoxim is an organophosphate that inhibits cholinesterase, Cyfluthrin is a pyrethroid affecting neural synapses and Amitraz acts on adrenergic and octopamine receptors in nerve cells.

Genetic resistance is defined as an inherited change in the genome of an organism that confers a selective advantage. PRM resistance to commonly used sprays, including pyrethroid and carbamate, has been reported in many countries within Europe (Beugnet, Chauve et al. 1997; Fiddes, Le Gresley et al. 2005; Meyer-Kühling, Pfister et al. 2007). This included 63% of mite populations in 29 studied farms in England showing rapid signs of population recovery after spraying with pyrethroid (Guy, Khajavi et al. 2004). Resistance to acaricides in PRM has likely been promoted by improper or overuse of single chemical sprays due to lack of education or option of effective alternative controls. Evidence of genetic variation between PRM populations is limited, however sequence differences have been reported for the ITS1 (internal transcribed spacer 1) region of the ribosomal DNA genes (Brannstrom, Morrison et al. 2008) and cytochrome c oxidase subunit 1 of different PRM populations (Oines and Brannstrom 2011).

Application of acaricides with certain active ingredients has also been limited by more stringent regulations and legislations within the E.U. No products have been registered for the use of PRM control in Sweden since 2000 (Chirico and Tauson 2002) and none in Italy between 2007 and 2010 (Marangi, Morelli et al. 2012). Licensing of new sprays may be influenced by reports of chemical residues over the legal limit found in commercially sold eggs and bird meat. In Germany, Hamscher, Prieß et al. (2003) reported carbamate propoxur residues at 101µg/kg in commercially sold eggs, more than double the 50µg/kg legal limit stated as healthy for human consumption in Germany. Carbamate is banned for use as a control within the U.K. In Italy Marangi, Morelli et al. (2012) sampled 45 hens destined for the slaughter house, including 225 samples of fat, liver, muscle, skin and kidney, and found 40% of samples to contain residues of carbaryl and 2% residues of permethrin. Reduction of resistant mite populations and relaxation of stringent policies can be encouraged by education of farmers. Teaching proper use and regulation of acaricidal sprays, such as that suggested by the HACCP

strategy written by Mul and Koenraadt (2009), and rotation of several chemical sprays in a structured programme (Zeman and Zelezny 1985) may reduce build-up of acaricidal resistance.

1.3.3 Desiccant dusts

In many places, such as organic farms or countries that do not allow use of acaricidal sprays, sorptive dusts are used as an alternative to chemical sprays. In general, these dusts are free flowing airborne particles, sized 3-9µm, made either synthetically from silica dust or from naturally formed dematiaceous earth (Maurer and Perler 2006). These materials target the waxy layer of the mite exoskeleton that aids in retention of water inside the mite body. The absorptive nature of the dusts removes this waxy layer rendering mites to be killed by desiccation. As this interaction is chemically neutral and the dust is inert, the likelihood of mite populations forming resistance against the dust is very improbable (Kilpinen and Steenberg 2009). Use of desiccant dust does however have its limitations. Problems with application has led to an increased use of desiccants in a liquid format (Maurer, Perler et al. 2009; Mullens, Soto et al. 2012) and with the addition of other ingredients such as plant oils (e.g. MPoux, Olmix, France). *In vitro* studies have shown a range of desiccant dusts to be highly effective at mite killing over 24 hours (Kilpinen and Steenberg 2009) however high humidity (>85%) in chicken farms appears to largely confine sorptive dust efficacy to localised regions.

1.3.4 Heat and temperature

Poultry red mite do not survive in temperatures outside of -25°C to 45°C (Nordenfors, Hoglund et al. 1999) and high mortality of PRM has been shown at 35°C (Tucci, Prado et al. 2008). Although PRM control by heat has been successful in combination with chemical controls in smaller units, temperature increases in larger units without combination control failed to reduce PRM numbers (Mul, van Niekerk et al. 2009). Cyclic changes in temperature can induce heat stress in birds and even constant but higher temperatures of >30°C have shown to reduce egg laying capacity and increase mortality rates in broiler birds (Mashaly, Hendricks et al.

2004). Whilst increased heating may work in some circumstances, due to inefficiencies in repeat PRM killing, effect on bird welfare and the low economic return for farmers, heat killing is not an effective overall control method for killing PRM.

1.3.5 Biological controls - Predators

Biological control seeks to control pests such as insects and mites via use of other living organisms, predominantly exemplified by predatory or parasitic insects of crop infesting pests. Within the poultry industry the house fly, *Musca domestica*, and other closely related flies, have been implemented as predators to varying success (Axtell 1999; Wales, Carrique Mas et al. 2010). Whilst conceptually facile, introduction of predatory or parasitic species into an enclosed system requires careful consideration. Many species of predatory mite are recognised to have capacity to feed on PRM, including *Androlaelaps casalis*, *Hypoaspis aculeifer*, *Hypoaspis miles*, and *Stratiolaelaps scimitus* (Lesna, Wolfs et al. 2009; Ali, George et al. 2012; Lesna, Sabelis et al. 2012). These species are commercially implemented as control agents against other mite species, such as *Varroa destructor*, though some researchers have voiced negative opinions on use of these predatory mites. Some authors suggest that *A. casalis* may also feed on blood from birds (Pacejka, Santana et al. 1996; Rosen, Yeruham et al. 2002). Some authors express concern that other, non-harmful, mite species may be predated on, or that the activity of predator species may be temperature dependant (Ali, George et al. 2012; Lesna, Sabelis et al. 2012). As highlighted by Axtell (1999), success of biological control integration into a poultry farm varies extensively depending on farming systems used, farm management, predatory and prey habitats and also presence of other species. To simplify matters, encouragement of species already residing within farming units may be a more suitable option for PRM control. The histerid beetle, *Carcinops pumilio*, is often encouraged by farmers, however as Lesna, Sabelis et al. (2012) mention, this beetle resides in manure and as an adult can be up to 2cm long. *C. pumilio* thus already habitats many poultry farms, but has

limited capacity to predate on PRM residing in cracks and crevices. Histerid beetles are currently available commercially as controls against PRM (Biobest, Netherlands), though results of this control have not yet been reported.

1.3.6 Biological controls – Fungi

Fungi have been used to control many species of mites, often via blockage of the respiratory tract and biocidal activity to eggs (Chandler, Davidson et al. 2000; Shaw, Davidson et al. 2002; Shi and Feng 2004). Commercialisation of mycopesticidial controls includes *Hirsutella thompsonii*, used against citrus feeding Eriophyoid mites, and *Beauveria bassiana* used against the two-spotted mite. Fungal control of PRM has to overcome several challenges before reaching commercialisation. It is important other invertebrate species are not affected, so PRM-specific fungi should be used. Fungal dissemination and fungal growth both require specific environmental conditions, such as high humidity, which may not be present in all farms. *In vitro* testing against PRM has shown *Beauveria bassiana*, *Metarhizium anisopliae*, *Trichoderma album* and *Paecilomyces fumosoroseus* to be particularly effective at killing PRM within the first 5-10 days of spraying (Steenberg and Kilpinen 2003; Kaoud 2010; Tavassoli, Allymehr et al. 2011). *In vivo* testing of fungi in chicken houses however has not been followed up, presumably due to difficulty in large scale fungal dissemination.

1.3.7 Biological controls – Endosymbiotic bacteria

Endosymbiotic bacteria reside within the body of many species of mite, profiting from optimal growth conditions in exchange for aiding with essential biological processes such as digestion of blood or nutrient poor meals (Konecka and Olszanowski 2015). Much research has focused on *Wolbachia* due to its unique role in essential functioning of cytoplasmic compatibility and feminisation of several species (Perrot-Minnot, Guo et al. 1996; Bouchon, Rigaud et al. 1998). As yet, *Wolbachia* has not been detected in PRM, however other species including *Candidatus cardinium*. *Rickettsiella sp*, *Schineria sp*. and *Spiroplasma* have been detected (De Luna, Moro

et al. 2009; Valiente Moro, Thioulouse et al. 2009). These species form part of the gut microbiota of PRM and represent potential targets for PRM control. Further study into microbiota population dynamics should be of primary research interest as biodiversity between bacterial populations in PRM varies between farms (Valiente Moro, Thioulouse et al. 2009).

1.3.8 Disrupting mating behaviour using micro-vibrations

Both females and males of various species of insects produce and react to micro-vibrations thought to be involved with mate attraction. Predominantly, this behaviour has been studied in tree and plant parasitizing species, including the American grapevine leafhopper *Scaphoideus titanus* (Mazzoni, Lucchi et al. 2009), the southern green stink bug *Nezara viridula* (de Groot, Čokl et al. 2010), the Asian citrus psyllid, *Diaphorina citri* (Rohde, Paris et al. 2013) and the southern pine beetle *Dendroctonus frontalis* (Aflitto and Hofstetter 2014). Studies have shown conspecific vibration patterns, such as those from competing males (Mazzoni, Lucchi et al. 2009; Rohde, Paris et al. 2013), or heterospecific patterns, such as those from a predator (de Groot, Čokl et al. 2010), can alter male behaviour resulting in reduced mating events. PRM are a colony-developing species and therefore mating may simply be a random process or pheromone-dependent rather than being directed by vibration (De Boer and Dicke 2004; Koenraadt and Dicke 2010). Mite activity is increased when PRM are exposed to substrate-borne microvibrations at 2 kHz (Kilpinen 2005), however this has not been directly related to mating behaviour. Further work into PRM reproductive behaviour and vibration sensing is needed to understand if this could be a potential route for population control.

1.3.9 Use of carbon dioxide / mite traps

PRM initially remain static in the presence of CO_2, although after 2 minutes exposure display higher rates of movement compared to those of unexposed PRM (Kilpinen 2005). This correlates to the behaviour of other haematophagous arthropods, such as mosquitoes and

ticks, where CO_2 induces movement based on evolution of host seeking behaviour. Carbon dioxide has also been considered for control of several species of phytophagous mites that feed on stored crops (White and Jayas 1991; Conyers and Bell 2003). Using levels of 50-60% CO_2 in enclosed storage units reduced mite numbers significantly, presumably by asphyxiation. The use of CO_2 at these levels however is not appropriate for PRM control in farming units due to simultaneous housing of poultry flocks. The use of local CO_2 gradients to attract PRM into the vicinity of already established PRM traps could be an alternative approach. Cardboard traps coated in compounds with acaricidal properties have proved to be a simple but effective control measure in trials in Sweden (Chirico and Tauson 2002) and could be further tested on a larger scale.

1.3.10 Lighting regimes

Mite populations that are openly exposed to hen flocks in illuminated areas are quickly pecked and eaten (James Pritchard, personal observation), which may be why PRM usually inhabit dark enclosed spaces and mostly feed at night time. Use of intermittent light regimes (4 hours dark, 2 hours light) to interrupt this behaviour lowered mite numbers captured in hen houses in Poland (Sokół, Szkamelski et al. 2008). Application of such lighting regimes in the commercial poultry sector may be subject to poultry welfare laws in each country. Poultry houses within the European Union require a statutory, continual eight-hour dark period to allow for bird sleep. It is therefore hard to envision how use of intermittent lighting could be implemented, at least within the European Union. The effect of lighting on mite feeding behaviour may be further limited as PRM are reported to parasitize and feed on birds also in the daytime, though at a lesser rate (Nakamae H, Fujisaki K et al. 1997).

1.3.11 Natural plant extracts

Plant derived essential oils possess repellent and even lethal characteristics towards PRM, with garlic and thyme oils being the most effective (Soon-Il, Jee-Hwan et al. 2004; George, Olatunji

et al. 2010; George, Sparagano et al. 2010). As reviewed by George, Finn et al. (2014), naturally derived essential oils benefit from low mammalian toxicity and short environmental persistence, indicating their potential future use as part of an integrated control strategy (Pritchard, Kuster et al. 2015). Oils also benefit from vapour release toxicity, allowing for penetration of mite habitats where manual spraying may not be effective (Kim, Na et al. 2007). Extraction of the active ingredients within these oils is thought to be a more realistic approach for use in future controls. Current commercially available PRM controls containing natural plant extracts include Red Mite Avian (Bugico S.A., Switzerland), a thyme-based additive for water, Breck-a-Sol (ECOspray, United Kingdom) and MiteStop (Felema, Switzerland), both garlic-based sprays.

1.4 Vaccines

Vaccination can be defined as the introduction of immunogenic, though not pathogenic, foreign agents into a host body to aid development of immunity against a particular agent. Vaccines are generally categorised into several types including live, live attenuated, killed, subunit, DNA vaccines and vectored vaccines. Development of vaccines against parasites in both the human and veterinary medical fields has been slow when compared to the relative success of vaccines against bacterial and viral pathogens (Vercruysse, Knox et al. 2004). Difficulty of *in vitro* culturing of many of the most important human and livestock parasites has historically hindered vaccine research (Lightowlers 1994). The following sections outline the development, advantages, disadvantages and current use of different types of vaccines in the veterinary field. Proteins from PRM discovered in the course of this project could be incorporated into a subunit, vectored or DNA vaccine so these concepts are discussed in more detail, particularly in relation to vaccination of chickens and against ectoparasites such as PRM.

1.4.1 Live and live-attenuated vaccines

Live and live-attenuated vaccines induce immune responses following introduction of a live pathogen in small doses (live) or a live pathogen selected for low pathogenicity (live attenuated). Traditionally attenuation is by serial passage of live pathogens through several generations, selecting for the least pathogenic organism. For example, Swanepoel and Coetzer (2004) carried out 118 serial passages of wild-type Rift valley Fever virus through mouse brain tissue, each time culturing strains which appeared less pathogenic to the mouse tissue. After further tests in hamster kidney cells (WHO/FAO 1983) a live attenuated vaccine for veterinary and human was commercially produced. Whilst attenuated strains of a pathogen offer protection, there is also a small chance genetic mutation can revert an attenuated pathogen strain back to a virulent strain. This was illustrated with a small outbreak of poliomyelitis in Dominican Republic and Haiti during 2000-2001 with 21 confirmed cases of polio occurred due

to administration of a reverted batch of the single oral vaccine (OPV) during 1998-1999 (Kew, Morris-Glasgow et al. 2002) . In the modern era, attenuation is achieved by inducing mutations into genes that code for proteins involved with pathogenesis or pathogen development. For example, a vaccine against *Salmonella typhimurium*, the causative agent of typhoid, targets genes encoding for amino acids containing aromatic rings that are required by the bacteria for growth. *S. typhimurium* cannot multiply in the gut, however remains viable long enough to induce immunity (Hoiseth and Stocker 1981; Tennant and Levine 2015).

1.4.2 Inactivated, subunit & toxoid vaccines

These vaccine types elicit an immune response without the ability to replicate inside the host. Inactivated vaccines, whether completely killed or partially impaired, are whole pathogens that have undergone either heat treatment (e.g. Hepatitis B vaccine (Desmyter, De Groote et al. 1983)), chemical treatment (e.g. Formalin treatment for Rabies vaccine (Kissling and Reese 1963)) or radiation (e.g. γ-irradiated influenza vaccine (Alsharifi and Müllbacher 2010)). These procedures target either protein structure or the breakdown of DNA nucleotide strands leading to the interruption of translation of proteins that are required for pathogen viability and reproduction. Storage and transport of inactivated vaccines does not require a 'cold chain' of low temperatures that live vaccines require to survive (Matthias, Robertson et al. 2007). Exposure of refrigerated (2°C - 8°C) or frozen (<0°C) live vaccine samples to warmer temperatures can reduce vaccine efficacy and lead to vaccine failure, something that may not be discovered until vaccine programme completion (Weir and Hatch 2004).

Subunit vaccines include the protective antigens expressed by a pathogen instead of a whole pathogen itself. Application of specific protective antigens requires previous knowledge of proteins that are protective against a pathogen. Whilst this is possible for well-established pathogens e.g. the tetanus toxoid vaccine (Clare, Rayment et al. 1991), other less well studied pathogens may lack the basic biological knowledge to select a recombinant vaccine target.

Subunit vaccination allows inoculation of a safer, non-replicating vaccine with less chance of adverse reactions occurring due to incorporation of fewer types of proteins than a whole pathogen vaccine. Counter-intuitively this selection of the most immunogenic antigens may actually make a subunit vaccine less immunogenic (Flower and Perrie 2012). Immune reactions are complex, involving many molecules and interactions. It is possible that a subunit vaccine, with only a few immunodominant epitopes, simply does not elicit sufficient immunological molecular reactions. Identification of subunit antigens can be time consuming and expensive, however the advent of bioinformatics and large repositories of genomic and proteomic data may make it possible in the future to identify subunit vaccine candidates quicker (Wizemann, Heinrichs et al. 2001).

Some bacterial diseases are pathogenic due to toxins secreted by bacteria which directly damage host cells and aid in infection (Gill 1982). In the presence of formaldehyde, lysine amino groups in toxin molecules breakdown, bringing change to the toxin structure and toxicity levels – now referred to as toxoid molecules. Toxin structure is preserved sufficiently to keep antigenic determining sites, however lowered toxicity allows toxoid molecules to be used as vaccine candidates. For example, the avian bacterium *Clostridium perfringens* causes pathology through necrotic enteritis using an array of toxins (Al-Sheikhly and Truscott 1977). Vaccination with the toxin NetB, a major virulence factor, showed minor protection in immunized birds against *C. perfringens* in studies (Keyburn, Portela et al. 2013). Addition of whole attenuated bacteria plus Net B displayed higher levels of protection in the same study. This exemplifies a disadvantage of toxoid vaccine development, that toxoids can only be used exclusively if toxins are the sole disease causing molecules. Most toxoid vaccines are combination vaccines with multiple antigens such as the, toxid vaccine developed against porcine *Actinobacillus pleuropneumoniae* using a combination of Apx toxins and transferrin-binding proteins (Van Overbeke, Chiers et al. 2001).

1.4.3 DNA vaccines and Recombinant-vectored

DNA vaccines represent a newer class of vaccine technology, yet to be fully integrated into veterinary medicine (Dhama, Mahendran et al. 2008; Redding and Weiner 2009) . A gene of interest encoding for a vaccine antigen is cloned into a plasmid and introduced into host tissue via electroporation, injection, using a gene gun or a mixture of these three options (Wang, Zhang et al. 2008). Target plasmids are taken up by host cells, transcribed and translated by cellular machinery to express the target protein. DNA vaccines offer advantages over live or subunit vaccines as DNA is stable in various environments and does not require cold conditions for transport. DNA vaccines also profit from their versatility in immunological exposure. As proteins are expressed intracellularly, subsequent infections produce both humoral and cellular mediated immune responses (Aldovini and Young 1991; Millan, Weeratna et al. 1998). Up regulation of cytotoxic T cells, as well as B cell antibodies, define a more effective and complete immune response. Though DNA vaccines are beneficial in many ways, vaccine trials have often shown a DNA vaccine to elicit an overall weak immune response (Gurunathan, Klinman et al. 2000). Furthermore, the need for injection may not be suitable for vaccination in all situations (Luo and Saltzman 2000). Currently there has only been one DNA vaccine commercialised for human use against Japanese encephalitis (Halstead and Thomas 2011) and one vaccine in veterinary use against equine West Nile virus (Davis, Chang et al. 2001). Commercialisation of DNA vaccines in the future is likely to advance towards diseases where current vaccines simply reduce symptoms, such as vaccines against foot and mouth (Redding and Weiner 2009). Alternatively, DNA vaccines could also be implemented where current vaccines are not effective due to variations in serotypes, such as in Porcine reproductive and respiratory syndrome virus (Meng 2000).

Vectored vaccines utilise attenuated pathogens to deliver either DNA or recombinant proteins of another targeted pathogen. This can be either similar to a DNA vaccine, where DNA is introduced into host cells, or the vector itself can act as an expression system. The most

common examples of used vectors are the vaccinia virus, used for many vaccines including Rabies (Brochier, Kieny et al. 1990), turkey herpesvirus used in chickens against Marek's disease (Sharma and Burmester 1982) and chimp adenovirus vectors used in humans (Farina, Gao et al. 2001). Recombinant technology in vector vaccines also has potential to induce vaccination against multiple diseases. Attenuated Newcastle disease virus also incorporating the VP2 protein sequence of Infectious bursal disease virus produced 90% protection rates in trials against both diseases (Huang, Elankumaran et al. 2004). Similar studies in attenuated *Salmonella enteritica* bacteria have protected *against Clostridium perfringens* (Kulkarni, Parreira et al. 2008) and in the *Eimeria tenella* parasite against *Campylobacter jejuni* (Clark, Oakes et al. 2012).

1.4.4 Vaccination of poultry

Vaccines in livestock, including poultry, are predominately aimed at increasing efficiency of meat and egg production. Healthier livestock and reduction in disease leads to higher profits (Hamra 2010). Within the UK, there are currently vaccines licensed and commercially available against 18 major pathogens of chickens including bacteria, viruses and parasites (See Table 1.3).

Pathogen type	Species
Bacteria	*E.coli* (D)
	Erysipelas (D)
	Mycoplasma gallisepticum (D)
	Pasteurella multocida (D)
	Salmonella enteritidis (L/D)
	Salmonella typhimurium (L/D)
Parasites	Coccidiosis (L)
Viruses	Avian encephalomyelitis (L)
	Avian reovirus (D)
	Avian rhinotracheitis (L/D)
	Chick anaemia disease (L)
	Egg drop syndrome 76 (D)
	Infectious bronchitis (L/D)
	Infectious bronchitis variants (L/D)
	Infectious bursal disease (L/D)
	Infectious laryngotracheitis (L)
	Marek's disease (L)
	Newcastle disease (L/D)

Table 1.3: Diseases of poultry for which vaccines are licensed and commercially available within the UK. (L) indicates a live vaccine, (D) indicates dead or inactivated vaccine. Adapted from RUMA (2015).

Such a range of vaccines in the commercial market leaves many decisions for poultry breeders in terms of what is economical to purchase for raising healthy livestock (Marangon and Busani 2007). Within the UK all poultry producers and handlers have a duty of care to raise animals as described by the Animal Welfare Act (2006), written by the Department for Environment, Food & Rural Affairs (DEFRA). This directive strongly recommends the introduction of 'core vaccines' in order to raise healthy poultry. Vaccination against Newcastle disease, caused by avian paramyxovirus (APMV), is highly encouraged. Newcastle disease currently has no treatment and three types of AMPV cause a range of pathology and clinical signs making diagnosis difficult (Miller, Decanini et al. 2010). Vaccination against *Salmonella enteritidis* is also strongly recommended, however due to the potential for transmission to and from humans as well as animals. Vaccination against *S. enteritidis* in the UK currently involves vaccinating two million pullets a month at a cost of £4 million a year (BEIC 2016). The level of research into vaccination against avian influenza has recently been stepped up worldwide in response to a sharp rise in the number of influenza outbreaks in countries including Italy, Netherlands and Canada as well as the Eurasian-African H5N1 epidemic (Capua 2007). There were eleven reported outbreaks from March 2006 – March 2015 within the UK (Siettou and Biosciences 2016) and Avian Influenza is considered a notifiable disease. Marek's disease vaccine, a related turkey strain of the herpes virus (Okazaki, Purchase et al. 1970), is injected to one day old chicks. Vaccination in the UK is usually not recommended, unless Marek's disease is present in a flock, as inoculation of small birds in great numbers by untrained staff can lead to bird damage or death. Though vaccine through injection requires a skilled vaccinator, it has a lower risk of 'missing' inoculation of individual chickens that can occur through other more widespread vaccine delivery techniques. Widespread vaccine distribution through water or feed (e.g. Paracox® against Coccidiosis (Williams, Carlyle et al. 1999)) and spraying directly onto to large flocks (Newcastle disease (Corbanie, Remon et al. 2007) and infectious bronchitis (Bande, Arshad et al. 2015)) is common in poultry vaccination.

1.4.5 Vaccines against ectoparasites

As noted by Willadsen (2006), to consider ectoparasites as an analogous group of organisms gives a false misrepresentation of homogeneity in behaviour and biology. Ectoparasite behaviour, for example, varies considerably in feeding time, habitat and host choice. PRM only transiently live on hosts during feeding though many other mite species, such as *Ornithonyssus sylviarum*, live almost exclusively on hosts throughout their entire life (DeLoach and DeVaney 1981). These differences between species can have a significant impact on the success of parasite control. The scabies mite *Sarcoptes scabiei* lives primarily on its human host, thus controls used for other parasites, such as fogging of environmental areas like carpets, would have little effect on *S. scabiei* numbers. Instead, a combination of direct treatment of infested people with scabicides, and washing of clothes and bedding at high temperatures is recommended (Bornstein, Mörner et al. 2001). TickGARD®, the only successfully commercialised ectoparasite vaccine, was developed against the Ixodid tick species *Rhipicephalus annulatus* and *Rhipicephalus microplus* (Willadsen, McKenna et al. 1988). Its success has been associated with the extensive period of time these ticks take to feed on their cattle host (Willadsen 2006). Long feeding time allows prolonged interaction of the bovine immune system and the tick body. Unlike *Rhipicephalus* species, PRM feeds for only short times, often less than an hour (Maurer, Bieri et al. 1988). Immune molecules targeting proteins expressed during feeding would thus have limited time to function. This includes salivary proteins which have been identified as potential vaccine targets in *Ixodes scapularis* (Xu, Bruno et al. 2005).

The physiology and anatomy between related parasite species can also hugely vary. The gut of *R. microplus* tick is at neutral/slightly acidic 6.3-6.5 (Mendiola, Alonso et al. 1996), allowing survival of antibodies and therefore opportunity to bind target molecules. Digestion occurs mostly intracellularly, therefore there is an absence of high levels of extracellular proteases

which could degrade immune molecules (Willadsen 2006). This is demonstrated by the action of antibodies, raised against the TickGARD vaccine, binding to BM86 midgut proteins in the *R. microplus* gut (Willadsen, McKenna et al. 1988). Other invertebrate ectoparasites, such as the buffalo fly, *Haematobia irritans exigua*, break down all immunoglobulin molecules in a blood meal within 4 hours of digestion. This is due to presence of extracellular proteases which act to presumably digest haemocytes and soluble hemoglobin proteins (Allingham, East et al. 1998). Anterior midgut epithelial cells in PRM ingest host blood cells and become highly swollen and vacuolated, suggesting digestion in PRM may be mainly intracellular (Mathieson and Lehane 2002). pH in PRM gut is unknown, but could be an important consideration in future vaccine studies. The peritrophic membrane, a lamellar structure of chitin surrounding a food bolus, protects some mite species from micro pathogens, as well as compartmentalizing food within the gut. It is present in the flour mite *Acaris siro* (Sobotnik, Alberti et al. 2008), however is absent in other mite species, such as *Macrocheles muscaedomesticae* (Coons 1978). Vaccination of peritrophic membrane extracts from *Lucilia cuprina* into host sheep was reported to reduce feeding parasite larval growth by 50% ($p<0.05$) (East, Fitzgerald et al. 1993). Whether the peritrophic exists or not in PRM and if it would affect function of immune molecules from a blood meal requires further research.

1.4.6 Whole parasite vaccination

Preliminary vaccine studies against ectoparasites often used whole parasite homogenates, comprising of 1000s of potentially immunogenic proteins. This is beneficial as, for example with tick salivary proteins, single immunogenic molecules vary between species making production of a universal vaccine problematic (Nuttall and Labuda 2004). Whole parasite fractions have been studied as vaccines in several species of mites including *P. ovis* (Smith and Pettit 2004), *O. sylviarum* (Minnifield, Carroll et al. 1993), *S. scabiei* (Tarigan and Huntley 2005) and *D. gallinae* (Arkle, Harrington et al. 2008; Wright, Bartley et al. 2009). For all these studies

whole extracts of a mite homogenate, or a partially soluble fraction purified by chromatography, resulted in a reactive serum in western blots, but no significant reduction in mite biting or host pathology. Minnifield, Carroll et al. (1993) showed no significant difference to control groups in survival rates of blood fed *O. sylviarum* mites when fed on vaccinated poultry. Similarly, both Wright, Bartley et al. (2009) and Arkle, Harrington et al. (2008) showed no significant mortality of PRM fed on poultry vaccinated with either whole mite fractions or soluble protein fractions. These examples demonstrate the difficulty and complexity of developing vaccines against ectoparasites, including mites and ticks. In initial trials for each species there has been much expectation placed on whole parasite vaccination, often without any prior in depth knowledge of parasite molecular biology or resultant experimental evidence. As clearly and honestly written by Willadsen (2006):

"*The application of current knowledge across parasite and host species needs to be explored but little has been done. In most cases, the path to commercial delivery is uncertain. Although many constraints and challenges remain, the need for vaccines and our capacity to develop them can only increase."*

1.4.7 Concealed and Exposed antigens

Proteins targeted as vaccination candidates against ectoparasites can be generally divided into two categories; those that are 'exposed' and those that are 'concealed'. Many parasite-expressed proteins are normally exposed to a host's immune system, such as tick salivary proteins during feeding, or are expressed on the outside of the parasite body, e.g. the mite exoskeleton. Other proteins are expressed internally inside the parasite body and thus are typically hidden, or 'concealed', from interacting with the host immune system. Currently, the only successfully commercialised vaccine against an ectoparasite species is the anti-cattle tick 'concealed' antigen vaccine TickGARD® (Willadsen, McKenna et al. 1988). This vaccine uses the midgut membrane protein BM86 of the Cattle tick, *Rhipicephalus microplus*.

Vaccination of animals against hematophagous ectoparasites using exposed and concealed antigens has the same end targets; to increase parasite mortality, reduce egg development/oviposition and reduce engorgement weight. The mode of action of how this is achieved however is different. Vaccination against exposed antigens, such as salivary proteins, aims to produce an anamnestic response in the host rather than creating an initial immune response against a "not seen before" protein (Nuttall, Trimnell et al. 2006). An exposed antigen vaccine boosts an already acquired immune response and primarily aims to reduce the immunomodulatory effects of the exposed antigens. For example, tick species produce a plethora of biomolecules in their saliva that have been shown to reduce T cell proliferation and downregulate Th1 cytokines (Ramachandra and Wikel 1992; Brossard and Wikel 2004). Furthermore, the acquired immunity of many host species has been shown to reduce tick attachment but not tick infestation, even after repeat exposure (Ribeiro 1987). It would therefore be useful to identify the interacting parasite and immune molecules in order to target enhancement of immune reactions.

Successful commercialisation of the TickGARD vaccine against the Cattle tick *R. microplus* (Willadsen, Riding et al. 1989; Willadsen, Bird et al. 1995) has given prospects to recent vaccine research in other parasitic species of veterinary importance using the concealed antigen approach (Manzano-Román, García-Varas et al. 2007; Gao, Luo et al. 2009). Using proteins extracted from the midgut of a haematophagous parasitic organism would, in theory, prove more immunogenic to the host compared to an antigen which had been already exposed to the host immune system. In the case of the TickGARD vaccine, the function of the BM86 midgut protein is still unknown. Its localisation on the surface of microvilli of gut cells and similarity in structural organisation to epidermal growth factor-like domains, suggests maybe BM86 has a role in the regulation of midgut cell growth (Liao, Zhou et al. 2007; Kamau, Skilton et al. 2011). The TickGARD vaccine mechanism is not fully understood either (Willadsen 2004). Tellam, Smith et al. (1992) summarised TickGARD mechanism as a 'resultant, weak midgut

structural integrity and a dependence on antibody presence'. Non-humoral immune molecules are shown to either play a secondary 'boosting' role in tick mortality, such as the complement system, or appear to have no effect at all, such as host leucocytes (Kemp, Pearson et al. 1989). It appears damage to the midgut does not, in the majority of cases, kill the adult tick. Rather TickGARD hinders development of eggs, perhaps through reduced nutrient uptake in engorged females. This is supported by the low mortality of feeding ticks on vaccinated cattle, typically <50% when compared to control groups (Willadsen 2004).

Concealed antigens have also been used in vaccination studies against the ruminant nematode endoparasite *Haemonchus contortus*. H11 is an integral glycoprotein expressed in the gut of *H. contortus*. Vaccination studies with H11 antigen have shown 99.8% reduction in faecal egg counts and up to 95% reduction in worm burden in sheep post mortem (Munn, Smith et al. 1997). Another group of membrane gut proteins, known as the *Haemonchus* galactose-containing glycoprotein complex (H-gal-GP), also show protective immunity both as a single antigens and in combination with H11 (LeJambre, Windon et al. 2008).

Some vaccine candidates have been suggested to be 'dual action' vaccines, combining both the natural immunity raised against exposed antigens, as well as the more protective immunity induced by concealed antigens. Trimnell, Hails et al. (2002) studied 64P, a putative tick biting cement protein expressed in *Rhipicephalus appendicalatus*. They reported that vaccination of tick-naïve guinea pigs, using a truncated form of the recombinant protein, stimulated significant protection without boosting. Vaccination resulted in nymph mortality of 48%, adult mortality of 70% and increased antibody titers with each tick infestation. Antibodies also reacted to tick gut and salivary tissue. These results led the authors to suggest 64P as a 'dual action' vaccine candidate. Follow up studies also revealed 64P to be cross protective against other tick species (Trimnell, Davies et al. 2005). No further publications have been published

regarding 64P, perhaps due to the presence of the already successful TickGARD® and TickGARD plus® vaccines.

1.4.8 A Vaccine against PRM

Several attempts have been made to develop a vaccine against PRM, using a variety of methods (see Table 1.4). Vaccine trials using *in vitro* feeding assays and *in vivo* bird feeding have produced varied but ultimately limited success. Initial vaccine research carried out by the Sparagano group in Newcastle (Arkle, Harrington et al. 2008), using whole mite homogenate inoculated into birds, produced no significant difference in mite mortality to that in mites fed on control adjuvant-only immunised birds. Subsequent fractionation of mite homogenate via application of detergent and ultra-filtration gave a much higher mite mortality compared to controls (Harrington, Din et al. 2009), though to a lower number of mites (n = 20). Fractionation of mite homogenate was implemented by the Nisbet group in Moredun, Edinburgh (Wright, Bartley et al. 2009) using detergent and ultracentrifugation. Again, no significant change in mite mortality after vaccination was seen when compared to control groups. This study was recently followed up (Bartley, Wright et al. 2015) by further fractionation of the soluble fraction of the PRM mixture by anion exchange chromatography, two-dimensional immunoblotting and immunoaffinity chromatography. Subsequent recombinant expression and vaccination of serpin, vitellogenin and hemelipoglycoprotein found these proteins increased mite mortality rates by 1.7-2.8 fold. Feeding trails were conducted using *in vitro* feeding assays (10 tube assays with total 326 mites) using heparinised blood taken from vaccinated chickens. Commercial success using these proteins is perhaps achievable however will require further evaluation in trials that are both *in vivo* (i.e. chickens – not tube assays) and with larger numbers of mites and birds. Other approaches have used inoculation of birds with recombinant proteins. Trials using homologous recombinant proteins from other ectoparasitic species (Harrington, Canales et al. 2009) and highly expressed

proteins of PRM during feeding (Bartley, Huntley et al. 2012), both induced small but statistically significant increases in mite mortality.

As with vaccine research in other complex organisms, purification of a single or small range of specifically immunogenic PRM target proteins is difficult. As noted by Wright, Bartley et al. (2009), a simple fractionation of proteins based on size or properties, such as hydrophobicity, still produces mixtures of 1,000s of target proteins. To overcome problems in complexity, single proteins can be expressed as recombinant proteins, such as Histamine Release Factor (Bartley, Nisbet et al. 2009) and Subolesin (Harrington, Canales et al. 2009).

Vaccine target	Immunoglobulin Response	% Mite Mortality (control mortality)	Difference to control mortality/ significant?	Mechanism of Identification	Reference
BM86	Increased IgY but not recognised on w/blot	41% (18%)	23% - not significant	Orthologue from ticks	(Harrington, Canales et al. 2009)
Subolesin	No IgY increase - recognised on W/blot	54% (18%)	36% - significant	Orthologue from ticks	(Harrington, Canales et al. 2009)
Soluble antigen mix (identified 3 proteins: tropomyosin/actin/myosin orthologues)	IgY increase. IgM constant	69% (18%)	51% - significant	Total mite protein injection	(Harrington, Din et al. 2009)
Proteolytic enzymes - Cathepsin Proteases (Cat L / Cat D) tested separately	IgY produced but also seen without injection for Cat L (naturally exposed)	Cat L - 18% / Cat D - 31% (11%)	Cat D - x4.42 more likely to die (significant) / Cat L - x2.08 (not significant)	Suppression subtractive hybridisation	(Bartley, Huntley et al. 2012)
Histamine Release Factor	N/A - monoclonal IgY produced	9% (3%)	6% - significant	Predicted from cDNA library	(Bartley, Nisbet et al. 2009)
PBS soluble protein fraction (unknown identity)	IgY produced	35% (25%)	10% - not significant	Fractionation	(Wright, Bartley et al. 2009)
PBS fraction further analysed (identified serpin, vitellogenin and hemelipoglycoprotein	IgY produced		x1.7-2.8 more likely to die	anion exchange chromatography	(Bartley, Wright et al. 2015)

Table 1.4: The various vaccine targets and methods used to date in vaccination experiments against the poultry red mite. Vaccine success is based upon the production of IgY in eggs and blood of vaccinated hens that reacts in western blots to mite homogenate. As well, a significant increase in mortality rates in mites feeding on vaccinated hens compared to non-vaccinated hens (i.e. the control groups) is a measure of vaccine success.

Future targets for PRM are likely to come from one of three methods commonly used to identify vaccine candidates. Firstly, identification via immunological response, such as the production of antibodies that interfere with the mite life cycle, is one possible way. This method however may cause confusion, as the production of antibodies is not always homologous to level of protection. This was shown by Bartley, Huntley et al. (2012) when IgY antibodies reactive on western blots against PRM Cathepsin D were found to exert only a minor effect on mite mortality. A second method involves targeting proteins that are vital for mite survival and function, which has worked well in development of acaricidal sprays, often by targeting neural receptors or mitochondrial respiration (Dekeyser 2005). This method however requires in depth knowledge of parasite biology in order to pre-select target proteins and is unlikely to bear fruit until more knowledge of PRM biology is available. Thirdly, PRM targets may be identified by stepwise fractionation, vaccination and challenge trials of progressively less complex mixtures of immunogenic proteins. This method was used to develop the TickGARD vaccine and several immunogenic candidates against *H. contortus*, however from initial experiments vaccinating with whole tick homogenate, to the purification of BM86 protein, took the Willadsen group almost two decades (Willadsen 2004). Most likely, a combination of these three approaches will be needed for a successful PRM vaccine. Combining knowledge of the biology of related tick and mite species, as well as generating PRM specific gene databases can aid in identification and prediction of protein expression and interactions. Much research has been carried out on the exoskeleton and salivary proteins of the dust mite (*Dermatophagoides* sp.) due to its role in human allergy (Van Bronswijk and Sinha 1971). Similarly, genetics of the reproductive traits of *Varroa destructor* are well studied due to the ability of the mite to colonise bee species at different levels of parasitism (Calderón, Van Veen et al. 2010). All recent PRM vaccine studies have used mite mortality and egg development as a measure of vaccine success. Whilst it is tempting to correlate production of IgY antibodies with protection, changes in mite mortality and egg counts are far more reliable

assays for measurement of vaccine success. The development of *in vitro* feeding mechanisms for PRM and established protocols for *in vivo* challenge trails in other mite species provide a foundation for the future testing of vaccine candidates.

1.5 Avian immunity and analysis of an antibody response induced by a PRM vaccine

The avian immune system refers to a system of biological structures, molecules and processes within a bird that recognises and elicits a defensive response against foreign, 'non-self' molecules in order to protect against disease. An immunological response can be functionally separated into two systems, the innate immune response and the adaptive immune response. Both systems overlap in many aspects, although in general innate responses are non-specific and immediate, whilst adaptive responses are highly specific and take longer (Davison, Kaspers et al. 2011). Poultry red mites are blood-feeding ectoparasites that can only be affected by chicken immune components found in an ingested blood meal. Vaccination against PRM aims to develop acquired immunity in the host through B cells and antibody production as demonstrated by analysis of IgY molecules in several PRM vaccine trials (Harrington, Din et al. 2009; Bartley, Huntley et al. 2012). Whilst innate immunity in birds is important for recognition of many other pathogens (Bar-Shira and Friedman 2006), only adaptive immunity will be discussed further to keep this chapter relevant to PRM control. Note is made however, that original inoculation of a vaccine activates antigenic presentation of pathogenic associated proteins by dendritic cells and macrophages that are part of the innate system. B cell development relies on such presentation, demonstrating one of the many complex interactions between innate and adaptive systems (Hoebe, Janssen et al. 2004). Similarly, cell-mediated cytotoxicity is an important aspect of adaptive immunity in birds, however is specialised in elimination of intracellular pathogens (Erf 2004) and thus will not be discussed in detail further. Vaccination of PRM antigens is likely to be through either recombinant subunit

or vectored vaccine as this has been successful in Cattle tick control (Willadsen, Riding et al. 1989).

1.5.1 Unique features of the avian immune system

Birds and mammals share a common ancestral lineage and are thought to have diverged no more than 350 million years ago (Hedges, Parker et al. 1996). Both share many common immunological features such as the creation of innate and adaptive immune responses; however, the avian immune system does include some unique immunological tissues and defence strategies. Principal unique features of avian immunity include the development of the Bursa of Fabricius, mechanisms of B cell differentiation and selection (McCormack, Tjoelker et al. 1991), generation of IgY antibodies and the generation of the B complex (Sayegh, Demaries et al. 2000). The bursa of Fabricius is a lymphoid organ required for the development of B cells. A similar organ does not exist in mammals as mammalian B cell development only occurs in the bone marrow. Also of note, the generation of IgY antibodies is unique to birds. IgY are passed down from mother to egg as part of maternal immunity (see '1.5.3 Avian immunoglobulins') . Finally, B cell differentiation is unique in birds, as avian B cells only include one gene region encoding for the variable light chain and three gene regions encoding for the variable heavy chain. To overcome the limitation of genetic variability associated with such comparatively small sequences, avian hypervariable gene regions are upregulated or downregulated in conjunction with other 'pseudogene' regions downstream of the variable region, in a process known as 'somatic recombination' (Reynaud, Anquez et al. 1985; Thompson and Neiman 1987) (see '1.5.2 B cells and antibody production').

1.5.2 B cells and antibody production

Cells of the lymph system, or lymphocytes, are categorised into either natural killer cells, T cells or B cells based on cell function, protein expression and origin of cell maturity. All lymphocytes originate from haematopoietic stem cells, located in the avian aorta in early

stages of chick development (Bertrand, Chi et al. 2010). Undifferentiated haematopoietic cells travel in the blood stream and, amongst other sites, can colonise the thymus (eventually becoming T cells) or the bursa of fabricius (eventually becoming B cells). Unlike T cells, B cell progenitors are not located in the bone marrow in later developmental stages. More specifically, colonisation of the bursa by B cell progenitors occurs by a single wave of cells during embryonic development (Reynaud, Imhof et al. 1992). B cell progenitors are thought to either lack the Notch 1 gene that T cell progenitors express or if Notch 1 is expressed it is involved with B cell arrest and apoptosis (Morimura, Goitsuka et al. 2000). B cells contain genetically variable regions for the light chains and heavy chains of the immunoglobulin molecules they produce. Unlike rodent and mammalian B cells, that have several families of genes encoding these regions, avian B cells only have one gene region encoding the light chain and three regions encoding the heavy chain (Reynaud, Bertocci et al. 1994). Functionally, this means genetic recombination of hyper variable regions in avian B cells is limited. Avian B cell immunoglobulins can however undergo somatic gene conversion with one of 25 non-functional, so-called 'pseudo' variable genes that are located upstream of the functional variable region (Ratcliffe 2006). This secondary genetic substitution event adds more variation to Ig gene sequences in the B cell, thus allowing avian B cells to have a genetic repertoire with amounts of variability similar to that of mammals (McCormack, Tjoelker et al. 1991). Progenitor B cells that fail to produce viable cell surface immunoglobulins, due to failed genetic recombination, do not migrate to the bursa and are eliminated by immune cells. Similar to T cells, B cell progenitors undergo a positive and negative selection stage, requiring binding to self-MHC and foreign antigens. B cells that do not reach this requirement do not reach full maturity and undergo apoptosis (Niiro and Clark 2002).

Mature B cells migrate to secondary lymphoid organs such as the spleen and lymph nodes in the digestive tract. Here, B cells interact with either free-floating antigens or antigens presented by dendritic cells/macrophages. These antigens are engulfed, intracellularly

processed and presented on MHC class II receptors on the B cell surface. T helper cells previously activated by the same antigen bind to the B cell MHC class II via the T cell receptor and promote B cell proliferation via the cell surface receptor CD40 and cytokines IL-4 and IL-21 (Crotty 2015). Activation also induces class switching, a process of genetic deletion and recombination of the constant region of the immunoglobulin. Here, a chicken can produce classes of immunoglobulin IgY, IgM or IgA – differentiated by their structure (Leslie and Clem 1969).

Other mechanisms for B cell activation are also possible. T-cell independent activation can occur through B cell binding to commonly expressed microbial toll like receptors on infected cells. B cell proliferation is quicker by this mechanism; however due to lesser specificity to antigen binding, antibodies generated by binding of toll like receptors tend to have a lower affinity and therefore reduced functionality (Liu, Zhang et al. 1991). Some activated B cells undergo differentiation into memory B cells, rather than into antibody producing plasma cells. Memory B cells function to create an accelerated antibody-mediated immune response in case of re-infection. Rapid cloning of memory B cells due to antigen binding during reinfection, known as clonal expansion, allows replication of many numbers of B cells from an initial few memory B cells (Masteller, Pharr et al. 1997). High rates of mutation during cloning result in some clones having a higher affinity in the B cell receptor to the invading pathogen. Thus, secondary immune responses often have a larger and quicker reacting antibody response due to increased specificity to the re-invading pathogen species (Janeway, Travers et al. 1997).

1.5.3 Avian immunoglobulins

Unlike mammalian B cells that produce five classes of antibody, avian B cells produce IgY, IgM or IgA but not an equivalent to IgD or IgE. Immunoglobulins are generally made up of a two heavy chain and two light chain peptide chains, connected by a disulphide bridge (see Figure 1.4). Each chain contains a constant region, which is similar in all antibodies, and a variable

region, which differs between individual antibodies. Variable chains contain three hypervariable regions, known as complementary determining regions, which express the antigen binding part of the antibody.

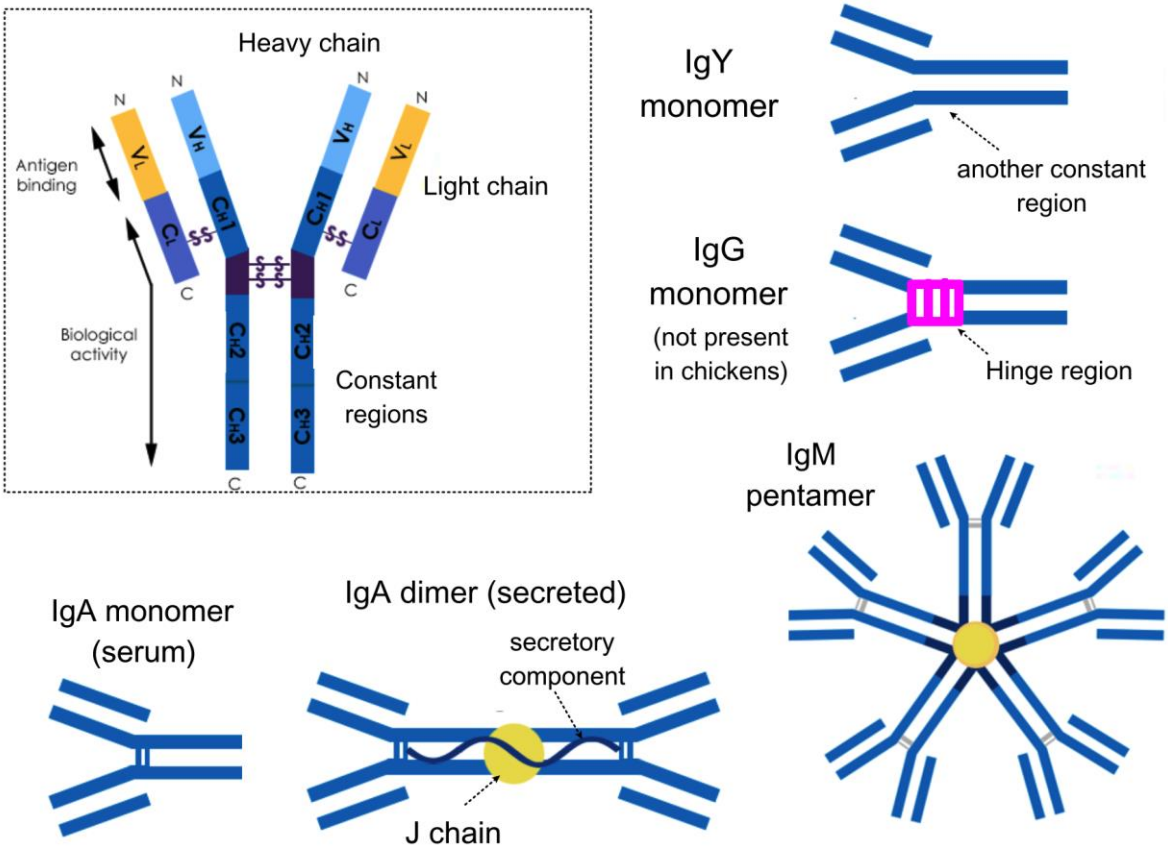


Figure 1.4: Structures of antibody classes found in avian immunity. 'General' antibody monomer structure is shown in detail (top left) indicating the heavy and light chains of the variable region as well as the constant regions. IgY monomer structure containing 4 constant regions is compared to the non-avian IgG monomer that instead contains 3 constant regions and a hinge region allowing molecular flexibility. IgM is a pentamer, whereas IgA is present as a monomer in serum or as a dimeric structure when secreted, incorporating a joining chain (J-chain) and a 'secretory component' polypeptide, which aids with transcellular transport and secretion of the immunoglobulin. Adapted from Abcam (2016).

Antibody classes vary in structure, depending on the number of constant regions in the heavy chain domain. Immunoglobulins can be either monomeric, dimeric or a polymer and can be soluble or membrane bound. IgM is typically found as a soluble pentamer, joined at the constant region by a small 'joining' peptide linker. IgM is the first immunoglobulin to evolve in embryonic development, detectable in the bursal tissue after 14 days. IgM is multivalent, containing 10 antigen binding sites, and so is an efficient tool in agglutination tests (Tizard 2002). IgA is expressed later at 18 days into development in the bursa. Structurally, IgA typically forms a dimer, connected by a joining chain at the constant region. IgA is classically found in bird external secretions including the intestinal glands, oviducts, and ocular glands. IgA is transported through epithelial cells in mucosal sites into external secretions by cells expressing a polymeric Ig receptor. Dimeric IgA is linked by disulphide bonds and surrounded by a carbohydrate-rich secretory component that protects the molecule form proteolytic enzymes found, for example, in the gut lumen (Lillehoj and Trout 1996). IgA is also found in the blood of chickens as both monomeric and polymeric molecules. IgA in blood occurs at levels of 0.33mg/ml without infection, representing less than 4% of total immunoglobulins (Lebacq-Verheyden, Vaerman et al. 1974). Of this, less than 20% are monomeric whilst most polymer molecules are trimeric or above.

IgY is not found in mammal immune systems, but rather in bird, fish, amphibian and reptile immune systems. Previously, IgG and IgY were used interchangeably in literature, however this is no longer regarded as appropriate. IgY molecules are structurally and functionally distinct from mammalian IgG molecules and do not cross react with antibodies raised against IgG molecules (Ohta, Hamako et al. 1991). IgY molecules have one more constant chain compared to IgG molecules and no linking peptide between constant regions (see Figure 1.4). Three isoforms of IgY have been discovered, including a truncated form in ducks that lacks the Fc

region for complement activation (Lundqvist, Middleton et al. 2006). This is thought to be why ducks do not display pathology of avian influenza as much as other bird species, as viruses are unable to infect macrophages through IgY binding (Magor 2011). IgY is found in high concentrations both in circulating blood and also in egg yolk. IgY is passed on from mother to chick as part of maternally-derived immunity. This has been exploited in the biotechnology industry as a non-invasive way of producing immunoglobulins, rather than extracting blood from vaccinated animals.

1.5.4 An antibody response to damage PRM and testing of vaccine efficacy

The success of the TickGARD vaccine through antibody production and targeting of midgut protein BM86 is due to antibody binding to epithelial cells. Sera reacting against the BM86 antigen binds the surface of tick gut cells and the digestive endocytotic activity of these cells is strongly inhibited (Willadsen, McKenna et al. 1988). Lack of blood digestion leads to reduction of tick engorgement weight and capacity of survived ticks to lay viable eggs. Vaccine targets in the PRM gut may act in a similar fashion if proteins exposed to the blood meal are targeted. Vitellogenesis, the process of egg formation from nutrients, is an example candidate pathway from digestion to egg production in PRM that can be exploited through vaccination (Bartley, Wright et al. 2015). In various vaccine studies against PRM mite weight, egg laying ability, feeding success and mortality are all monitored to determine target vaccine antigen success (Wright, Bartley et al. 2009; Wright, Nisbet et al. 2011; Bartley, Huntley et al. 2012). Detrimental effect of poultry vaccination against red mite can therefore be measured either individually or at a population level, through a static mite population or over multiple generations. Studies have shown PRM feeding on blood containing antibodies against histamine release factor increased mite mortality over a 5 day period from 2.74% to 9.24% (Bartley, Nisbet et al. 2009). Other studies vaccinating with whole mite protein samples, or soluble fractions, analysed mortality and oviposition over 2 weeks, allowing for monitoring of

change in mite egg laying (Arkle, Harrington et al. 2008). Similar testing was used when testing vaccination of BM86 in Cattle ticks (Willadsen, Riding et al. 1989) and Subolesin in Deer ticks (Almazan, Kocan et al. 2005), that showed reduced rates of oviposition by 35% and 45% respectively over time. Though these values were acceptable for small trials in *in vitro* studies, larger studies in commercial farming settings would aim to monitor mite numbers, poultry health and weight, as well as the number of eggs produced. Average layer hen weight is 1.1-1.3kg in a healthy adult hen, and a typical layer hen can produce 300 eggs per year depending on species and type of farming system used. Mite numbers could be detected using simple sticky traps or corrugated cardboard, such as the traps used by (Nordenfors and Chirico 2001).

1.5.6 In vitro feeding assays

Success of vaccines against PRM have largely been based on *in vitro* feeding assays containing small numbers of mites feeding on blood mixed with IgY specific to the vaccine candidate protein. Vaccinated chickens were subjected to the target protein and IgY was extracted from their eggs. Though in some cases this was mixed with blood of vaccinated rabbits (Bartley, Nisbet et al. 2009), most studies have solely relied on the efficacy of IgY preparations. It is likely however; these 'IgY only' blood meals are missing factors required for efficient induction of an immune response and therefore may not be a true reflection of a 'natural' immune reaction. Other problems faced with *in vitro* feeding assays include low numbers of successfully fed mites. Assays designed by Bruneau, Dernburg et al. (2001), Harrington, Canales et al. (2009) and (Wright, Bartley et al. 2009) vary slightly but fundamentally involve a small tube/chamber containing starved mites exposed to a skin/membrane and a blood reservoir above (see Figure 1.5). Red mite are believed to be attracted to the surface skin lipids from alive chicken hosts (Zeman 1988) and low feeding numbers are reported when PRM are fed through artificial membranes lacking the natural biological attraction. More recent studies (Bartley, Nisbet et al. 2009; Wright, Bartley et al. 2009) have used 1 day old chick skin stored

from 4°C in ethanol which provides better, but still limited, feeding of below 50% of mites even after multiple rounds. Harrington, Guy et al. (2010) showed similar feeding rates using the synthetic membrane Nescofilm® (Nippon Shoji Kaisha Ltd, Osaka, Japan) combined with a skin extract (32.3%) when compared to using 1 day old chick skin (38.8%).

Many assays have limited space for mites, ranging from 20-200 mites per study, and often with large variability in feeding rates between experimental repeats (Harrington, Din et al. 2009; Wright, Bartley et al. 2009). Bruneau, Dernburg et al. (2001) suggested creation of mite colonies adapted to membrane feeding after high mortality in first round feeding would produce higher feeding rates. *In vitro* colonies of PRM have however yet to be established for a long period in a laboratory.

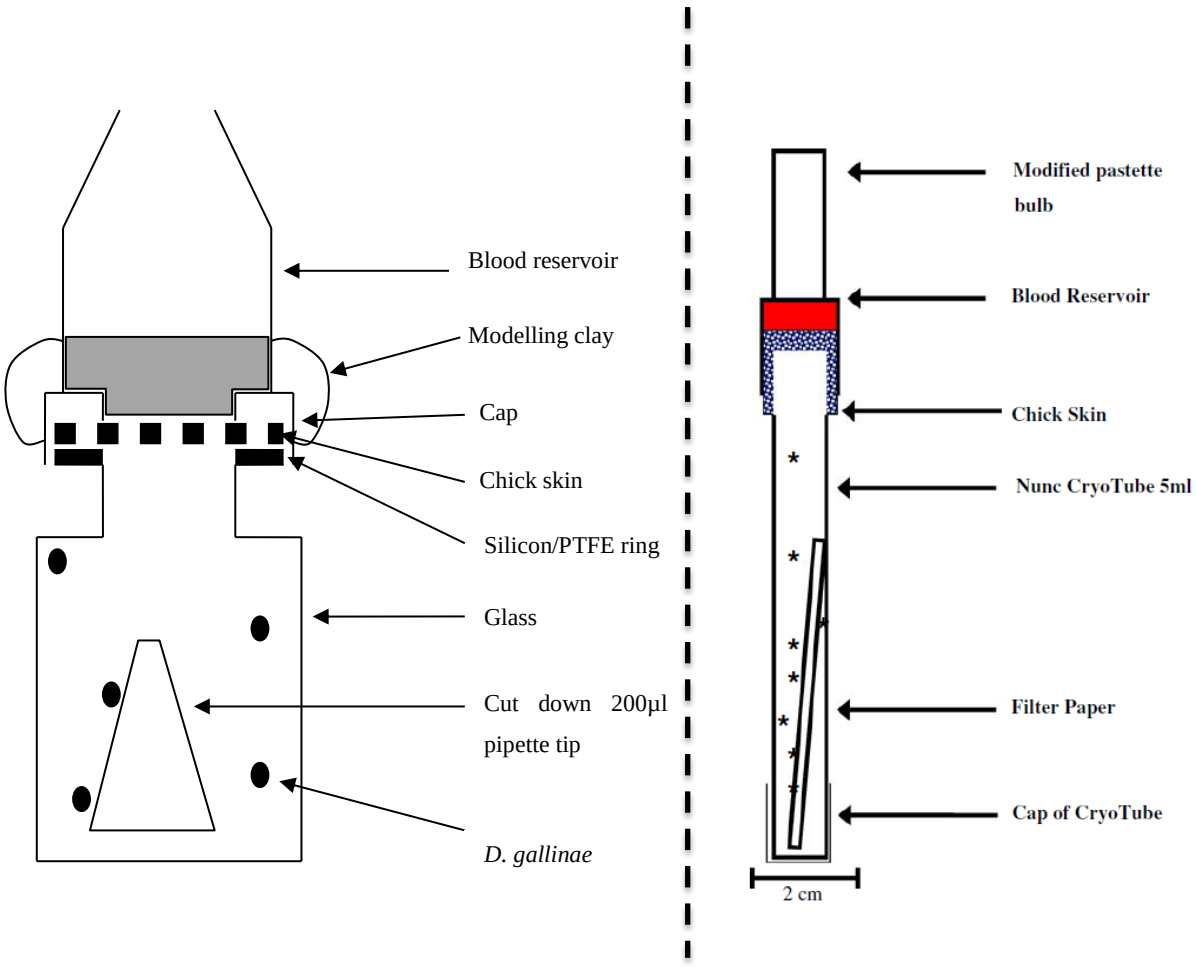


Figure 1.5 *In vitro* feeding assays for PRM using a blood reservoir. Assays designed by Harrington, Robinson et al. (2010) (left) and Wright, Bartley et al. (2009) (right). Mites are enclosed in a tube and exposed to fresh blood from a vaccinated chicken through the skin of a one day old chick. Blood reservoirs are held in place by a pipette tip and chick skin is tightened by addition of a PTFE ring. PRM are transported in and out of the tube by being placed on either a pipette tip or filter paper.

1.5.7 In vivo feeding

To date, very few *in vivo* feeding experiments have been carried out in PRM research due to mite small size, ability to escape and the logistics of mite collection and handling. Maintaining colonies of PRM by their feeding on bird hosts is possible (Tucci 1997) however this requires complex protocols for mite handling and bird welfare. As shown by Kilpinen, Roepstorff et al. (2005), poultry exposed to increasing mite numbers in a laboratory setting show increased feather pecking, grooming, generally reduced welfare, reduced packed cell volume (PVC) of red blood cells and death by anaemia. Such pathology would have to be controlled and test birds would be monitored closely. PRM are cosmopolitan in their host choice, however to our knowledge, no work has been carried out on alternative hosts. Reports of PRM infestations in both wild (Gaaboub, Donia et al. 1982) and domesticated rodent populations (Lucky, Sayers et al. 2001) suggests mice or rats could be used as experimental animals. Caution should however be taken with such reports, as there is no direct evidence that secondary hosts, such as rodents, can support a PRM population over several generations. Furthermore, though PRM could potentially be tolerated by such species, consideration would be required to identify how to feed and collect mites from cages and animals containing fur or hair.

1.6 Summary and conclusions

The poultry red mite (PRM) is an economically important ectoparasite of egg laying hens. Current controls are not completely effective in PRM removal and cases of acaricidal resistance suggest there is a need for alternative controls. Commercial success of the 'concealed protein' vaccine approach in the cattle tick, *R. microplus*, may also be possible in PRM. In particular, target proteins must be expressed in the digestive tract and have an opportunity to bind antibodies. Earlier studies investigating soluble proteins in PRM midgut, such as proteolytic enzymes, showed limited success (Bartley, Huntley et al. 2012). For this reason, membrane proteins of gut epithelial cells may be seen as an alternative vaccine target, especially as the TickGARD antigen BM86 is a gut membrane protein. The following chapters include details of experimental design, methodology, results and discussion of results from a project aimed at targeting immunogenic, membrane proteins expressed specifically in the gut of PRM. Overarching project aims and the corresponding chapters are discussed below:

1. **To break up live PRM into a sub-cellular homogenate that can be further enriched for membrane proteins.**

To analyse single proteins as vaccine candidates, whole mites must be broken up into a sub-cellular homogenate from which proteins can be extracted. Homogenisation protocols differ, involving various freezing temperatures to kill mites and exploit various cell characteristics and homogenisation tools to lyse tissue to a sub-cellular state. To enrich this complex mixture for membrane proteins various types of centrifugation and detergent can be employed by separating proteins based on hydrophobicity, density, size and cellular location. Chapter 3 describes such methods of homogenisation and membrane protein enrichment that were applied and tested during the project.

2. **To select a library of single chain variable fragments (scFv) that bind to immunogenic PRM proteins and can be further used in investigation of target proteins.**

Vaccine target proteins must interact with antibodies in the blood of the chicken during vaccination as well as in the PRM gut during feeding. Proof of protein binding to an antibody library *in vitro* provides evidence that selected proteins are potentially immunogenic before expensive and time consuming vaccine trials occur. Biopanning can be used to isolate a library of single chain variable fragments (scFv) that specifically bind immunogenic PRM proteins and can then be utilised in downstream experiments to isolate such target proteins. Chapter 4 details the introduction, method, results and discussion of implementing biopanning of a single chain variable fragment (scFv)/phage library (which is analogous to an antibody library) against red mite proteins. An end library of PRM binding scFv/phage would be used in later chapters to investigate vaccine targets further.

3. **Incubation of individual scFv/phage with mite tissue sections in immunohistochemistry to identify which scFv/phage bind specifically to proteins expressed in the PRM gut.**

Vaccine target proteins should be expressed in the gut of the PRM in order to interact with antibodies in the blood meal of the mite. The scFv/phage library selected to bind immunogenic PRM proteins could be further selected to only bind gut expressed immunogenic proteins. ScFv/phage that bind proteins matching these criteria could later be used in further experiments for protein isolation. Chapter 5 describes the use of immunohistochemistry to identify which scFv/phage stain gut regions of the PRM, hence binding to gut-expressed proteins.

4. **Isolation of vaccine target proteins via scFv/phage and immunoprecipitation columns.**

Proteins that have a good chance of being gut expressed, immunogenic and membrane-bound match the criteria this project requires of a vaccine candidate. Chapter 6 describes the potential use of immunoprecipitation using bound scFv/phage from chapter 5 to isolate

vaccine proteins from a complex PRM subcellular protein mixture. Protein identification via mass spectrometry and potential use in future vaccine trials is also discussed.

Chapter 2: General Materials and Methods

2.1 Mite manipulation

2.1.1 Mite collection

Poultry red mite were collected from either of two commercial egg-producing farms based around the South and West of England. Both farms were known to have a history of PRM infestation. Collections were carried out all year round however less PRM were found during winter months. The first collection site was an organic, medium sized, free-range farm containing 6,000 birds per barn and 3 barns. Broiler chickens slept in metal boxes connected to wooden perches and had access to outside grassland throughout hours of daylight. The second collection site included a more high-through put system of caged birds, 80 birds per cage and 230,000 birds per barn. Stacked cages consisted of a wire mesh flooring and metal roosting platforms. The first site was an organic farm, controlling PRM using dusting of diatomaceous earth (Hemexan, RME organics Ltd) and introduction of parasitic wasps. Contrarily, controls in the second site included the spraying of various types of residual insecticide or knockdown products, often containing permethrin, at a period of approximately once every 3 months. Mites were collected similarly from both sites using a metal spatula. Mites were scrapped from cracks and crevices in caged areas into a weighing boat (~0.1g each time) and were transferred into a 75cm^3 conical flask (Corning, UK) with a vented cap firmly screwed on. Collection was repeated until a flask become one quarter full, containing roughly 1-2g of mites. Each collection lasted 4-6 hours and collected roughly 10-20g of mites. Once in a laboratory setting, flasks were stored at 4°C upright in a tray of soapy water to prevent any mite escape. Mites were used in experiments within 2 weeks of capture or more specifically after 7 days of capture if used for homogenization or immunohistochemistry. To separate mites, a flask was brought to room temperature to allow the congregation of mites in the screw cap. The cap

containing mites was carefully removed and screwed onto the top of a 10ml bijou tube. Banging the bottom of the bijou tube on a flat surface forced mites to the bottom. The screw cap was returned to the culture flask and the bijou lid screwed tightly back on the bijou tube. This was repeated with several flasks until the required amount of mites was obtained. Mites were killed by placing the bijou tube at -20°C for one hour or placed in liquid nitrogen (see 3.2.1 Preparation of mite homogenate). Killed mites were either kept at -20°C for several weeks or at -70°C for long term storage.

2.1.2 Mite quantification

10mg (±1mg) of a mixed population of freshly killed mites 7 days after capture were weighed on an analytical balance (KERN ALJ 220-4NM, John Morris scientific). Mites were resuspended in 0.15ml 1x PBS and placed into the counting field of a Macmaster cell counter (Vetlab, UK). Mite numbers within the square field were counted using 4x microscopy (Eclipse N2000, Nikon). This was repeated four times and the average taken. Averages of 317 mites were found per 1mg indicating 1g of mites was approximately 31,700 mites if measured as part of a partially fed mixed population.

2.1.3 Controlling mites in a laboratory setting

Transfer of live mites in a laboratory setting was carried out on benches sprayed with Total mite kill (Nettex) containing 0.23% (w/w) permethrin and inside an area of the bench bordered with double sided sticky tape. Gloves and lab coats were worn and gloves, bottles, and spatulas were sprayed with Total mite kill spray and washed in soapy water once used. Waste was double bagged and autoclaved after each time live mites were transferred. Mites were stored at 4°C in culture flasks sprayed on the outside with Total mite kill and placed in a container surrounded by a layer of soapy water at the base.

2.2 Protein manipulation

2.2.1 Bradford assay

5µl of each protein sample was added to 250µl of Bradford reagent (Sigma) in a 1.5ml Eppendorf tube, mixed gently and incubated at room temperature for 10-15 minutes. 2µl of each mixed sample was then pipetted onto a platform of a NanoDrop 1000 Spectrophotometer (Thermo Scientific) and the absorbance measured at 595nm. Protein concentration was calculated against a 0.006-2.0mg/ml BSA standard curve.

2.2.2 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Protein samples were diluted with dH_20 to similar concentrations, typically 5-50µg total protein in 15µl total volume. Each sample was added in a 1:1 mixture with 15µl 2x Laemmli's loading buffer (see Appendix) (Sigma-Aldrich, UK). Samples were denatured for 5 minutes at 95°C in a dry heating block (VWR). A precast agarose gel (NuPAGE™ Novex™ 4-12% Bis-Tris Protein Gel) was placed in a XCell SureLock™ Mini-Cell tank (Invitrogen) as per the manufacturer's instructions. 1x MOPS buffer (see Appendix) (Invitrogen) was poured until wells were covered. The comb was removed and 5µl of ladder (Precision Plus™ Dual Colour ladder for SDS-PAGE; or PageRuler™ Prestained NIR Protein Ladder, Thermo Scientific, for western blotting) was loaded into the first well. Protein samples were centrifuged at 13,000rpm for 5 seconds to remove insoluble debris and 15-20µl of the supernatant was loaded per well. The gel was electrophoresed at 190 volts for 50-60mins or until the leading dye front reached the bottom of the gel. The gel was removed, and stained with Coomassie Brilliant Blue (Bio-Rad) for 30 mins at room temperature. In a fume cupboard the Coomassie was removed and replaced with sufficient volume of destaining solution (see Appendix) to cover the gel. This was repeated after 1 hour then the gel was left on a rocker at room temperature overnight. The gel was photographed using ultraviolet light.

2.2.3 Western Blotting

SDS-PAGE protein gels were electrophoresed according to the '2.2.2 SDS-PAGE' section, however gels were placed in 10ml 1x CAPS transfer buffer (see Appendix) in a weighing boat rather than Commasie Blue. A scalpel was used to remove excess gel at the comb end. A PVDF membrane (Life technologies) was placed in methanol for 30 seconds, then in dH_2O for 1 minute then finally into 1x CAPS transfer buffer. Two sets of one filter paper and two blotting pads (sized 7.5cm x 8.4cm, Life technologies) were placed in x1 transfer buffer for 5 minutes. Inside a tray one filter/blot set was placed inside an XCell II™ Blot Module (Invitrogen) then the gel was placed on top of this, followed by the PVDF membrane and then the second filter/blot set. The whole 'sandwich' was placed inside an XCell SureLock™ Mini-Cell tank (Invitrogen) and submerged in transfer buffer. Protein transfer was carried out at 30 volts for 1 hour 15 minutes. The PVDF membrane was removed from the Blot Module and placed in blocking buffer (see Appendix) for 1 hour at room temperature or overnight at 4°C. Blocking buffer was removed and the PVDF membrane was washed in wash buffer (see Appendix) 3x5 minutes. Primary antibody (diluted in wash buffer) was added to the PVDF overnight at 4°C. This antibody solution was removed and the membrane was washed 5x5 minutes then incubated in secondary antibody for one hour at room temperature. Secondary antibody was removed and the membrane was washed a further five times. This was repeated for a tertiary antibody if required. Final washes were followed by 3x5 minute washes in PBS (pH 7.0). Bound antibodies were visualised using Luminata™ Forte Western HRP Substrate (Merck Millipore) and photographed using a G:BOX Chemi XX6 (Syngene).

2.3 DNA manipulation

2.3.1 Phage DNA extraction

Phage infected TG-1 E. coli cells were grown up at 37°C overnight from -70°C stocks in 2ml of LB broth containing 100µg/ml Ampicillin and 50µg/ml Kanamycin. Samples were centrifuged at

13,00rpm for 10 mins on a bench top microcentrifuge. DNA was purified from cell pellets using a Quaiprep Spin Miniprep Kit (Quiagen) according to manufacturer's instructions. Briefly, bacteria were lysed, releasing plasmid DNA which was absorbed onto a silica membrane in the presence of high salt. Contaminants were passed through the column in wash buffer and DNA was eluted in 50µl water.

2.3.2 DNA quantification

DNA samples were quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) according to the manufacturer's instructions. 2µl of each sample was analyzed under UV light, reading absorbances at 260nm and 280nm wavelength.

Chapter 3: The preparation and evaluation of subcellular fractions enriched for membrane-bound proteins of the poultry red mite

3.1 Introduction

A vaccine is created from using either a whole pathogen or a purified version of certain highly immunogenic proteins expressed by the pathogen. Whole pathogen vaccination against PRM has resulted in no significant mortality in feeding mites, and has limited potential (Arkle, Harrington et al. 2008). Further PRM vaccine studies, including this project, have targeted enriched fractions containing a subset of specific proteins (e.g. soluble proteins (Wright, Bartley et al. 2009)), or a small number of defined recombinant proteins (Harrington, Canales et al. 2009). To study a particular set of proteins, such as the gut membrane proteins that are targeted in this project, separation of proteins from other subcellular material including other non-targeted proteins is beneficial. Homogenisation of whole organisms into a subcellular mixture, followed by centrifugation to separate subcellular compartments containing proteins, is a common methodology used in vaccine studies (Sheeler 1981; Ahmed 2004).

3.1.1 Homogenization of whole mites into a sub-cellular mixture

Homogenisation of mites sized from 200-1000μm to a sub-cellular mixture of organelles, proteins, lipids etc. (generally <2-5μm) can be carried out through several techniques. Live PRM do not survive well at low temperatures and in particular, -20°C is shown to be lethal to PRM (Nordenfors, Hoglund et al. 1999). Freezing of mites is a common way to kill PRM without interrupting protein secondary and tertiary structure (Bartley, Nisbet et al. 2009; Wright,

Bartley et al. 2009). Following mite freezing, homogenisation is carried out to reach a subcellular state. PRM are invertebrates with a hardened exoskeleton composed of chitin polymers and a hydrophobic layer of wax which protects them from desiccation (Di Palma, Giangaspero et al. 2012). This layer can be removed through physical force, for example by using a mortar and pestle or a tissue homogeniser, or through chemical 'softening' by boiling specimens in sodium or potassium hydroxide (Barbosa 1974). Chemical treatment for extended periods however, does lead to dissolving of internal soft tissue (Álvarez-Padilla and Hormiga 2007). More commonly, electronically powered 'mechanical' tissue homogenisers and 'manual' mortar and pestle grinding are used to break open Acari species bodies (Lutsky, Teichtahl et al. 1984; Alasaad, Rossi et al. 2009; Campbell, Burdin et al. 2010). Mortars and pestles are inexpensive and easy to use, however failure to clean sufficiently causes regular contamination issues. If used with liquid nitrogen frozen samples, smaller samples may even be precipitated in the surface of the mortar (Burden 2008). Electronic disruptors such as the Quiagen TissueRuptor® (ID 9001273) are more expensive; however, they are very effective at cell disruption due to the high sheer forces they can create. Bead beating is another alternative that offers various sizes of beads depending on cell/tissue sample sizes and is common in most research laboratories. Bead beating is reported to be less effective than other methods as larger samples or hard structures may not be broken up by the force of the beads (Byreddy, Gupta et al. 2015). Two-step processes incorporating more than one disruption method are more time consuming, but are also common. Physical disruption of tissue or whole organisms can be followed by techniques that can disrupt individual cells a if sample is not already in subcellular state. Sonication utilises ultrasonic sound waves (>20 kHz) to disrupt cell membranes and has been successfully used to break open tick cells and isolate DNA during genome sequencing of the blacklegged tick, *Ixodes scapularis* and Southern cattle tick, *Boophilus microplus* (Ullmann, Lima et al. 2005). Osmotic lysis, the induction of cell lysis

through osmotic imbalance between cell and extracellular environment, is also an option but not common in mite/tick protein purification studies.

3.1.2 The potential for dissection of PRM

Investigation of proteins from a specific organ or tissue, such as the midgut, could be significantly enhanced by dissecting out the relevant region of the mite before proceeding with tissue homogenisation. Most mites are considered too small for dissection (for example *Dermatophagoides pteronyssinus* is 200-300μm (Arlian, Rapp et al. 1990)) although in theory some species are big enough to attempt dissection, such as *Psoroptes ovis* (750μm (Wall and Shearer 1997)). Tick species of similar size, such as *Amblyomma maculatum* (700-900μm), have been successfully dissected (Edwards, Goddard et al. 2009), as have mosquitoes and flies (Meadows 1968; Strome, DeSantis et al. 1980; Wu and Luo 2006). Dissection of the poultry red mite, to the best of our knowledge, has not yet been described. Dissection of ticks such as *A. maculatum* is carried out in hardened wax so this protocol is not suitable for fragile mite midgut dissection. Midguts of mosquitoes are routinely separated from outer bodies, using aqueous conditions (e.g. PBS), dissection pins and forceps (Strome, DeSantis et al. 1980; Coleman, Juhn et al. 2007). Removal of the posterior part of the abdomen, whilst simultaneously pinning down the anterior head and thorax, allows extraction of ovaries, gut tissue and salivary glands. If midgut dissection is possible, homogenate generated from dissected material would be highly enriched for gut-specific proteins and would increase the chance of successful identification of a gut expressed vaccine target.

3.1.3 Enriching for membrane proteins by centrifugation

Centrifugation is the process of separating substances of varying densities by the use of centrifugal force. The less dense a substance or solvent is, the higher the centrifugal force

required to sediment it through a fluid medium. Centrifugation is referred to as 'differential centrifugation' when separating organelles or proteins in a stepwise manner. Differential centrifugation of insects and Acari has been previously used to separate and identify potential drug and vaccine target proteins in a large range of studies. This includes research into midgut proteins of *Errinyis ello* caterpillars (Santos, Ribeiro et al. 1986), neural secretory proteins of *Periplaneta Americana* cockroaches (Evans 1962) and even *Plasmodium* sporozoite proteins from infected mosquitoes (Ozaki, Gwadz et al. 1984). Early vaccine studies of the Cattle tick *Rhipicephalus microplus* used differential centrifugation to separate fractions of homogenised ticks. This led to the successful purification, identification and commercialization of the BM86 antigen TickGARD vaccine (Willadsen, McKenna et al. 1988). Similar studies have also been carried out in other Acari, including *Psoroptes ovis* (Smith and Pettit 2004) and PRM (Wright, Nisbet et al. 2011), however so far with limited success.

Table 3.1 shows the centrifugal spin times and gravitational forces (g forces) required to sediment an organelle type, or specific protein fraction, during centrifugation. These times and speeds are referenced from large scale reviews that report 'generally accepted' spin times/speeds (Ahmed 2004; Wilson and Walker 2010) combined with publications where centrifugation has been carried out specifically on PRM homogenate (Wright, Nisbet et al. 2011; Bartley, Wright et al. 2015). All spin times and g forces are based upon using a 1XPBS buffer (see Appendix).

Protein/organelle purified	Centrifugal force	Spin time	Detergent
Whole cell (pellet)			
***E. coli* cell**	500g	10 minutes	-
Organelle specific fractions (pellet)			
Nucleus	1000g	10 minutes	-
Mitochondria	3,000g - 10,000g	10-30 minutes	-
Cell membrane / Endoplasmic reticulum	80,000g - 100,000g	1 hour	-
Ribosome	150,000g	Several hours	-
Enriched fractions (supernatant)			
Soluble proteins	25,000g	25 minutes	-
Peripheral membrane proteins	25,000g	20-25 minutes	0.1% Tween 20
Integral membrane proteins	100,000g	45 minutes - 1 hour	1% Triton X-100
Insoluble proteins	100,000g	20 minutes	10M Urea

Table 3.1: Details of centrifugal forces and spinning times required to separate either a particular organelle or protein fraction from a homogenised cell mixture during centrifugation. Whole cells and organelles are purified by sedimentation at these speeds (noted as 'pellet'). In an alternative approach, fractions 'enriched' for proteins based on detergent interaction are contained within the supernatant. Detergents added during 'enriched' fraction purification are labelled in the final column. Less dense organelles / proteins require higher centrifugal forces to sediment. Centrifugal forces are measured in g-force and all values are based on using 1xPBS (see Appendix) as a solvent. Times/speeds

referenced from various sources (Graham and Higgins 1997; Ahmed 2004; Wilson and Walker 2010; Wright, Nisbet et al. 2011).

Centrifugation can also be carried out by separating a sample through a buffered gradient of various densities. This density gradient centrifugation is further categorised as 'rate-zonal' or 'isopycnic'. Rate-zonal centrifugation separates particles based on mass, i.e. more massive objects can pass through more viscous solution. Because the density of the particles is greater than the density of the gradient, all particles will eventually pass through the gradient and form a pellet. Isopycnic centrifugation separates particles based on their density, rather than mass. Particles are spun through different gradient density layers until they reach centrifugation buffer that has equal density to the particles. Gradients of varying buoyant density ranges are generated, depending on the medium used.

3.1.4 Membrane proteins and the effect of detergent on the cell membrane

Membrane proteins are defined as proteins that interact with, or are part of, a biological membrane such as the cell plasma membrane. Between 20-30% of all genes in most genomes, encode membrane proteins (Wallin and Heijne 1998). Membrane proteins are classed as either integral membrane proteins, that are permanently attached to the membrane, or as peripheral proteins, that are transiently attached through non-covalent, protein-protein interactions (Lodish, Berk et al. 2000) (see Figure 3.1). Integral proteins typically transverse a whole membrane bilayer, anchored via amino acid hydrophobic side chains interacting with fatty acyl groups of membrane phospholipids in the hydrophobic membrane core (Sachs and Engelman 2006). Peripheral proteins do not interact with the hydrophobic interior however interact with membrane integral proteins or the phospholipid polar head groups. Glycosylphosphatidylinositol (GPI) anchored proteins have characteristics of both peripheral and integral proteins. The GPI protein is covalently attached to a lipid that interacts with the hydrophobic core, however these proteins do not span the entire lipid bilayer (Ferguson MAJ, Kinoshita T et al. 2009).

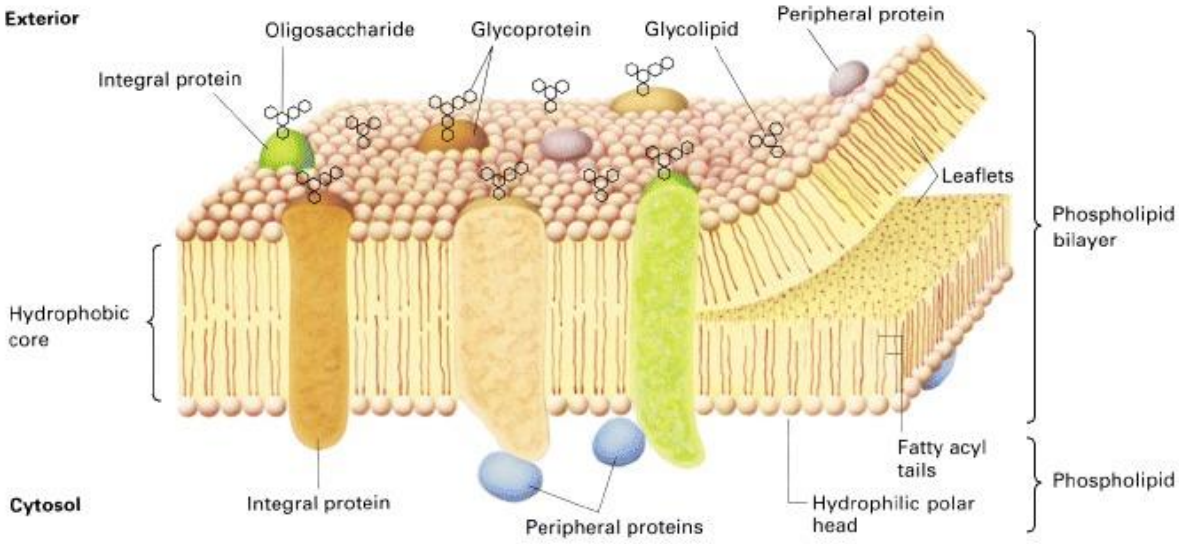


Figure 3.1: The arrangement of membrane proteins in a cell membrane, taken from Lodish, Berk et al. (2000).

Detergent molecules are amphipathic, containing a polar head group and a non-polar hydrophobic tail group. This allows detergents to interact with the hydrophobic core of a bilayer cell membrane. Detergents thus can be utilised in the extraction, purification and manipulation of membrane proteins (Takács 2000; Scopes 2013). Physical/chemical properties of different types of detergents govern the effect each detergent will have on a membrane. Detergents can be classified based on whether the hydrophilic head is ionic, non-ionic or zwitterionic (i.e. containing both a positively charged group and negatively charged group). Ionic detergents are considered the most 'harsh' of detergents in protein purification as charged groups interact with both intra and inter-molecular protein-protein interactions. Ionic detergents such as sodium dodecyl sulphate (SDS) are effective at total membrane disruption, however they also disrupt protein structure and so are not recommended if downstream functional assays are required (Ragan 2012). Non-ionic detergents are considered less harsh as they do not interact with protein-protein interactions however can disrupt protein-lipid bonds (Anatrace 2015). This makes non-ionic detergents useful for membrane protein purification, allowing membrane disruption whilst keeping intact protein structure. Zwitterionic detergents are considered an intermediate between ionic and non-ionic detergents. Zwitterionic molecules are electrically neutral however have capacities to break protein-protein boding which results in a mild to strong disruption of protein/membrane structures. Two commonly used non-ionic detergents in biological research are Tween 20 (i.e. Polysorbate 20, Croda International PLC) and Triton X-100 (Dow Ltd). Triton X-100 contains a hydrophilic, aromatic alkyl phenyl ring head connected to a polyethylene oxide tail. Tween 20 also has a polyethylene tail, but has a hydrophobic fatty acid moiety head group (see Figure 3.2). The bulky head group of both molecules do not penetrate into water soluble proteins and therefore do not disrupt native structure of water soluble proteins (Seddon, Curnow et al. 2004). Tween 20 is commonly used to remove peripheral proteins from membranes (used at 0.05%-0.1%) or in conjunction with Bovine Serum Albumin as a blocking solution in ELISA

experiments (0.05%-0.2%) (Steinitz 2000).Triton x-100 is a more harsh detergent as the alkylphyl group on the Triton X-100 is more hydrophobic than the fatty acid ester group on the Tween 20 allowing for disruption of a plasma membrane. Triton X-100 is commonly used to purify integral membrane proteins from membrane sheets (used at 1%).

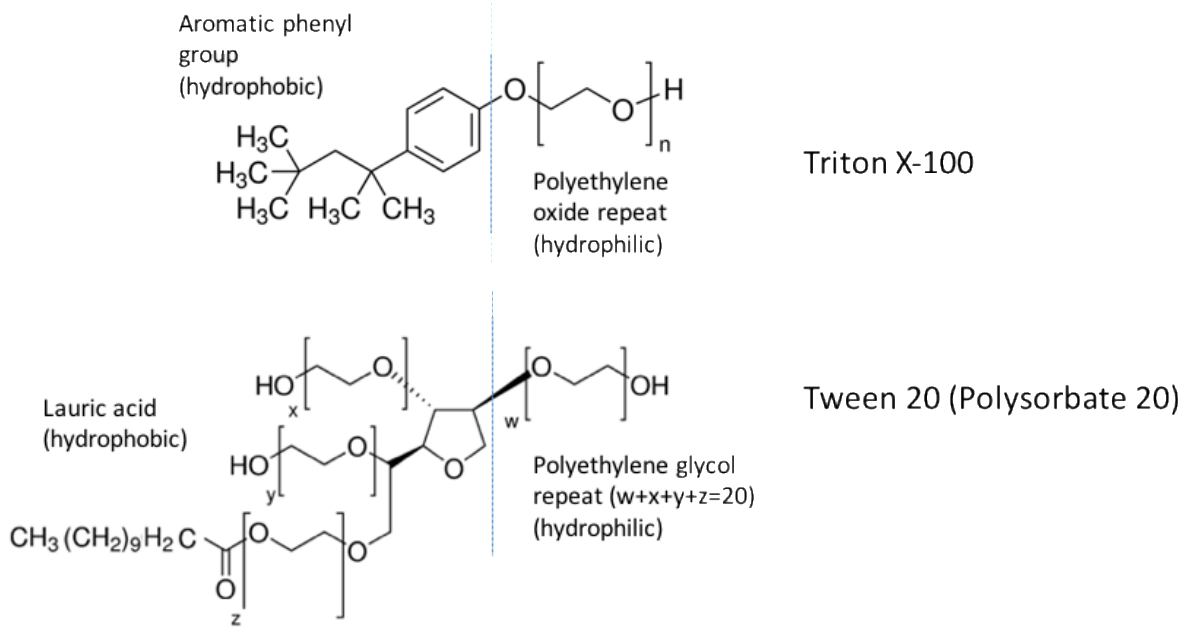


Figure 3.2: The chemical structures of non-ionic detergents Triton X-100 and Tween 20 (Polysorbate 20). The dotted blue line separates the hydrophobic tail group (left) and the hydrophilic head group (right). Modified from Sigma-Aldrich (2016).

At a sufficiently high enough concentration in aqueous conditions, known as the critical micelle concentration (CMC), detergent monomers cluster together to form a more thermostable structure termed a 'micelle'. Hydrophilic interactions between the polyethylene head groups and water molecules force hydrophobic parts of monomers to interact forming a hydrophobic core and hence forming a micelle. At concentrations at or slightly below the CMC, detergents interact with hydrophobic parts of cell membrane lipid bilayers and the hydrophobic regions of integral proteins located within a membrane. Cell membrane is disrupted and the integral proteins are solubilized as part of the detergent micelles. At concentrations much lower than the CMC, monomers partition into membranes without completely solubilising membrane proteins (Bhairi 2001). This understanding of a detergent's CMC is important when considering protein purification. All detergent monomers added to a detergent concentration above the CMC form micelles. Increase in detergent concentrations above the CMC therefore does not increase level of membrane protein solubulisation. Many non-ionic detergents, especially Triton X-100, also interfere with downstream quantification as the aromatic head group interferes with ultra violet light analysis techniques (Tiller, Mueller et al. 1984) and chemically with Bradford assay reagent (Friedenauer and Berlet 1989). Detergent concentrations in protein purification should therefore be close to the CMC but not at or over it.

3.1.5 Aims & Hypothesis

Vaccination studies against PRM are yet to identify suitable protein targets that can induce an immune response that significantly increases mite mortality rates. Gut membrane proteins may be suitable vaccine targets against PRM, as the mite is an obligate blood-feeding parasite. This chapter describes the assessment of several methods for the homogenisation of PRM followed by centrifugation to enrich for membrane proteins. Enrichment increases the chance that immunogenic proteins identified in downstream techniques will be membrane proteins.

These are the class of proteins that are most likely to interact with antibodies in a PRM blood meal and are hence potentially important vaccine targets. The primary objectives of this chapter were:

1. To determine the most efficient way to homogenise poultry red mite into a subcellular mixture of organelles and proteins.

To enrich for membrane proteins, a whole mite sample was homogenated into a subcellular level homogenate. PRM have a strong chitinous exoskeleton and are most likely too small to dissect out the mid gut region. Various methods of mite homogenisation were analysed via microscopy and the protein amounts obtained were measured by Bradford assay. Results indicated which method would be used to create a subcellular mite homogenate required in future experiments.

2. To identify via SDS-PAGE which technique could produce repeatable results in fractions enriched for membrane proteins.

Centrifugation can be employed to enrich a mixed sub-cellular sample for plasma membrane fragments that contain membrane proteins This is based on utilising variable properties of organelles and cell fragments such as size and density. Many types of centrifugation are available using various speeds, buffers and potentially adding detergents. We tested protocols for differential centrifugation, sucrose gradient centrifugation and centrifugation using a commercially bought membrane protein extraction kit (Proteoprep® kit, Sigma #PROTMEM-1KT). Membrane protein enriched fractions were compared by SDS-PAGE. The technique that resulted in the most visible and repeatable results in SDS-PAGE was used to enrich for membrane protein homogenate for further experiments. This homogenate was also analysed by mass spectrometry to determine amount and function of membrane proteins present inside the final enriched homogenate fraction.

3**. To investigate the use of detergents in membrane protein purification at varying concentrations.**

Detergent effect on protein quantification and binding to ELISA plates was investigated as one protocol for differential centrifugation (objective 2) used 0.1% Tween 20 and 1% Triton X-100 to enrich for membrane proteins. Detergents were deemed acceptable for use at such concentrations if they did not affect binding and were below the respective CMC level for each detergent.

3.2 Methodology

3.2.1 Preparation of mite homogenate

Various methods of mite killing and homogenization were compared, each starting with 0.1g of a mixed population of live red mite 7 days after collection. Approximately 0.2g of live mites was placed in a 10ml bijou tube and stored at -20°C for 2 hours, allowing mites to freeze. As well, ~0.4g of mites was placed in 2 x 2ml cryovial tubes (Simport, UK) and the tubes were immersed in ~10ml of liquid nitrogen (<-190°C) for 30 seconds. Frozen mites from -20°C and liquid nitrogen were weighed and separated into six aliquots of 0.1g using an analytical scale (Ohaus, USA ±0.01g). One aliquot from each freezing technique was added to 1ml chilled PBS+PI (see Appendix) (SigmaFAST, Sigma #S8820-20TAB) in a mortar and pestle, that was placed on ice. Mites were crushed into a homogenised mixture using manual force for 5 minutes. Another aliquot from each freezing technique was added to 1ml chilled PBS +PI in a 5ml bijou tube on ice and homogenized mechanically using an electronic tissue disruptor (Qiagen) for 5 x 1 minute on full power with one-minute rests on ice in between to cool homogenates. A final aliquot of mites frozen by liquid nitrogen in 1ml chilled PBS + PI was placed in a bead beating tube (VWR), with 0.1mm beads in a 1:1 ratio and beaten for 3 minutes on full power. All samples were incubated on ice, and the protein concentrations measured by Bradford assay (see Chapter 2) using a 1:10 dilution of homogenate in dH_20. All samples were photographed by light microscopy at x40 and x200 magnification (Olympus BX60 microscope, Axiovision software).

3.2.2 Dissection of midguts from poultry red mite

Two days after a mite collection and been stored at room temperature in a T-75 flask on a laboratory desk, ~100 live mites were suspended in 300µl of 1XPBS for 30 minutes on ice. The live mites/PBS were placed on a slide (VWR) and observed by light microscopy at x40

magnification. A dissection pin was used to hold down a single blood fed mite by the anterior part of the body whilst forceps pulled away the posterior end of the mite. This was repeated with nine different mites. A further ten mites were dissected by alternatively pinning down the posterior and pulling away the anterior section. Released internal organs were positioned away from the exoskeleton body to identify individual morphological structures and photographed using Axiom software.

3.2.3 Differential centrifugation and addition of detergents to enrich for membrane proteins

15ml of mite homogenate produced by immersion in liquid nitrogen followed by tissue disruptor homogenization (see '3.2.1 Preparation of mite homogenate') was centrifuged at 25,000g for 20 minutes at 4°C (L-80 XP Beckmann Coulter ultracentrifuge, SW32 Ti swingout rotor). The supernatant, containing the 'PBS-soluble' protein fraction, was removed and stored at -20°C. The pellet was resuspended in 15ml chilled 1x PBS+PI, 0.1% Tween 20 (Sigma) and centrifuged at 25,000g for 20 minutes at 4°C. This supernatant, containing peripheral membrane proteins solubilised by Tween 20, was removed, labelled the 'Tween soluble' fraction and stored at -20°C. The pellet was resuspended in 15ml chilled 1x PBS+PI, 1% Triton X-100 (Sigma), incubated on ice for 15 minutes then centrifuged at 100,000g at 4°C for 1 hour in an ultracentrifuge (Sovrall MX+). The supernatant was aliquoted into 1.5ml Eppendorf tubes and stored at -20°C. This supernatant was hypothetically enriched for Triton X-100 solubulised integral membrane proteins and was labelled the 'Triton soluble' fraction. The pellet was stored at -80°C and labelled the 'insoluble' fraction as this contained insoluble proteins such as proteins found in undisrupted lysosomes. 15µl samples of the supernatants and the resuspended final pellet were electrophoresed using SDS-PAGE.

3.2.4 Differential centrifugation to enrich for 'organelle-specific' fractions (no detergent added)

15ml of mite/PBS+PI homogenate (see '3.2.1 preparation of mite homogenate') was centrifuged in a 15ml falcon tube at 1,000g, for 10 minutes at 4°C to remove whole cells and intact nuclei in the pellet (Multifuge X3-FR centrifuge, F15 fixed angle rotor). Supernatant was centrifuged at 10,000g for 30 minutes at 4°C to sediment mitochondria and other similar sized organelles. The resultant supernatant was further centrifuged at 100,000g for 1 hour at 4°C (L-80 XP Beckmann Coulter ultracentrifuge, SW32 Ti swingout rotor) to sediment membrane proteins that were bound to cell membrane fragments. All pellets were resuspended in 1ml of PBS+PI and were electrophoresed using SDS-PAGE.

3.2.5 Low Speed centrifugation without the use of detergent or harsh physical homogenisation

This protocol was adapted from Scott, Schell et al. (1993) and Graham and Higgins (1997) to isolate cell membrane sheets which are larger than broken up bilayer fragments or solubilised single membrane proteins. 0.2g of mites and 2ml of chilled 1xPBS+PI was homogenized via mite freezing in liquid nitrogen and mechanical homogenisation (see '3.2.1 Preparation of mite homogenate'). Using a 2ml Pasteur pipette, 0.5ml of the homogenate was transferred to a dounce homogenizer (GPE Ltd. 0.025mm clearance) and forced through the shaft by pushing up and down on the pestle ten times. The homogenised mixture was transferred into a 2ml Eppendorf tube on ice and the process was repeated a further three times until all mite and PBS+PI solution was homogenised. This sample was centrifuged at 250g for 10 minutes; the supernatant was removed and stored at -20°C. This supernatant included already soluble proteins such as cytosolic proteins. The pellet was resuspended in 2ml PBS +PI and centrifuged at 1500g for 10 minutes. This supernatant hypothetically contained cell membrane sheets and

membrane proteins. The supernatant was removed and the pellet resuspended. The resuspended pellet was centrifuged at 3,000g for 10 minutes, the supernatant removed and the pellet resuspended. This supernatant hypothetically contained mitochondria and other 'heavy' organelles. 15µl of all samples were electrophoresed in SDS-PAGE.

3.2.6 Sucrose gradient centrifugation

0.8g of mites were frozen in liquid nitrogen and homogenised in 7ml PBS+PI (see 3.2.1 Preparation of mite homogenate). A 15ml centrifuge tube was layered with a discontinuous gradient of sucrose solution in 1x PBS, layering 1.6ml of 1.8M sucrose solution at the bottom of the tube and consecutively adding 1.8ml of four more layers at 0.2M density intervals up to 1.0M sucrose. 6ml of the homogenate solution was layered on top of the 9ml sucrose gradient and centrifuged at 100,000g for 1 hour 30 minutes at 4°C (L-80 XP Beckmann Coulter ultracentrifuge, SW32 Ti swingout rotor). The tube was removed and transferred to a standing clamp. A sterile needle pierced the bottom of the tube and 12 fractions of the sample were collected in separate 1.5ml Eppendorf tubes via gravitational force. Samples were electrophoresed in SDS-PAGE.

3.2.7 Membrane protein extraction using a commercially bought Proteoprep® kit

Mite homogenate was purified using a Sigma Proteoprep® membrane extraction kit. The methodology for protein purification was carried out following the manufacturer's instructions. This meant using 2mg/ml of protein rather than the 200mg/ml used in the other centrifugation techniques.

Briefly, 20mg of mites frozen in liquid nitrogen for 20 seconds was added to 10ml of protein extraction reagent containing detergent and cells were disrupted by sonication (Soniprep 150,

MSE) at 10 microns for 2 minutes on ice. Lysed cells were stirred, again on ice, using a magnetic stirrer (SB169, Stuat Plc) for 1 hour with a further addition of 30ml of protein extraction reagent. Samples were centrifuged at 115,000g, 1 hour at 4°C (L-80 XP Beckmann Coulter ultracentrifuge, SW32 Ti swingout rotor) to remove unbound cytoplasmic and 'loosely bound' peripheral membrane proteins. The pellet was twice resuspended in distilled water and recentrifuged at 20,000g for 20 minutes at 4°C to remove residual salts in the buffer. Sedimented material was resuspended in a chaotropic buffer and sonicated four times at 10 microns for 20 seconds to break open membrane sheets and release membrane proteins. The solution was centrifuged at 14,000g for 45 minutes at 15°C to sediment cell debris. The soluble proteins in the supernatant were reduced by addition of 50µl of tributylphosphine, allowing reduction of disulphide bonds, and alkylated using 60µl iodoacetamide for 1.5 hours, preventing reformation of disulphide bonds. The sample was centrifuged a final time at 20,000g for 5 minutes at room temperature to remove cell debris. Supernatant containing solubilized membrane proteins was electrophoresed on an SDS-PAGE gel along with samples from other stages of the process.

3.2.8 Determining sensitivity and accuracy of Bradford and Bradford Ultra reagents in the presence of 1% Triton X-100

A dilution series of concentrations between 2mg/ml and 62ug/ml of Bovine Serum Albumin (BSA) was set up using a stock of 1g of BSA in 50ml dH20. An initial 1:5 dilution (200µl BSA: 800µl dH2O) was followed by a two fold dilution series until 62µg/ml was reached. 5µl of each BSA dilution was added to 250µl of Bradford or Bradford Ultra solution, mixed by hand and incubated at room temperature for 15 minutes. Absorbance of 2µl of each sample at 595nm was measured by Nanodrop (Thermo scientific) to create a standard curve of concentration vs absorbance for Bradford and Bradford Ultra. This process was carried out on five

independently generated dilution series and averaged to form an overall standard curve for both Bradford and Bradford Ultra.

To measure influence of Triton X-100 on Bradford assay quantification, three further stocks of 1000µg/ml and 500µg/ml BSA in dH2O were made with and without addition of Triton X-100 (final volume 1% v/v). The absorbance of each sample was measured in triplicate at 595nm using both Bradford and Bradford Ultra reagents. Triplicate absorbance values for each reagent/concentration mixture were averaged and plotted against the averaged standard curves for both the Bradford and Bradford Ultra reagents.

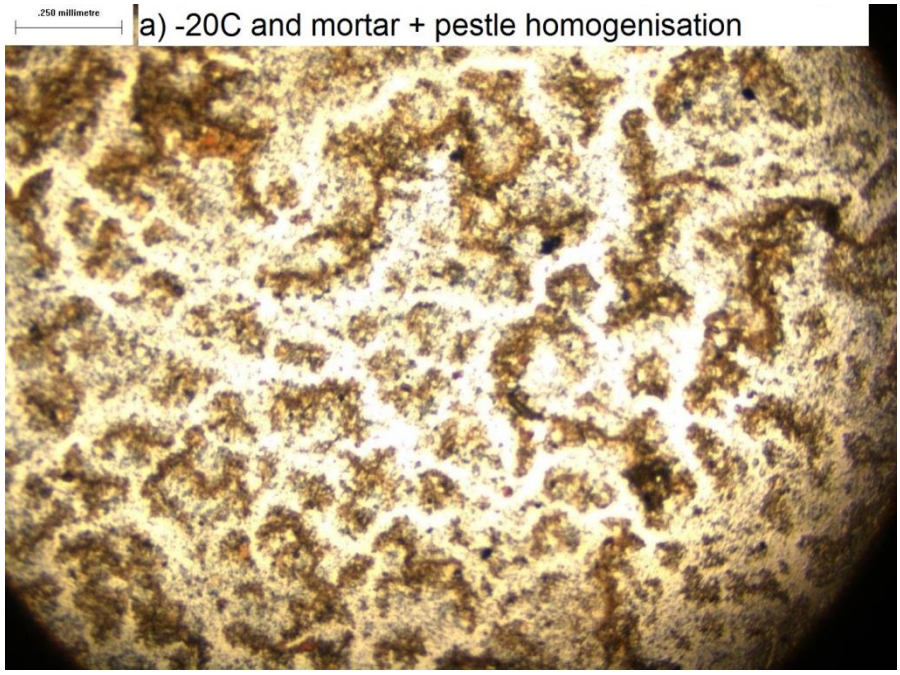
3.3 Results

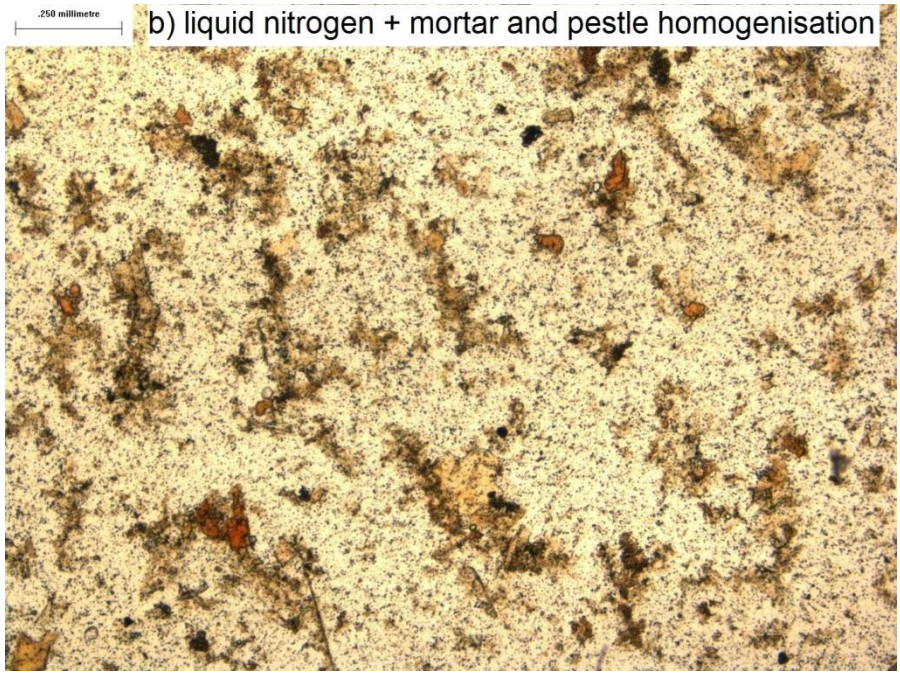
3.3.1 Comparison of freezing and homogenisation techniques

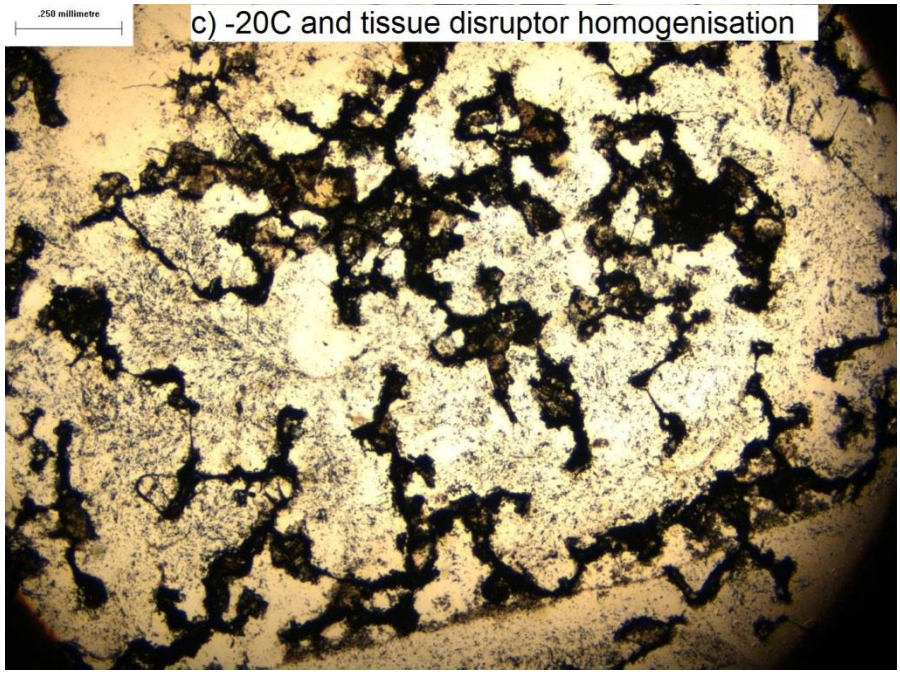
To identify the most efficient way of homogenizing mites to a sub cellular mixture, mites were frozen either at -20°C or by immersion in liquid nitrogen (-190°C). Frozen mites were then broken up by manual homogenization, via a mortar and pestle, or by mechanical homogenization using a tissue disruptor or bead beater. Success of homogenization for each technique was determined by Bradford assay (Table 3.2), to measure protein concentration, and by microscopy at x40 magnification (Figure 3.3) and x200 magnification (Figure 3.4) to determine cell lysis. Table 3.2 indicates that freezing of live mites in liquid nitrogen and homogenisation by a tissue disruptor produced much larger amounts of detectable protein (14.34mg/ml) than other methods (4.86-9.23mg/ml). This was supported by microscopy, shown in figure 3.3c, which shows larger amounts of sub-cellular material compared to other homogenisation methods. Figure 3.4b, magnified at x200, shows that most of the material homogenised by the liquid nitrogen and mechanical tissue disruptor method was sized <1µm and therefore at a subcellular level as required. Bead beating was the least effective method at homogenisation (4.86mg/ml), supported by observation of whole, intact mites in microscopy (Figure 3.3e). Based on the results obtained by Bradford assay and microscopy, the method of choice for all future mite homogenate preparation was to use mite immersion in liquid nitrogen and homogenisation by the mechanical tissue disruptor.

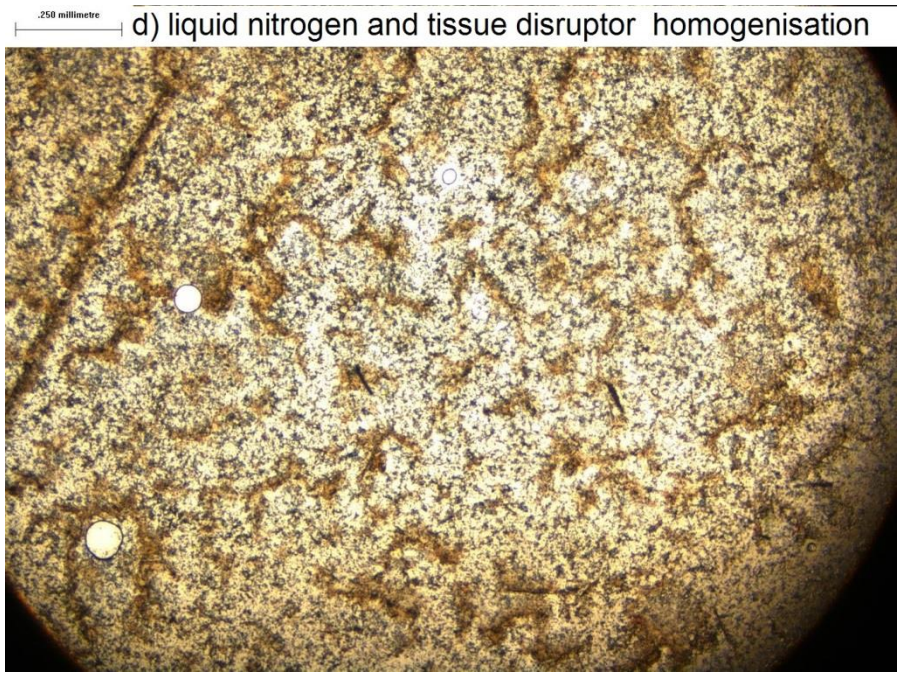
Freezing technique	Homogenization technique	Protein concentration (mg/ml)
Frozen at -20°C	Manual disruption	9.23
	Tissue disruptor	7.95
Frozen in liquid nitrogen	Manual disruption	7.65
	Tissue disruptor	14.34
	Bead beating	4.86

Table 3.2: Protein concentrations of homogenised mite samples quantified by Bradford assay. Each homogenised mite sample was measured by Bradford assay in a 1:10 dilution to fit the standard curve range of 0.006-2.0mg/ml. Liquid nitrogen immersion and use of a mechanical disruptor (14.34mg/ml) resulted in the highest amount of protein recovery compared to all other treatments.


.250 millimetre
a) -20C and mortar + pestle homogenisation


.250 millimetre
b) liquid nitrogen + mortar and pestle homogenisation


.250 millimetre
c) -20C and tissue disruptor homogenisation


.250 millimetre
d) liquid nitrogen and tissue disruptor homogenisation

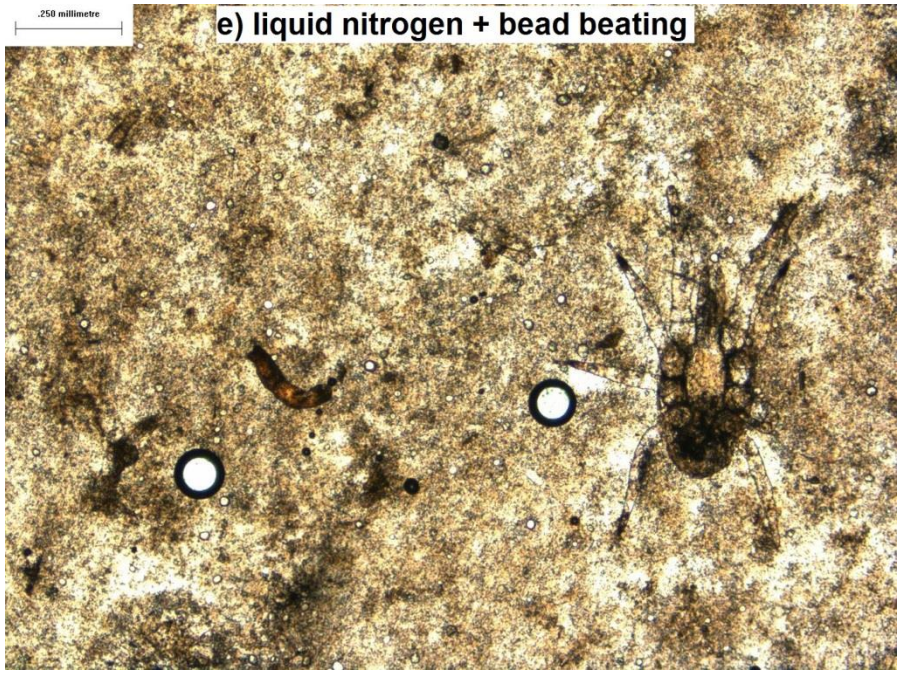


Figure 3.3: **Homogenised mite tissues viewed by light microscopy at x40 magnification**. 0.1g of mites were frozen then homogenised either (a) at -20°C and broken up by manual disruption using a mortar and pestle, (b) by liquid nitrogen and mortar and pestle, (c) at -20°C and using tissue disruptor (d) by liquid nitrogen and using tissue disruptor (e) by liquid nitrogen and bead beating. All methods broke up tissue to sub-cellular sizes (<1μm), however some larger tissue and cell 'clumps' remained. Immersion of samples in liquid nitrogen appeared to improve sub cellular homogenization (b, d), compared to samples frozen at -20°C (a,c). Bead beating appeared to be the least effective homogenization technique as even whole mites could be seen (e). Scale bars = 250μm.

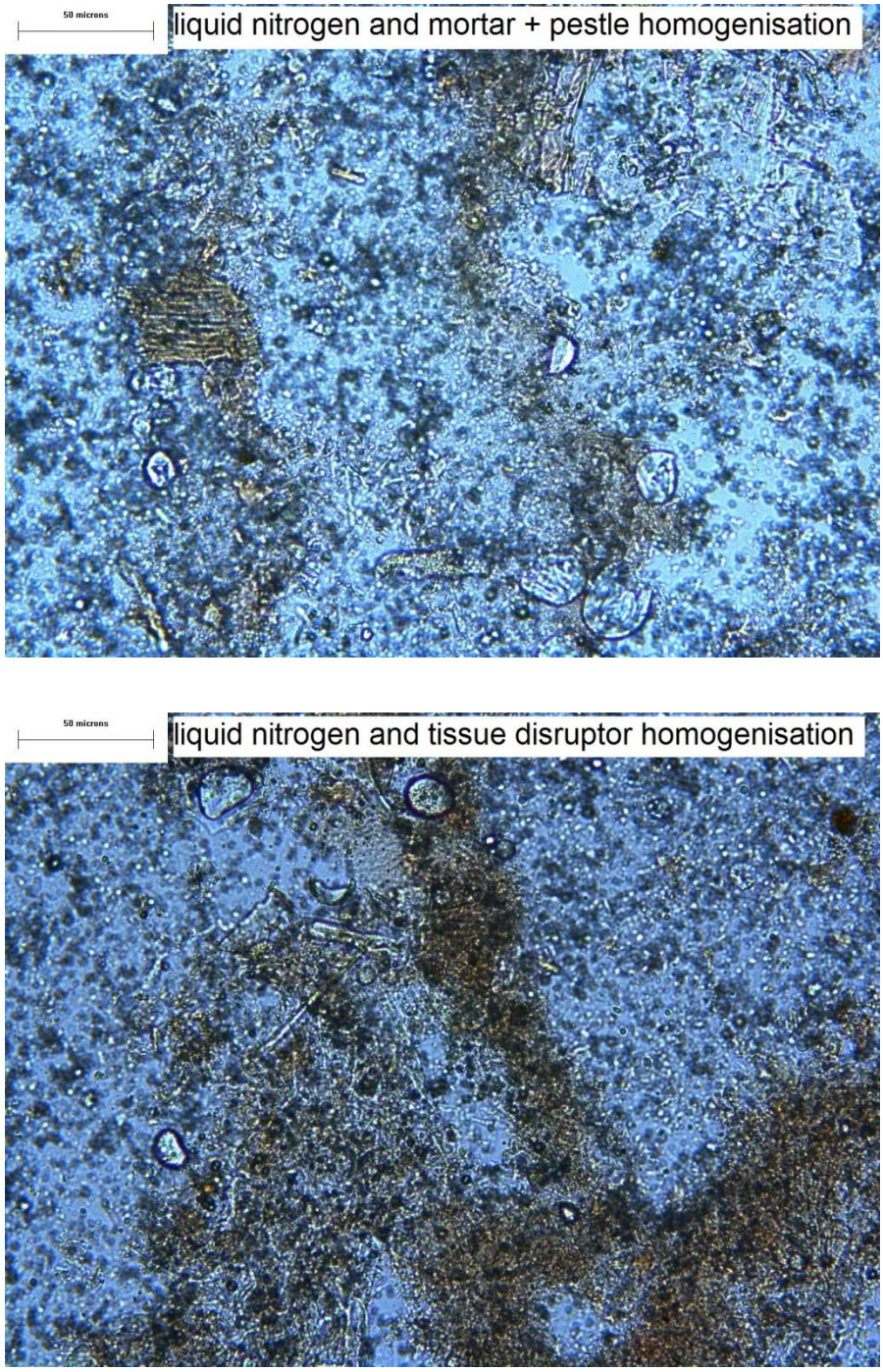


Figure 3.4 Homogenised mite tissue viewed by light microscopy at x200 magnification. Mite samples immersed in liquid nitrogen then homogenised either by mortar and pestle (top figure) or by tissue disruptor (bottom figure). Both techniques produced cell to tissue sized homogenate (10-50μm) and sub cellular (<2μm) material, however comparatively more sub cellular material was seen in samples homogenized by the tissue disruptor (b). Scale = 50μm.

3.3.2 Manual dissection of PRM to extract midguts

Manual dissection of the PRM midgut under x40 light microscopy was first attempted on ten mites by pinning down the anterior part of the mite in 1xPBS to a slide surface and removing the posterior body with forceps. These attempts were not successful; it was not possible to break the mite gnathosoma exoskeleton into several pieces without damaging internal structures (not shown). Alternatively, dissection of another ten mites was attempted by pinning the posterior body and removing the anterior parts of the head and body. This process allowed some internal structures, such as the ovaries (Figure 3.5, green arrows) and malpighian tubules (red arrows), to be removed intact. The gut structure however could not be dissected intact (Figure 3.5, blue arrows denote midgut cells). Consequentially, dissection was not further implemented in this project.

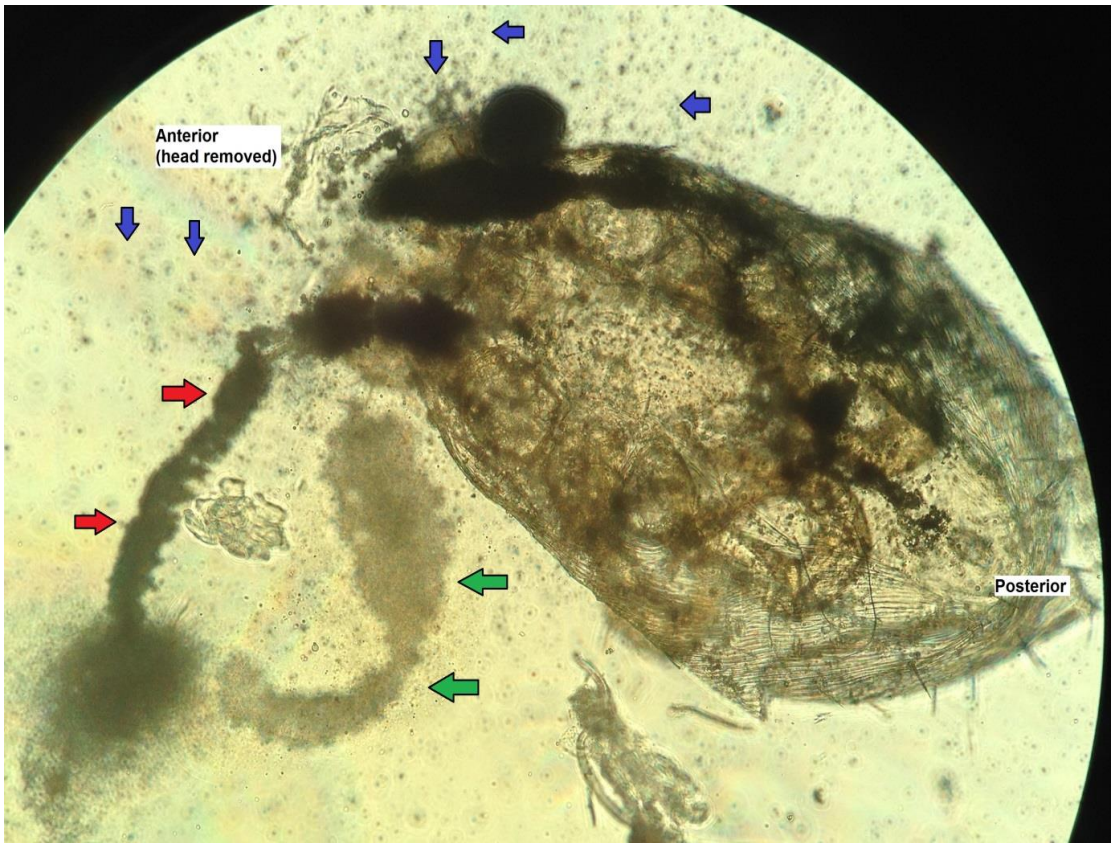


Figure 3.5: Separation of internal structures from a mite body by removal of the head during dissection. Removal of a head by forceps then squeezing on the posterior part of the gnathosoma separated malpighian tubules (red arrows) and ovaries (green arrows) away from the exterior mite body. Midgut tissue was also removed, however broke up into a soluble cell mixture (blue arrows). Posterior and anterior parts of the mite are labelled. Microscopy was carried out at x40 in 1xPBS.

3.3.3 Triton X-100 effect on protein quantification using the Bradford assay

Bradford reagent used to quantify protein concentrations between 62ug/ml and 2mg/ml has been previously reported to be less accurate in the presence of 1% Triton X-100. Bradford Ultra, a newer formulation of the Bradford reagent, was sold as producing a more accurate result in the presence of the detergent. Average absorbances of triplicate 1000µg/ml and 500µg/ml BSA samples in dH2O, with or without 1% Triton X-100, quantified by Bradford or Bradford Ultra reagent, were plotted against standard curves of BSA/dH2O (63µg/ml to 2000µg/ml) (see Figure 3.6).

Absorbance values for both reagents, Bradford and Bradford Ultra, were close to absorbance values recorded by the respective standard curves at 500µg/ml when no Triton X-100 was present (and respectively). In the presence of 1% Triton X-100 however, absorbance values for 500µg/ml were much higher for both reagents (and respectively). For example, the absorbance of Bradford Ultra in the presence of 1% Triton X-100 (0.101) was more than double that of Bradford Ultra without Triton X-100 (0.047). Absorbance values of 1000µg/ml BSA in dH20 were higher than corresponding standard curve values for both reagents in the presence of 1% Triton X-100. Without Triton X-100, the Bradford Ultra absorbance for 1000µg/ml (0.054) was much lower than the corresponding value from the standard curve (0.075). Conversely, Bradford regent without Triton X-100 (0.035) was larger than the standard curve value (0.029).

Overall, Bradford assay values appeared to be inconsistent. Average replicate values did no correspond with standard curve values at 500µg/ml and 1000µg/ml even without presence of detergent in the case of 1000µg/ml Bradford Ultra. This demonstrated the variability of such colorimetric tests when using low numbers of repeat samples as would be analysed when quantifying homogenate samples in future experiments.

Bradford reagent appeared to produce comparatively more consistent absorbance values at 500µg/ml and 1000ug/ml than Bradford Ultra however addition of 1% Triton appeared to almost double absorbance values when present with either reagent. Bradford reagent (not Bradford Ultra), therefore appeared to be an overall better reagent to quantify protein concentrations in future mite homogenate experiments.

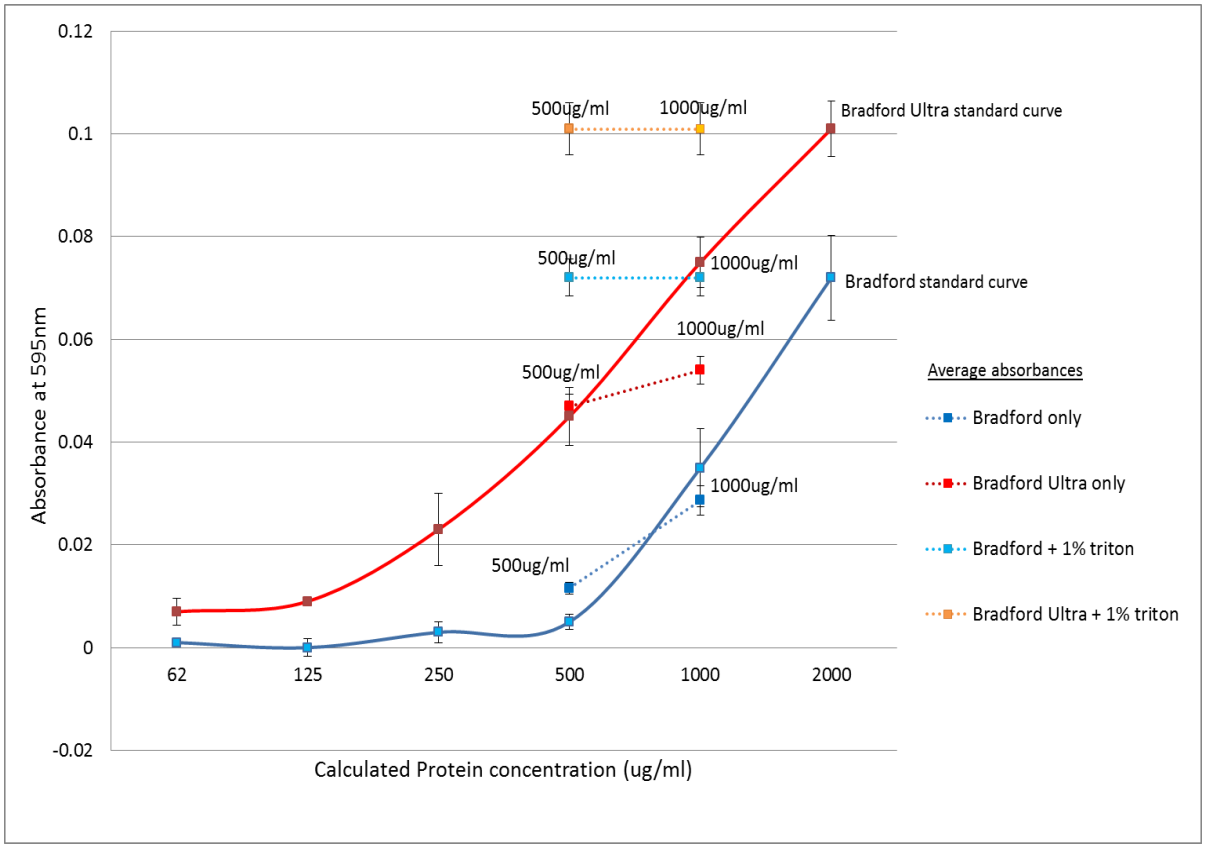


Figure 3.6: Comparison of absorbance values at 595nm for BSA samples of 500µg/ml and 1000µg/ml, with and without 1% Triton X-100, using standard curves created using Bradford reagent and Bradford Ultra reagent. Absorbance values of triplicate 5µl samples of 500µg/ml and 1000µg/ml BSA either with our without 1% Triton X-100, in either 250µl Bradford reagent or Bradford Ultra, were averaged and plotted against the respective standard curves. At 500µg/ml and 1000µl in the presence of 1% Triton X-100, both Bradford (···■···) and Bradford Ultra (···■···) reagents produced absorbance values much higher than the respective standard curve absorbance values. Without presence of Triton X-100, Bradford reagent values (···■···) were similar to that of the standard curve, however Bradford Ultra absorbance at 1000µg/ml (···■···) appeared much reduced. Standard curve values were created from five replicate dilutions from BSA/dH2O serial dilutions of 2000µg/ml to 64µg/ml. Error bars are standard deviation values from replicate values.

3.3.4 Validation of SDS-PAGE and Western blot

SDS-PAGE gels and Western blots were used to identify differentiated fractions separated by centrifugation experiments. To validate the sensitivity of the methodology used for both techniques (see 'Chapter 2: General methodology') homogenised mite and fruit fly protein mixtures were electrophoresed and either stained with Coomassie blue (Figure 3.7a + b) or transferred to a PVDF membrane. Transferred mite homogenate was probed with anti-Cathepsin D chicken serum/1xPBS (1:10,000 – a kind gift from Dr Alistair Nisbet) then mouse anti-chicken HRP/1xPBS (1:3000, Abcam #ab97135). Transferred fruit fly homogenate was probed with rabbit anti-Nervana 1/1xPBS (1:10,000, Abcam #ab127908) then goat anti-rabbit/1xPBS (1:3,000, Sigma, #A0545). Both blots were detected with HRP substrate (Figure 3.7c + d).

Coomassie blue stained SDS-PAGE gels showed multiple bands as expected from a complex protein sample. Western blots showed single dominant stained bands at the sizes expected for PRM Cathepsin D (75kDa) and *D. melanogaster* Nervana (38kDa) (Figure 3.6c+d). Anti-Cathepsin D showed binding to some additional PRM bands, however additional bands may have represented cross-reactivity to additional, related proteases within the PRM. These results validated the western blot protocol for future experiments and that both anti-Cathepsin D and anti-Nervana 1 could be used as working positive controls. Each antibody was species specific (not shown). Anti-Nervana 1 did not react with transferred mite homogenate and anti-Cathepsin D did not react with transferred fruit fly homogenate.

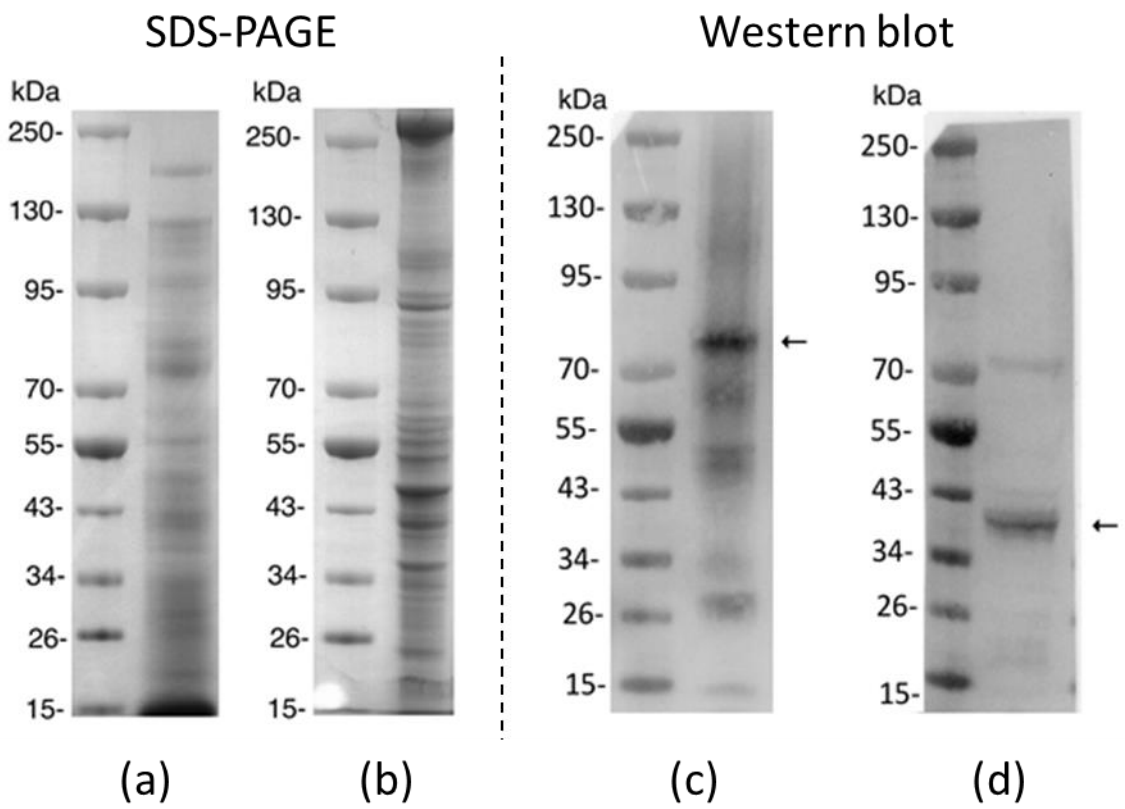


Figure 3.7: Validation of SDS-PAGE and Western blot protocols using mite and fruit fly homogenates, probed with anti-Cathepsin D or anti-Nervana 1 antibodies. Images show SDS-PAGE of (a) PRM and (b) fruit fly homogenate protein mixtures detected by Coomassie blue; and western blots of PRM and fruit fly homogenates probed with (c) anti-Cathepsin D or (d) anti-Nervana 1 respectively. SDS-PAGE of PRM and fruit fly homogenates (a+b) demonstrate clear separation and detection of multiple bands as expected for a complex protein sample. Western blots of both homogenates (c+d) showed dominant bands at sizes expected (indicated by arrows) for Cathepsin D (c - 75kDa) and Nervana (d – 38kDa) proteins. Some background staining was seen in the Cathepsin D blot that may be due to anti-Cathepsin D cross-reacting to additional related proteases within the PRM.

3.3.5 Purification of membrane protein enriched fractions separated by various methods of centrifugation

Several methods incorporating centrifugation were compared to identify which method could reliably produce distinct fractions of proteins from a starting mite homogenate. Separated fractions, concentrating particularly on fractions hypothetically containing membrane proteins, were analysed by SDS-PAGE for variability in protein banding. The reliability of each technique to produce the same banding pattern over repeat experiments was analysed.

i) Differential centrifugation including addition of detergents

This technique produced a protein-banding pattern by the membrane-bound protein fraction (indicated by red stars – Figure 3.8) that was reproducible in three repeat experiments. This banding pattern also showed prominent bands at sizes not seen in other fractions (marked as red arrows). Some bands however did appear in multiple fractions (indicated by blue arrows), demonstrating the limited capacity of this technique to fractionate completely 'pure' fractions.

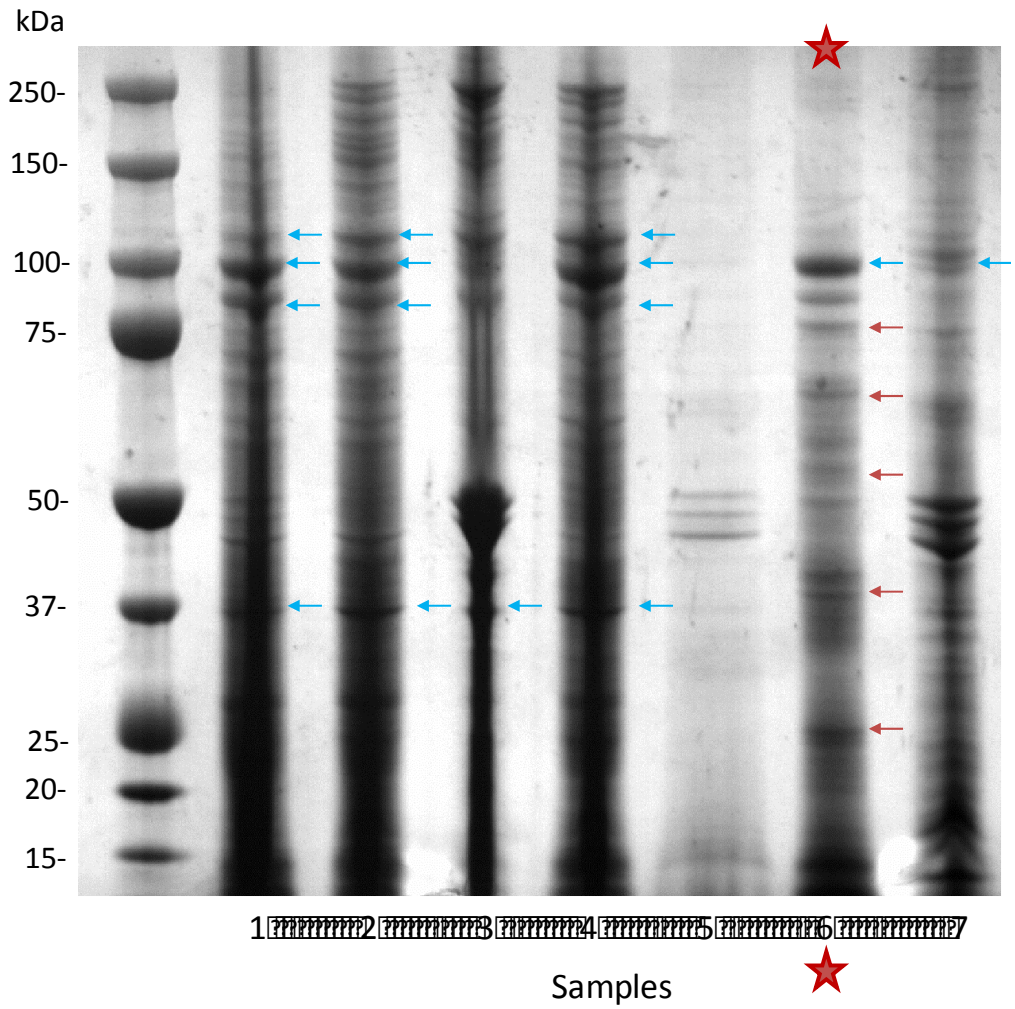


Figure 3.8: SDS-PAGE of fractions separated using 'differential centrifugation and addition of detergents' technique. Lane 1 – PRM homogenate, lane 2 – supernatant of 'PBS soluble' fraction, lane 3 – pellet of 'PBS soluble' fraction, lane 4 – supernatant of 'Tween soluble fraction, lane 5 – pellet of 'Tween soluble' fraction, lane 6 – supernatant of ''Triton soluble' fraction hypothetically enriched for membrane proteins, lane 7 – pellet of 'Triton soluble' fraction. Red stars indicate the fraction where integral membrane proteins are expected and red arrows indicate protein bands that appeared unique to that fraction. Blue arrows indicate examples of bands that appeared in multiple fractions. kDa – kiloDaltons.

ii) Differential centrifugation without the presence of detergents

PRM homogenate was fractionated by step-wise centrifugation at increasingly higher g forces. This technique was based on the hypothesis that membrane proteins remain attached to membrane fragments/particles that were sedimented at 100,000g. Though differentiated banding patterns between the 1,000g pellet and the 10,000g pellet fractions (indicated by red arrows, Figure 3.9) were observed, few differences between the 10,000g pellet, 100,000g pellet and the 100,000g supernatant were observed. This fractionation process required separation by just centrifugation and it appeared there was little difference between fractions. This indicated that though cells were lysed, many proteins appeared to have been only partially solubulised during 10,000g and 100,000g spins.

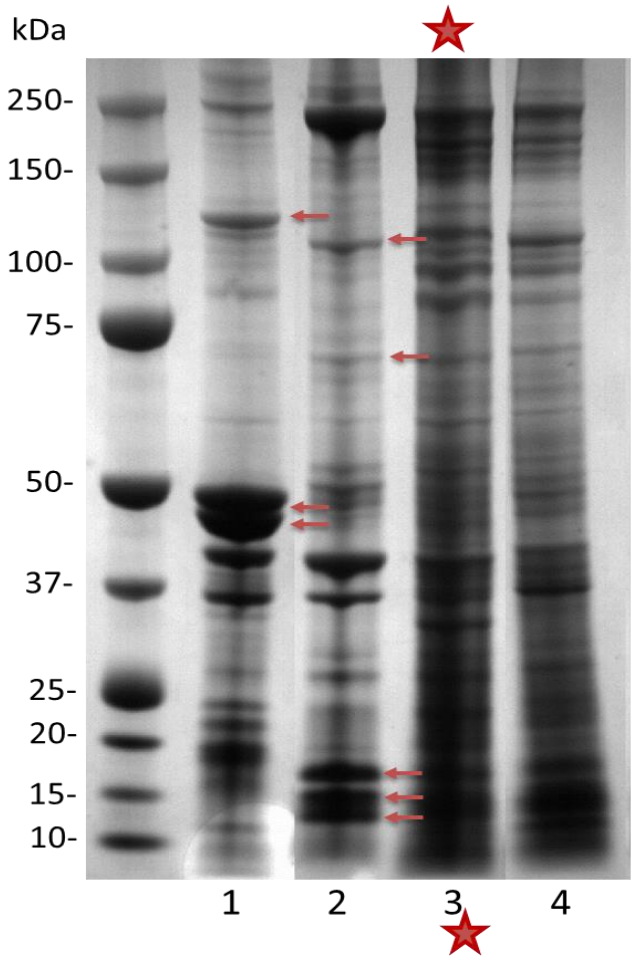


Figure 3.9: SDS-PAGE of fractions separated using the 'differential centrifugation without detergents' technique. Lane 1 – 1,000g pellet containing unlysed cells and nuclei, lane 2 – 10,000g pellet containing mitochondria, lane 3 – 100,000g pellet containing membrane fragments, lane 4 – 100,000g supernatant containing ribosomes and lysosomes. Most bands that appeared in the 100,000g pellet also appeared in the 100,000g supernatant, therefore producing a similar banding pattern. Fractionation appeared to have worked between the 1,000g fraction and the 10,000g fraction, producing distinct bands in both fractions (represented as red arrows). Fewer differences were noted between the 10,000g pellet and the 100,000g pellet, both having similar patterns. All samples produced many bands, indicating that all fractions were still complex mixtures. Red stars indicate the membrane protein enriched fraction. kDa – kiloDaltons.

iii) Dounce homogenisation and low speed centrifugation

This technique was based on the theory that a gentler approach to homogenization using a dounce homogenizer would lyse cells leaving membrane sheets, rather than much smaller individual membrane proteins/fragments (Scott, Schell et al. 1993; Graham 2002). An initial 250g spin hypothetically sedimented unlysed cells, followed by a 1500g spin of the supernatant that sedimented broken up membrane sheets. A final 3000g spin of the 1500g supernatant sedimented mitochondria.

Similar to results of centrifugation using detergents (Figure 3.8), the low-speed centrifugation method produced a banding pattern that was unique to the fraction hypothetically containing membrane proteins (Figure 3.10, lane 5 – indicated by red stars). Many bands were however seen in multiple fractions (blue arrows), demonstrating lack of purity in fractions. Unique banding pattern of the membrane sheet enriched fraction was due to lack of bands that were otherwise present in other fractions, rather than presence of bands unique to that fraction.

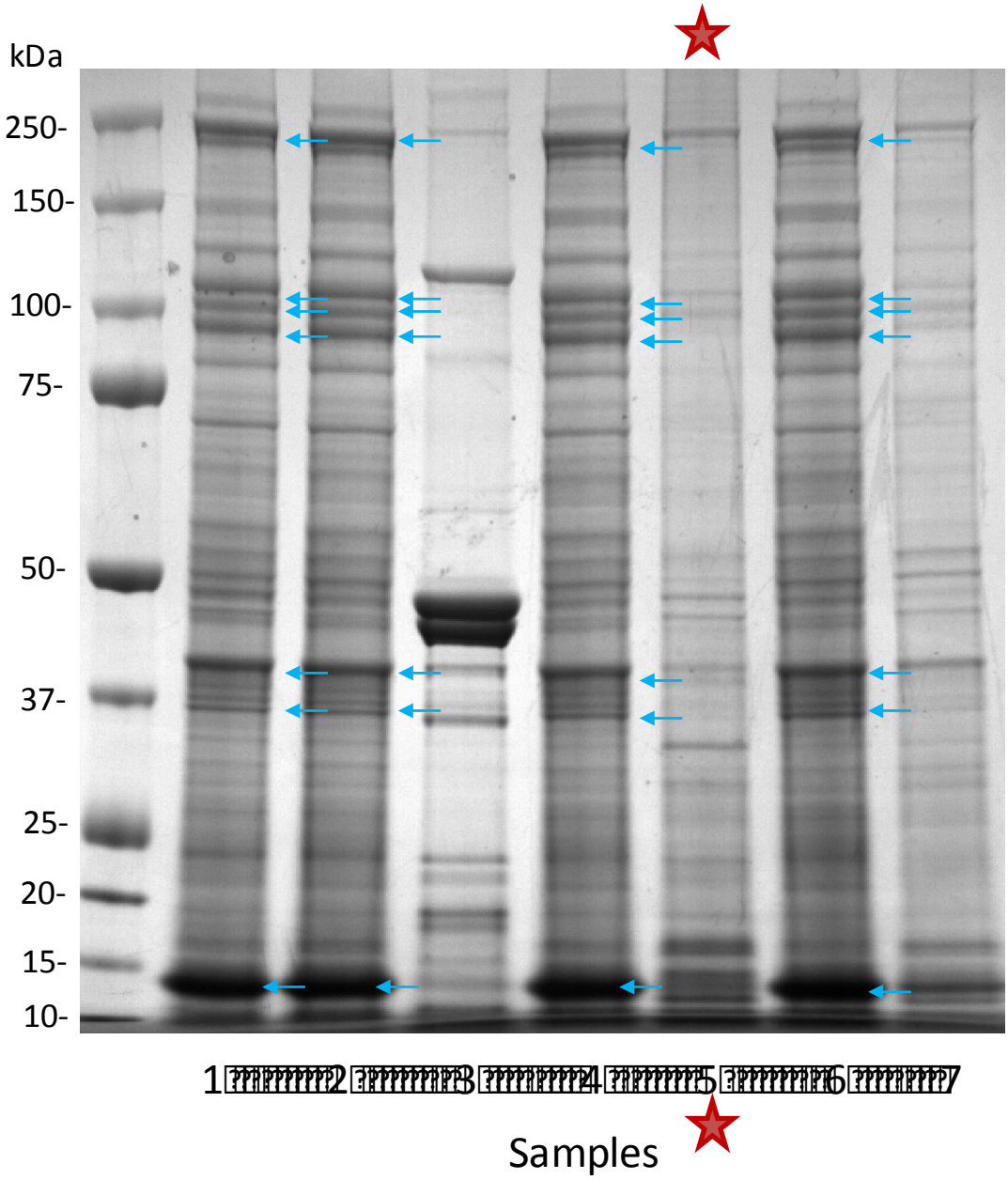


Figure 3.10: SDS-PAGE of fractions separated using the 'low speed centrifugation' method. Lane 1 – PRM homogenate, lane 2 – 250g supernatant, lane 3 – 250g pellet (containing unlysed cells), lane 4 – 1500g supernatant, lane 5 – 1500g pellet (containing membrane sheets), lane 6 – 3000g supernatant, lane 7 – 3000g pellet (containing mitochondria). Red stars indicate 'membrane sheets' enriched fraction, blue arrows indicate examples of protein bands that appeared in multiple fractions. kDa – kiloDaltons.

iv) Sucrose gradient centrifugation

Rate-zonal centrifugation through a sucrose gradient was used to separate subcellular components based on size. The resulting layers of subcellular fractions in the centrifugation tube were visible by different shadings of brown (e.g. Figure 3.11 fractions 7 and 8) or by layers of insoluble material (e.g. fractions 2 and 12). Much of the homogenate appeared not to enter even the top part of the sucrose gradient suggesting that it was composed of low-density material (figure 3.11 fractions 9-11). Other fractions appeared almost absent of subcellular material (fractions 4-6). This observation was supported by the comparatively low amounts of protein shown in the less concentrated sucrose fractions in SDS-PAGE (Figure 3.12, fractions 9-11). Protein banding patterns were similar throughout all fractions, despite observational colour differences in the gradient. Fractionation through the gradient therefore appeared to have shown limited capacity to separate subcellular material.

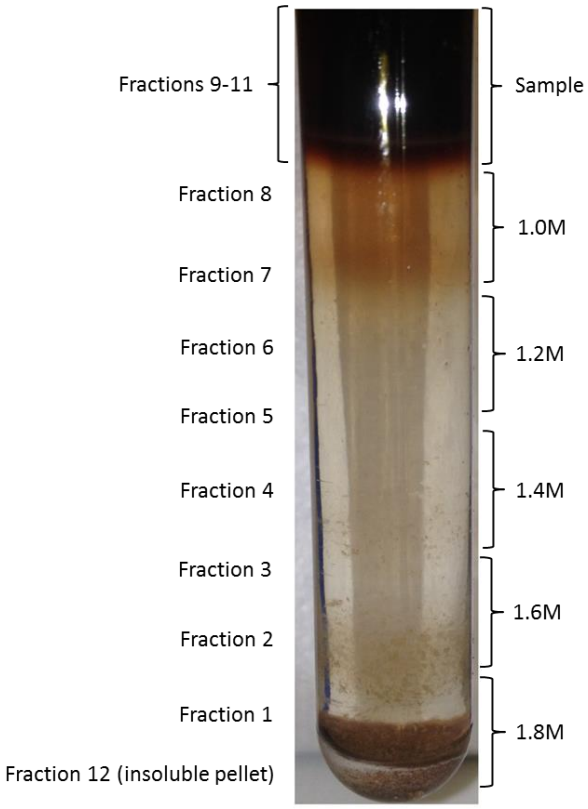


Figure 3.11: Fractions of PRM homogenate separated throughout a sucrose gradient after centrifugation. This tube was a result of a sucrose gradient layered from five concentrations of sucrose solution (1.0M – 1.8M) with a PRM homogenate (sample) layered on top. Centrifugation appeared to separate several fractions of subcellular material (fractions 1-12) which was extracted via piercing of the bottom of the tube. Some soluble proteins and small fragments remained on top of the gradient (fractions 9-11) whilst some larger fragments appeared to separate through only the 1.0M sucrose solution (fractions 7+8). Several layers of seemingly solid parts of the homogenate appeared at various bands in the gradient (between fractions 1 and 2 and between 3 and 4) whilst much of the sample sedimented at the bottom of the tube (fraction 12). 1.0-1.8M = 1.0-1.8 Molar sucrose solution.

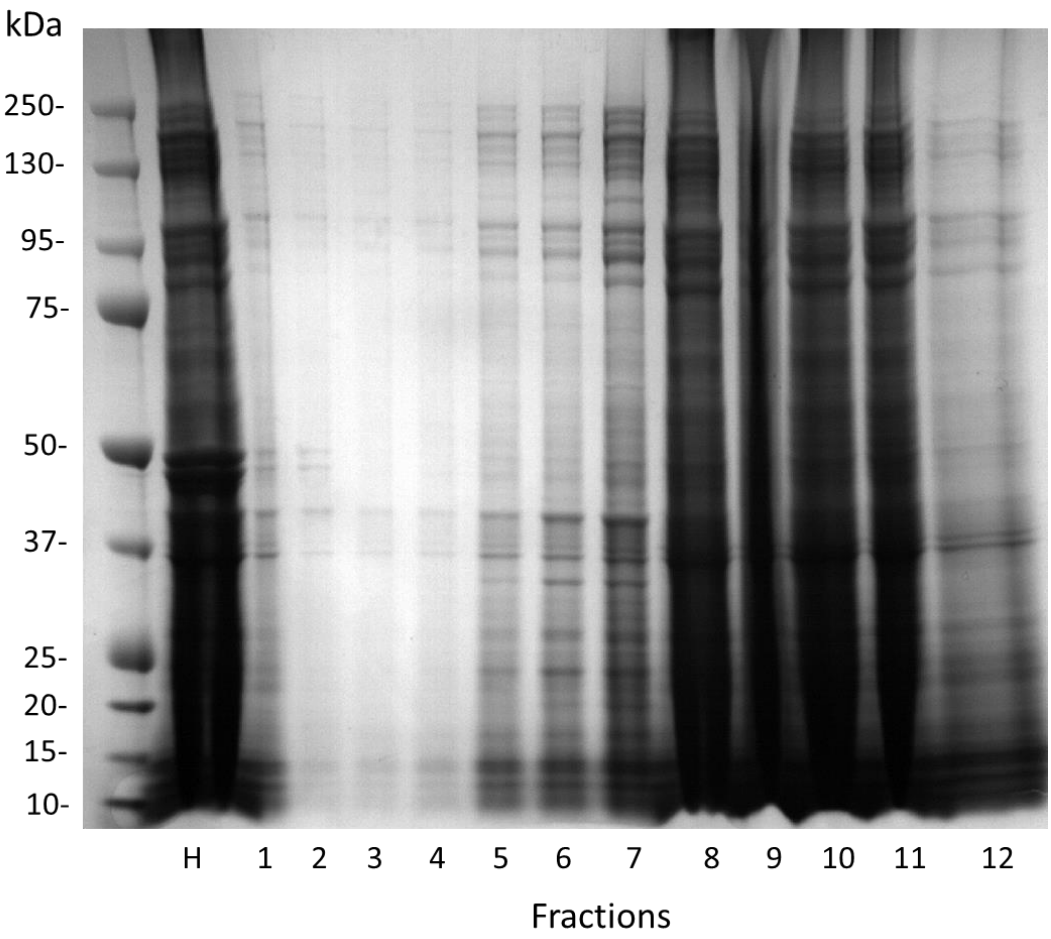


Figure 3.12: SDS-PAGE of fractions separated from sucrose gradient centrifugation. H – Homogenate, 1-12 = fractions 1-12. All fractions appeared to have very similar banding patterns and much more protein appeared in the sample that did not filter through the sucrose gradient (fractions 8-11). SDS-PAGE thus suggested that fractionation failed to differentiate proteins of varying densities. kDa – KiloDaltons.

v) Sigma Proteoprep membrane extraction kit

PRM homogenate was processed through a commercially bought Proteoprep extraction kit (Sigma) that is used to purify membrane proteins from a complex mixture. Fractions from various stages of the protocol were electrophoresed by SDS-PAGE (Figure 3.13). The end point sample, which in theory contained membrane proteins (Figure 3.13, lane 5, indicated by red stars), appeared to have little protein. SDS-PAGE gels appeared wide at the gel front and smeared in the centre, perhaps due to addition of detergents, making photography difficult. Such contaminants, could have affected downstream use of the membrane protein enriched mixture. Replicate purification experiments using the Proteoprep kit gave variable banding patterns (not shown) so this technique was unsuitable for extracting membrane proteins in future experiments.

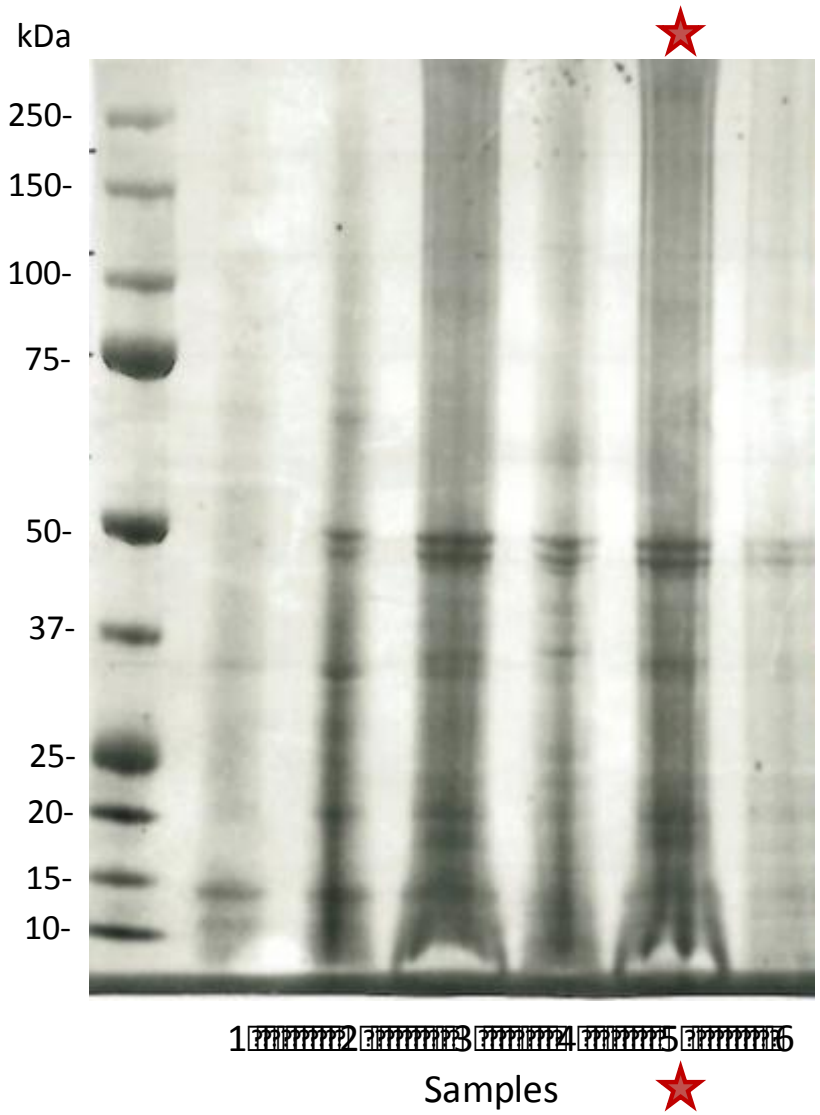


Figure 3.13: SDS-PAGE of PRM homogenate at various steps of the protocol of Sigma Proteoprep membrane extraction kit. 1 – supernatant after 115,000g spin containing non-membrane proteins, 2 – supernatant after 20,000g wash spin containing 'loosely bound membrane proteins', 3 – supernatant after addition of chaotopic buffer, 4 – pellet of 20,000g spin containing sonicated cell debris, 5 – supernatant after alkylation step containing plasma membrane proteins, 6 – pellet after alkylation step containing insoluble proteins. The fraction theoretically containing membrane proteins (fraction 5 – indicated by red stars) appeared to have a similar banding pattern to other non-membrane protein fractions (fractions 2+3).

Fraction 5 appeared to also contain material that influenced the run of the gel, most likely being detergent.

3.3.6 Mass spectrometry

The membrane protein enriched 'Triton soluble' sample produced by differential centrifugation and detergent (Fig 3.8, lane 6) was subjected to Matrix-assisted laser desorption/ionization (MALDI) mass spectrometry (Dr Dominic Kurian, University of Edinburgh). From mass/charge ratios analysed in the sample, hits were obtained against 11,186 contiguous RNA sequences (contigs) derived from PRM transcriptome data. These contigs were compared to NCBI protein databanks using BLASTx (nucleotide sequence to protein) to identify similarities with sequenced proteins in publicly accessible databases. From this 1,662 contigs (14.8% of the 11,186 total) were aligned with confidence to fully sequenced proteins from other organisms (e value<$1x10^{-50}$ and homologous identity >70%). The putative function of these homologous proteins was analysed via Uniprot.org and for each of the PRM contigs, a protein type (e.g. integral membrane, soluble) and expression location (e.g. mitochondria, cell membrane) was then inferred (Figure 3.14). Using this methodology, integral membrane protein function was inferred for 236 sequences of the 'aligned with confidence' contigs (14% of 1662). 448 contigs aligned with bacterial proteins (133 or 8%), chicken proteins (7 or <1%) or proteins of unknown function (308 or 19%) were removed from analysis (Figure 3.15). This resulted in a total 1214 contigs of which 19% were aligned to integral membrane PRM proteins. Of the remainder, 29% of contigs were inferred to encode cytoskeletal or cytoplasmic proteins.

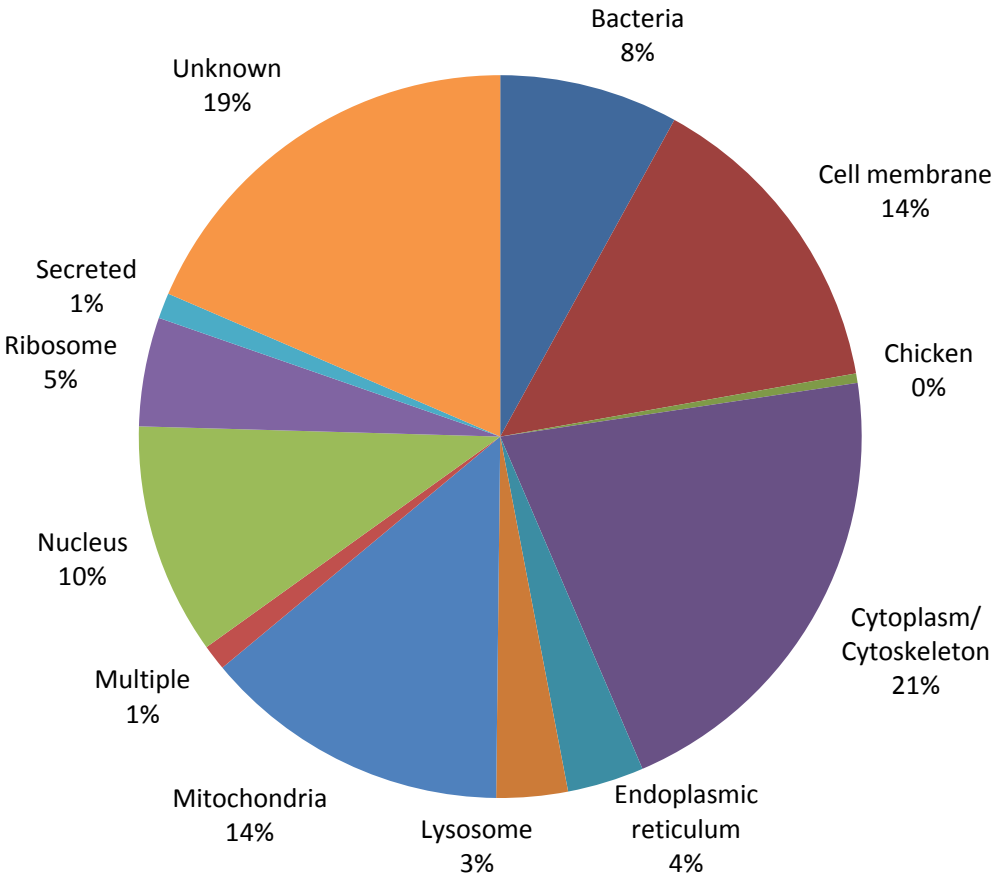


Figure 3.14: Categorisation of peptides in the membrane 'enriched' fraction, identified by mass spectrometry. Of the 1,662 PRM contigs that aligned with confidence to NCBI protein sequences using BLASTx, 19% aligned to proteins of unknown function, 21% were classed as cytoplasmic or related to the cytoskeleton; 14% were classed as membrane proteins, 14% as mitochondrial proteins and 10% as nuclear proteins. Less than 9% of peptides were categorised as 'contaminating' meaning an origin from bacteria, virus or chicken.

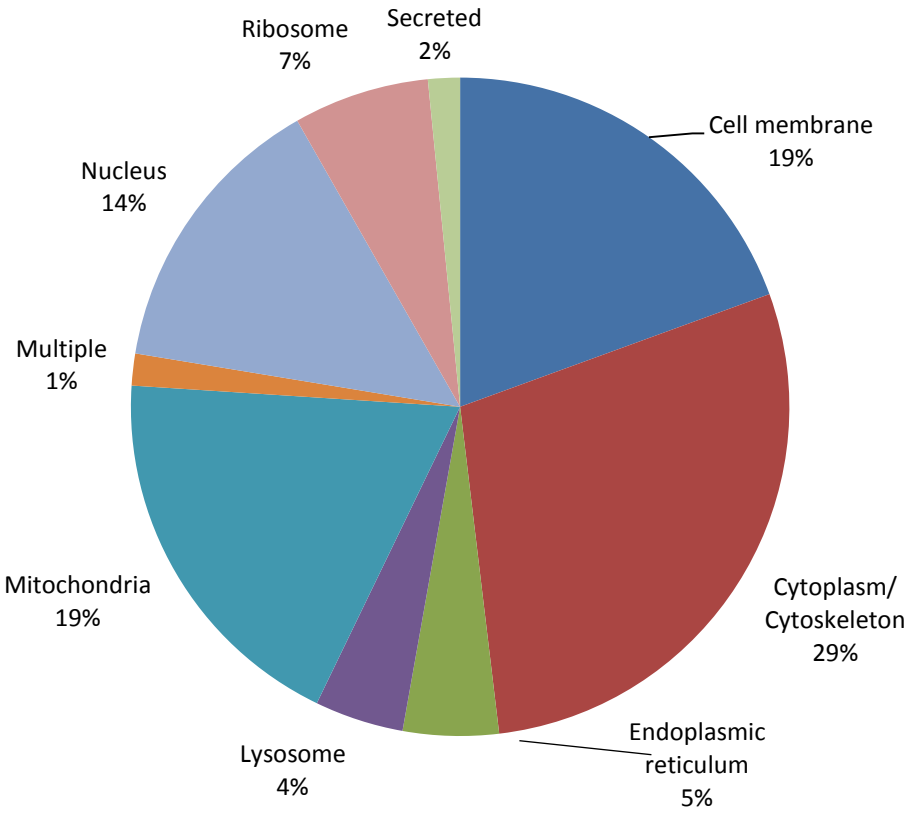


Figure 3.15: Categorisation of peptides in the membrane 'enriched' fraction, identified by mass spectrometry without inclusion of proteins expressed from chicken and bacterial origins or of unknown function. Removal of proteins from an unknown or non-PRM origin such as bacteria or chicken provided a more accurate description of PRM proteins in the fraction. This was a removal of 448 contigs to give a final number of 1214 PRM-originated contigs of known function. Similar to Figure 3.14, cytoplasmic and cytoskeletal proteins appeared most abundant (29%), whilst mitochondrial proteins (19%) and cell membrane proteins (19%) were slightly less abundant. Less than <1% of proteins were predominantly expressed in multiple cellular locations.

Of the 236 contigs aligned to cell membrane proteins, 28 showed over 90% sequence homology to a protein homolog sequence in NCBI datasets, defined by a '% identity' value in BLASTx (Table 3.3). Of note, Guanine nucleotide-binding protein was aligned with four contig sequences at >95% homology. Similarly, thirteen other proteins were aligned to three or more of the contigs via BLASTx, indicating a sign of apparent abundance in the fraction of each protein (Table 3.4). Laminin, V-type proton ATPase and Glutamate receptor 1 were the most common proteins to align with contigs from the Triton-soluble mixture with 11, 7 and 7 aligned contigs respectively (Table 3.4).

Hit description	% Identity
Membrane-associated protein, putative	98.93
Guanine nucleotide-binding protein subunit beta-2-like	98.53
Guanine nucleotide-binding protein G(q) subunit alpha-like	98.16
Synapsin-like	97.12
Syntaxin-binding protein 5-like	97.01
Guanine nucleotide-binding protein-like 1-like	96.59
Protein prickle-like	96.31
Sodium/hydrogen exchanger 2-like	96.05
Guanine nucleotide-binding protein subunit beta-2-like 1-like	95.76
VIP36-like protein-like	94.93
V-type proton ATPase 116 kDa subunit a isoform 1-like isoform 1	94.84
Acetylcholinesterase-like	94.67
Beta-parvin-like	94.39
23 kDa integral membrane protein-like	94.24
Synaptic vesicle membrane protein VAT-1 homolog-like isoform 2	93.49
Solute carrier family 22 member 6-like	93.37
Neuroglian-like	92.85
Vacuolar protein sorting-associated protein 52 homolog	92.79
Glutamate receptor 1-like	92.73
Rab GDP dissociation inhibitor beta	92.03
Fasciclin-1-like	91.81
Gamma-glutamyltranspeptidase 1-like	91.72
Vinculin-like isoform 1	91.5
GILT-like protein C02D5.2-like	91.46
Laminin subunit alpha-1	91.46
Scavenger receptor class B member 1-like	91.06
B(0,+)-type amino acid transporter 1-like	90.43
Innexin inx2-like	90.3
Y+L amino acid transporter 2-like	90.26

Table 3.3: Proteins with >90% identity values in BLASTx alignment to contig sequences identified from mass-charged ratios from the mass spectrometry of the membrane protein enriched fraction. % Identity indicates the percentage of homology a PRM RNA contiguous sequence has to peptide sequences of known proteins found in NCBI databases when aligned via BLASTx. PRM contig sequences are derived from mass-charge values of proteins in the 'Triton soluble', membrane protein enriched fraction when analysed against the PRM transcriptome. All protein homologues derived from the *M. occidentalis* transcriptome (Hoy, Yu et al. 2013) apart from Membrane-associated protein, putative which derived from the *Ixodes scapularis* transcriptome (Hill and Wikel 2005).

Hit description	Number of Sequences
Laminin subunit	11
V-type proton ATPase 116 kDa subunit-like	11
Glutamate receptor 1-like	7
Guanine nucleotide-binding protein-like	6
Innexin	5
AP-2 complex subunit alpha	4
Charged multivesicular body protein	4
Integrin alpha	4
Acetylcholinesterase-like	3
Aquaporin	3
Coatomer subunit delta	3
Scavenger receptor class B member 1-like	3
Transmembrane protease serine 3-like	3

Table 3.4: BLASTp matched protein functions aligned to >2 contig sequences defined from mass spectrometry data of the membrane protein enriched fraction.

3.3.7 Summary of results

- Freezing PRM in liquid nitrogen and homogenisation using a tissue disruptor is the most effective method to develop a sub cellular mixture. This can be verified by microscopy and also by Bradford assay to determine homogenate protein concentrations (average 14.34mg/ml in 15ml from 1g live mites).

- Live mites can be manipulated using ice, forceps and aqueous conditions to slow movement. PRM however are too small to dissect and in particular the structure of the gut is too fragile and cannot be removed without rupture.

- Quantification of protein concentration by Bradford assay is affected by the addition of 1% Triton x-100, using either Bradford or Bradford Ultra reagent. Sample absorbance values at 595nm are roughly doubled at protein concentrations 0.5mg/ml and 1mg/ml when in the presence of 1% Triton X-100.

- From all the centrifugation methods analysed the most reliable in terms of generating reproducible results and differential protein profiles was the 'differential centrifugation with addition of detergents' method. This method has been carried out previously for protein fractionation of PRM with similar reports of successfully enriched fractions, demonstrated in SDS-PAGE (Wright, Nisbet et al. 2011).

- Analysis of the membrane-enriched fraction from the differential centrifugation and detergents protocol by mass spectrometry identified peptide hits to 11,186 contigs of RNA sequence from PRM transcriptomic data. A subset of 1,662 contigs were analysed and 236 were found to align to proteins in the NCBI database that are defined as membrane proteins.

3.4 Discussion

3.4.1 Homogenisation and centrifugation of live PRM to a subcellular membrane protein enriched sample.

Freezing in liquid nitrogen followed by tissue disruptor homogenisation proved the most efficient way to break open mite cells (Table 3.2, Figures 3.3 and 3.8). This was therefore the method taken forward in later experiments to develop a membrane protein enriched PRM homogenate. Mite tissue was quickly frozen in liquid nitrogen (-190°C), becoming hard and brittle. Instant freezing is suggested to exert tension and stress on tissue (Smucker and Pfister 1975; Volossiouk, Robb et al. 1995) making homogenisation much easier than samples that were slowly frozen to -20°C. Snap freezing in liquid nitrogen has also been shown to be beneficial in maintaining protein confirmation and structure when compared to freezing protein over longer periods of time (Carpenter and Crowe 1988). Slow freezing at -20°C produces ice crystals between spaces in the protein structure and over time can create sheer force that may disrupt protein structures (Mazur 1984). Insufficient space for solute molecules due to water crystals within a protein or salt structure can also alter the buffer pH or precipitate proteins and salts, further damaging protein structure (Strambini and Gabellieri 1996).

Lysis of tissue/cells using a tissue disruptor was carried out in less space than using a mortar and pestle (5cm^2 tube vs. 130cm^2 pestle). This increased the chance of cells being placed under sheer forces and may explain why tissue disruptor homogenisation was more successful. Reproducibility of grinding using a mortar and pestle was operator dependent and inefficient compared to the tissue disruptor. This is in line with similar studies that report mortar and pestle to have a more gentle approach to cell lysis (Goldberg 2008). Bead beating produced sub cellular fractions from some broken up mites but left other mites intact (Figure 3.3)

resulting in low amounts of soluble subcellular material (table 3.2). This may have been explained by the use of 100μm beads that are designed to break open cells but not whole organisms. A more effective approach would have been to use a stepwise approach using several bead sizes – however as mentioned by Burden (2008) optimisation of bead size, tube size and beating time can be unnecessarily difficult.

Use of alternative buffers to PBS, including a hypotonic buffer or an alternative RIPA cell lysis buffer (see Appendix), made little difference to cell lysis analysed by microscopy at x40 and protein concentration by Bradford assay (data not show). Repeat liquid nitrogen freezing and disruption of the same homogenate, extension of homogenisation times (2 x 5 minutes, full power) and sonication of already mechanically homogenised samples (5 x 2minutes, 15Amps) also did not improve amount of cell lysis (not shown). Lack of change in final protein concentration or apparent cell lysis by these additional homogenisation steps therefore suggested the liquid nitrogen + tissue disruptor method for cell lysis (described in section 3.2.3) was already suitably efficient to use in future experiments. Homogenisation of 1g frozen mites produced 0.215g protein before enrichment steps (14.34mg/ml x 15ml) and between 0.018 – 0.051g protein (1.17-3.38mg/ml x 15ml) after differential centrifugation enrichment steps. SDS-PAGE required 5-50μg total protein in 15μl total volume and biopanning required 100μg in 100ul/well. Given these requirements, proteins from this membrane enriched fraction could be clearly detected in a protein gel and used in biopanning experiments. Homogenisation of 1g of mites was larger than the 0.1g tested in experiments, however larger volumes of 1g PRM batch purification allowed fewer homogenisation experiments and samples could also be stored in aliquots at -80°C.

Pre-homogenisation dissection was attempted on PRM, however it was not possible to extract intact midguts. As shown by Patton, Dietrich et al. (2012), midgut dissection from even a much larger blood fed *Ixodes scapularis* Deer tick can be extremely difficult without rupturing the

midgut. If dissection of red mite was pursued further, either dissection similar to mosquito midgut dissection (removing midguts attached to a posterior part of the exoskeleton (Coleman, Juhn et al. 2007)), or the use of laser dissection could potentially be incorporated. Laser microdissection has been used to dissect midguts from mosquitoes (Hong, Kang et al. 2011) however it is normally used for smaller organisms such as endoparasitic worms or flukes (Jones, Randall et al. 2004). Our downstream techniques required large volumes of fractionated homogenate and so the large amounts of successful dissections required would have been unrealistic.

3.4.2 Triton X-100 – requirement and limitations

Bradford and Bradford Ultra reagents showed inaccurate protein quantification in the presence of 1% Triton X-100, possibly due to detergent molecules facilitating non-ionic interactions between dye and protein. Triton X-100 however, was required to solubilise membrane proteins in differential centrifugation experiments. To extract the most membrane proteins from PRM homogenate it was important to add sufficient Triton X-100 without going over the critical micelle concentration (CMC). Further addition in Triton X-100 past the CMC could have negatively affected protein quantification, as well as protein binding in biopanning, without increasing the amount of membrane protein solubilised. Triton X-100 CMC is 0.24 mM (0.0155%, w/v) and membrane fractions were used in biopanning at a 1:100 dilution. Assuming membrane enriched fractions included a maximum of 1% Triton X-100 (w/v), due to all detergent micelles not pelleting, then a 1:100 dilution would bring Triton X-100 concentration to 0.01% (w/v). This was close to, but below the 0.0155% (w/v) CMC. This suggested therefore that 1% Triton X-100 was an optimal amount of detergent to use. In theory, other protein quantification assays, such as Bicinchoninic Acid (BSA) assay, are more robust in the presence of detergent (Walker 2009). Time constraints, due to optimisation of the membrane protein purification technique, did not allow time to test the BSA assay sufficiently, as is recommended

if detergents are present (Brown, Jarvis et al. 1989). The effect of detergent on protein quantification was originally challenged by using Bradford Ultra reagent, that was marketed as being usable for solutions containing 1% Triton X-100. This however proved more inaccurate than Bradford reagent (Figure 3.6) and so was not used in further experiments. Bradford Ultra is marketed at utilising a 0.1-1.5mg/ml standard curve, however is mentioned to…

'…show excellent linearity, but within a narrow range of protein concentrations that needs to be well-defined with standard spectrophotometers'. (Biocompare 2008)

Though the 0.5 and 1.0mg/ml samples used (see figure 3.6) are within this concentration range, the spectrophotometer was calibrated to run Bradford but not Bradford Ultra, perhaps leading to discrepancies in absorbance value read out accuracy.

1% Triton was substituted with various other detergents including Tween 80, SDS and CHAPS (3-((3-cholamidopropyl) dimethylammonio)-1-propanesulfonate), however following differential centrifugation, SDS-PAGE banding patterns were similar for all detergents (data not shown). Detergent removal was also attempted, using a FASP protein digestion kit (Expedeon #44255), as Triton X-100 could affect mass spectrometry analysis of membrane enriched homogenates. This technique used filters to remove detergent from the sample and hypothetically, some membrane proteins were part of the removed detergent micelles. The kit was also expensive and designed for small volumes (100µl). The FASP kit was therefore considered inappropriate for large scale detergent removal during differential centrifugation.

3.4.3 Purification of a membrane protein enriched fraction

Analysis of SDS-PAGE results from each of the centrifugation protocols to produce a membrane protein enriched fraction revealed differential centrifugation with addition of detergents (see section 3.2.3) to be the most successful technique. This method was the only one that generated a unique banding pattern in the membrane protein enriched fraction

(Figure 3.7) which could be reliably reproduced in replicate experiments. Other techniques showed comparatively little variation between fraction banding patterns (Figures 3.8 and 3.10) or were not reproducible (Figure 3.11). Low speed centrifugation to purify membrane protein sheets actually fulfilled the criteria of unique and repeatable banding, however the unique banding came from absence of bands compared to other fractions, rather than presence of bands unique to that fraction (Figure 3.9). Reasons for failure to separate proteins in sucrose gradient centrifugation may have been due to homogenate requiring a lower concentration gradient. Much homogenate did not pass through even the first 1.0M sucrose layer and other particulate and aggregated material formed as a pellet at the bottom (see figure 3.11 fraction 12). This indicated the inclusion of too much higher molecular mass material and therefore suggested this technique needed further homogenisation of the PRM sample. Lack of fractionation in differential centrifugation without presence of detergent only failed to differentiate banding patterns between supernatant and pelleted material at 100,000g. Interestingly, this may provide indirect evidence for the necessity of Triton X-100 for solubilisation of membrane proteins.

A limitation of all of the centrifugation techniques was the lack of definitive purification in each fraction. All SDS-PAGE gels contained some protein bands that were present in all fractions (red arrows, Figures 3.7–3.12). Fractions derived from a complex protein mixture containing 1000s of proteins and so 100% purification or removal of all proteins in each fraction would have been unrealistic. In an attempt to increase purity in the membrane-enriched fraction, duplicate spins of 25,000g and 100,000g were carried out on the supernatants of the first 25,000g and 100,000g spins. Protein bandings were the same after these repeated centrifugations (not shown) suggesting further enrichment did not occur.

Many other methods of protein purification do exist, including hydrophobic or ion exchange chromatography for example. Without a specific known target protein, centrifugation was

applied due to previous success of differential centrifugation from Acari (Willadsen, McKenna et al. 1988; Wright, Nisbet et al. 2011) as well as capacity of centrifugation to produce large volumes compared to column-based methods. Initial start-up cost is expensive in comparison (ultracentrifuge, centrifugation tubes), however running costs are low as no further expensive reagents, preparative costs or equipment are required.

3.4.4 Mass spectrometry of the membrane protein enriched fraction

Transcriptomic analysis of a mixed stage population of PRM carried out by Schicht, Qi et al. (2013) identified 7,361 contigs (13.4%) of a total 55,129 contigs as putative *D. gallinae* membrane proteins. Richards, Stutzer et al. (2015) identified only 878 putative transmembrane proteins (6.5% of total) from 13,456 contigs of a *Rhipicephalus microplus* transcriptome. In an unfractionated homogenate of a 'typical' eukaryote, membrane proteins make up approximately 30% of the overall transcriptome or genome (Nugent and Jones 2009). Our fraction identified 236 (19%) contigs of a subset of 1214 PRM contigs aligned to putative membrane proteins. This was an increase in membrane proteins when compared to the non-fractionated transcriptome of Schicht, Qi et al. (2013), from 13.4% to 19.4%. The true success of membrane protein enrichment in fractionation however was difficult to quantify. Our project specifically aimed for identification of integral membrane proteins of the cell membrane. The 236 contigs described here are expected to be mainly integral membrane proteins, as peripheral membrane proteins were hypothetically removed at the Tween 20 stage. Some peripheral proteins were however still present, such as Syntaxin-binding protein 5 (see Table 3.3. and (Fujita, Shirataki et al. 1998)).

The PRM transcriptome used in this analysis was limited in its annotation due to large amounts of highly repetitive regions and production of short reads which limited alignment success. Overall, the transcriptome was composed of 214,263 clustered short Illumina 454 reads,

maximum contig size of 16,595 bp and N50 value of 1,056 (i.e. 50% of the entire assembly was contained in contigs equal to or greater than 1,056 bp).

Of the sequences identified, contigs aligned to Laminin, V-type proton ATPase and Glutamate receptor 1 were the most numerous (Table 3.4). Laminin is a common component of the extracellular matrix involved in cell adhesion and cell signalling. Laminin is present in most tissues in arthropods however has been specifically associated to downregulation of macrophages and other immunosuppressive functions in the saliva of blood feeding ticks (Ganapamo, Rutti et al. 1996). V-type ATPases are essential proton pumps in many organisms and are ubiquitous in their expression across tissues. Though not yet targeted by vaccination in Acari, many acaricidal sprays disrupt ATPase function, specifically targeting respiration and neural pathways (Desaiah, Cutkomp et al. 1973; Kadir and Knowles 1991; Ishaaya and Horowitz 1998). Glutamate is a neurotransmitter that exerts an inhibitory effect on the muscle activity in insects and mites. Glutamate receptors are specific to arthropods and so if they were targeted as potential vaccine candidates, they would pose a low risk of producing autoimmune diseases in poultry during vaccination. A Guanine nucleotide-binding like protein was aligned to six contigs, of which four had a >90% sequence homology (the '% identity', Figure 3.15) to sequences found in the *Metaseiulus occidentalis* transcriptome (Hoy, Yu et al. 2013). Guanine nucleotide binding proteins (G proteins) and their receptors form part of one of the most prevalent membrane-associated signalling pathways in eukaryotes. G protein receptor signal transduction to intracellular G proteins is associated with a range or processes, including hormone regulation and cell growth (Roberts and Waelbroeck 2004). Bosch and Siderovski (2013) mention "G proteins or their receptors may make up to 30% of cellular targets for all modern day drugs". This is a large family of receptors so further work would be required to study the exact role and function of the sequences aligned with the G-protein receptor indicated in Figure 3.15.

Of the initial 1,662 contigs, 8% were homologous to bacterial proteins (Figure 3.14). These were considered contaminating and were likely endosymbiotic bacteria, or surface contaminants. Bacteria from PRM blood meal may have also been present in the Triton soluble fraction, however <1% of contigs were aligned to chicken origin suggesting contents of the blood meal were successfully broken down during 7 day digestion before homogenisation. Time until complete PRM digestion of blood was not previously fully known, however sectioning of blood fed mites in consecutive days after feeding (see Figure 1.3 in Introduction) saw blood dissipate after 5-7 days. Fractionation was carried out 7 days post capture, allowing full digestion of blood from PRM, however allowing for presence of proteins expressed during blood digestion to remain. PRM are known to carry many species of bacteria, both internally and externally (Moro, Thioulouse et al. 2009), and blood meal can take up as much as 70% of the body cavity when a mite is fully fed. Such is the presence of blood meal, and most probably bacteria too, and then 8% of a total homogenate consisting of this 'contaminating' source seems an acceptable amount.

3.5 Conclusions

This chapter has described the homogenisation of PRM into a sub cellular material that was further purified to create a fraction hypothetically enriched for PRM membrane proteins. Various techniques and protocols were tested to ensure the method used in future experiments to purify a membrane fraction was efficient and reliable. Further experiments including biopanning, immunoprecipitation and mass spectrometry overall required large amounts of membrane enriched homogenate and so, to this extent, the production of such large amounts of fraction was a success. Enrichment of membrane proteins appeared to be a success also, however the full extent of enrichment would require further analysis of all the membrane proteins in the fraction.

Chapter 4: Selection of single chain variable fragments specific to mite membrane proteins

4.1 Introduction

This chapter describes the use of biopanning a single chain variable fragment (scFv) library against the mite membrane protein-enriched mixture purified in chapter 3. A library of $1.37x10^8$ scFv variants attached to M13 filamentous bacteriophage were incubated in the presence of the mite protein fraction. ScFv molecules that bound specifically to proteins in the fractionated mite sample were purified by repeated binding and washing steps. Because of their antigenicity in the biopanning, the molecular targets of these purified scFv molecules were hypothesised to be potentially good vaccine candidates that may elicit strong antibody mediated immune responses in chickens. In addition, the purified scFv molecules were proposed be useful tools to pinpoint target protein localisation and expression in the mite body using immunohistochemistry. Because the overarching aim of this thesis is to identify membrane-associated proteins expressed within the digestive system of the PRM, only scFv that bind to the gut region of mites would subsequently be targeted as vaccine candidate proteins (see Chapter 5 and final discussion).

4.1.1 Phage & the phage replication cycle

A bacteriophage, or 'phage', is a virus that infects and replicates inside a bacterium. A phage comprises a single stranded DNA (ssDNA) plasmid genome encapsulated by a

protein coat. Upon infection of a bacterium the coat proteins envelop integrates with the cell membrane and the ssDNA enters the cytoplasm (see Figure 4.1 - step i). RNA polymerase synthesises an RNA template initiated at the origin of replication which then acts as a template for DNA polymerase to synthesise dsDNA molecule (step ii). The production of dsDNA is required both for transcription and translation of new phage proteins and as a 'replicative intermediate' for the amplification of single strand DNA to be packaged as new phage. Phage-derived F1/M13 origin of replication allow the replication of ssDNA whilst a colE1 origin of replication for dsDNA.

There is then a temporal switch and dsDNA is transcribed and translated into new phage proteins by the cell (step iii). Phage proteins pII, pV and pX mediate packaging of the ssDNA molecules and phage proteins pI, pIV and pXI form a transport complex (step iv). Packaged ssDNA is transported to the cell membrane by the transport complex and combined with pVIII major coat protein particles in the membrane to form a new phage structure that is secreted into the extracellular environment ready to infect a new cell (step v).

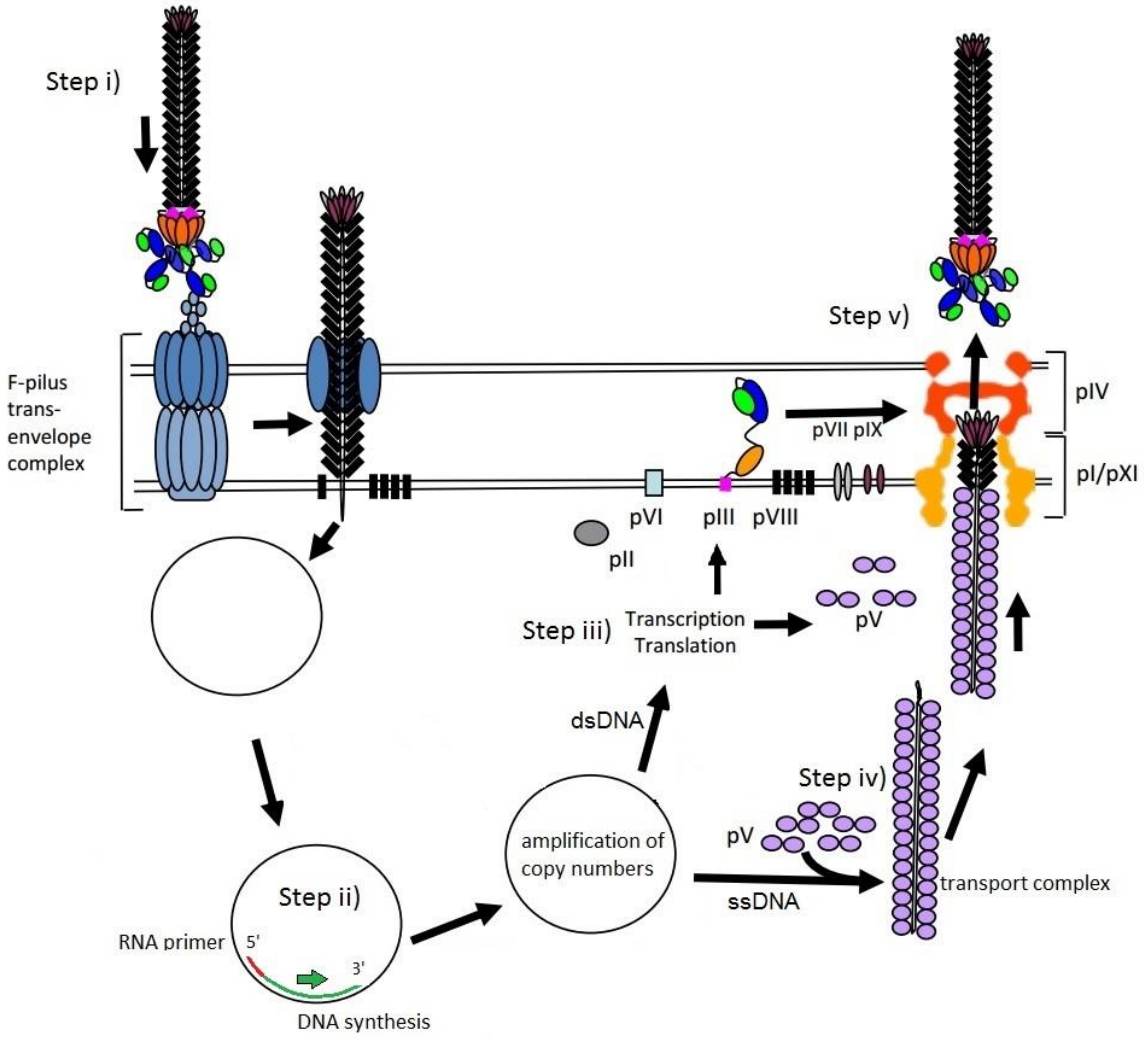


Figure 4.1: The reproduction cycle of a filamentous bacteriophage, adapted from (Rakonjac 2012). Phage replicate by invasion of a bacteria cell and use of bacterial cell machinery. The replication cycle can by lytic or lysogenic to the cell. Step i) A filamentous phage molecule binds to the bacterial cell and injects phagemid ssDNA into the cell cytoplasm through mechanical penetration. Step ii) Phage ssDNA and dsDNA is replicated using a DNA polymerase bound to an RNA primer. Step iii) dsDNA transcribed and translated into phage proteins. Phage proteins pII, pV and pX mediate packaging of dsDNA molecules and phage proteins pI, pIV and pXI form a transport complex. Step iv) A transport complex encapsulates a single ssDNA phagemid and

transports the sequence to the cell membrane Step v) A ssDNA strand is enveloped by pVIII phage coat proteins residing in the cell membrane and the whole structure is exocytosed to the extracellular space as a new phage molecule. Most filamentous phage (as seen here in step v), including the M13 phage used in the red mite project, follow a lysogenic replication cycle and thus do not lyse host cells.

4.1.2. Phagemids and phage libraries

Phage are used as clonal vectors in molecular biology to transport DNA fragments into a foreign cell. Phage plasmids, or 'phagemids', contain an origin of replication (ORI), such as F1, allowing the production of ssDNA from a plasmid and packaging of the ssDNA into a phage particle. An ORI region is recognised and unwound by a pre-replication complex inside a bacterium and is the site where phagemid replication begins.

Insertion of DNA fragments into a phagemid can not only be used for straight forward cloning, but also to study protein-protein interactions. A gene of interest inserted into one of the coat protein regions of a phagemid will be transcribed, translated and displayed on the surface of a phage as a coat protein fusion (see Figure 4.2 step iii)). Insertion of highly variable genetic regions, such as that of the V (D) J gene regions in B cells, can be extremely valuable in developing a library of genetically unique phage variants. Such genetically diverse libraries are widely applied in many aspects of drug discovery, protein engineering and antibody therapeutics. Insertion of DNA taken from human antibody repertoires, for example, has been exploited in the isolation of phage-bound antibodies that specifically bind to TNF-alpha (Jespers, Roberts et al. 1994). This has led to the commercialisation of HUMIRA (Cambridge Antibody Technology), a TNF-

alpha specific antibody used as an anti-inflammatory. Other major studies that have implemented phage libraries include identification of tumour specific antibodies (Kupsch, Tidman et al. 1999), identification of ligands that block HIV-1 cell entry (Ferrer and Harrison 1999) and discovery of human hepatitis B virus peptides that stimulate humoral response (Folgori, Tafi et al. 1994). Due to the diverse genomic-protein link in a theoretically simple and straightforward model, this makes biopanning protein mixtures using phage-bound antibodies a powerful tool for detection of single targets in a complex mixture. This is the reason behind using a phage library to detect specifically immunogenic, antibody-binding proteins in the complex poultry red mite mixture purified in chapter three.

4.1.3. Single chain variable fragments (scFvs)

Insertion of whole antibody gene sequences into phage plasmids results in improper folding and aggregation of translated peptide sequences in the bacterial cytoplasm (Boss, Kenten et al. 1984; Cabilly, Riggs et al. 1984). An alternative system by Skerra and Pluckthun (1988) of inserting only the antigen binding domain developed overcame this problem by expressing a much smaller polypeptide. Genes encoding the variable light and heavy regions of the antigen-binding domain of antibodies can be inserted into a phagemid and displayed as a single fusion protein (see Figure 4.2). Insertion of genetic sequences of these smaller fusion proteins, known as single chain variable fragments (scFv), compared to larger monoclonal antibodies, is less toxic to cells.

An advantage of using a scFv library in biopanning includes purification of scFv to a specific target being quicker than using antibody libraries, normally only taking two or

three rounds of selection (Hoogenboom 2005). Furthermore, mice are not required to produce hybridoma cells as are required in whole antibody libraries, consequently reducing animal welfare issues (Pasqualini and Arap 2004). There is also less risk of an unspecific immune response developing to a delivered scFv fragment than to a larger, whole antibody molecule (figure 4.2 step i) due to the smaller size of the scFv protein (Figure 4.2 step ii) (Carmen and Jermutus 2002).

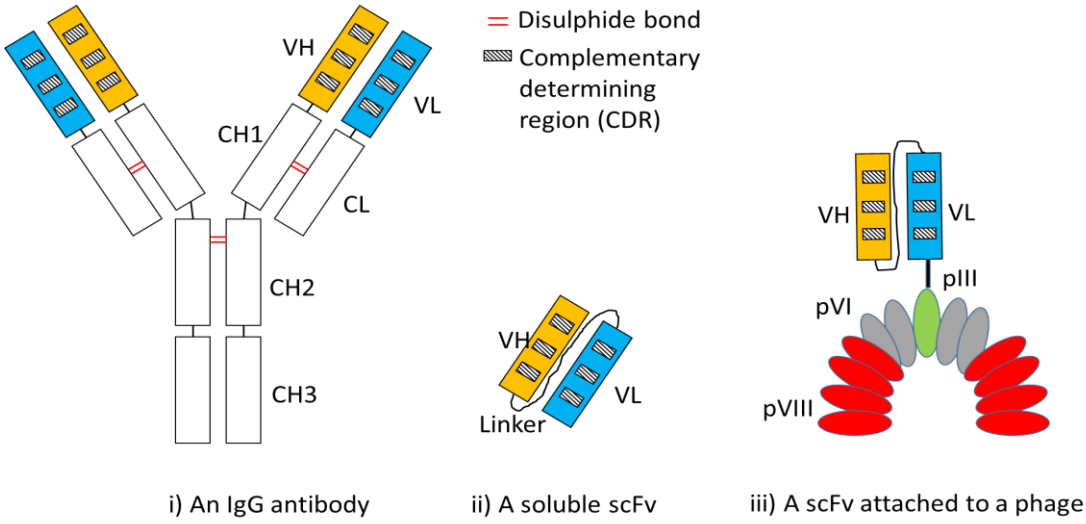


Figure 4.2: The structure of an antibody, soluble scFv and scFv displayed by a phage. An IgG antibody molecule (part i) contains two heavy chain molecules, each consisting of three constant domains (CH1-CH3,) and a variable domain (VH). As well, IgG has two light chains, each consisting of one constant domain (CL) and one variable domain (VL). These chains are crosslinked by disulphide bonding and each variable domain contains three complementary determining regions (CDR). A scFv (part ii) consists of a fusion between the heavy and light variable domains only, linked together by a glycine rich 'linker' polypeptide. Glycine linkers are chosen due to the ability of glycine molecules to rotate freely, therefore allowing exposure of scFv binding regions to potential target proteins. When a scFv gene is inserted into a phagemid (part iii), scFv genes are expressed as a single polypeptide fused to the pIII coat protein of the phage. Phage also have several other coat proteins, of which pVI and pVIII are shown here, though noting the full phage structure is not completely shown.

4.1.4. The Tomlinson J library

The group of Greg Winter at the MRC (Cambridge, UK) have cloned genes conferring a single chain variable fragment, based on the variable regions of human antibody heavy and light chains, into an ampicillin-resistant pIT2 phagemid (Figure 4.3). This created a scFv/phage library, referred to as the 'Tomlinson J library', that displays $1.37x10^8$ variations of a single variable fragment. The pIT2 plasmid also contains myc and his tag sequences for downstream purification of the phage, a ribosome binding site for initiation of protein translation, a pelB leader that signals phage peptides to a host cell periplasm and a phage gene III (gIII) that allows scFv fusion to the phage coat. pIT2 has an M13 phage origin of replication allowing packaging of ssDNA and a colE1 plasmid origin of replication for dsDNA replication. An amber stop codon between the scFv region and gene pIII means pIT2 plasmids can only be translated to polypeptides in bacteria that carry an amber supressing mutation in the corresponding tRNA molecule. The amber supressing bacterial strain used in conjunction with the Tomlinson J scFv library is *Sup E*, which translates a glutamine peptide in place of translational termination.

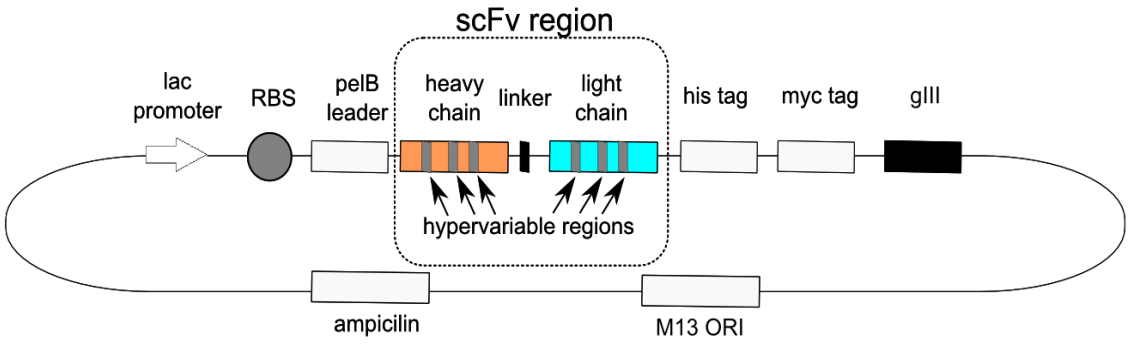


Figure 4.3: Vector map of pIT2 phagemid implemented in biopanning, including scFv region. RBS – Ribosome Binding Site, ampicillin – ampicillin resistance gene, M13 ORI – origin of replication. ScFv variable heavy and light chain fragments were inserted between the pelB leader and his tag sequences by restriction enzyme digestion (restriction sites not shown) and held together by a glycine linker (shown). The pelB leader sequence directs pIT2 translated peptides to a cell periplasm and secretory pathway. M13 ORI allows the plasmid to be replicated as ssDNA. The lac promoter and RBS aid in initiation of transcription and translation of the downstream scFv region. The region between the scFv region and the myc tag contains an amber stop codon, thus the scFv-gene III fusion protein in pIT2 can only be expressed in amber supressing bacteria.

The pIT2 scFv in the Tomlinson J library consists of a variable light chain and variable heavy chain of human origin, normally found on an antibody (Figure 4.2 step i)). ScFv variation is defined by variation in polypeptide sequences located in the six hypervariable regions of the heavy and light chains. Synthetic scFv libraries acquire such variation by the use of degenerate oligonucleotide primers and site directed mutagenesis. Degenerate primers are able to bind multiple loci of the ssDNA phage template DNA thus during PCR of the template can amplify products with different hyper variable region sequences.

4.1.5. The theory of biopanning poultry red mite (PRM) proteins

This project aimed to purify scFv from the Tomlinson J library that bound strongly to membrane proteins expressed in the digestive tract of PRM. Biopanning of the scFv library against a membrane-enriched fraction of red mite proteins and elution of non-bound scFv would, over several rounds, purify a more mite specific scFv library. ScFv variants from this library could then be cloned individually and exposed to whole mite sections in immunohistochemistry experiments (see Chapter 5). Gut-binding scFv could then be used as a tool to identify gut expressed proteins by techniques such as Western blotting and immunoprecipitation (see Chapter 5). In theory, such proteins would be good vaccine candidates, likely to induce strong antibodies in vaccinated poultry, because the *in vitro* scFv library mimics antigen binding regions found in an avian antibody repertoire *in vivo*.

4.1.6. Aims & Hypothesis

Included in this chapter are experiments carried out to purify scFv that bind to mite proteins. The aims of this work were to:

1. Select a scFv library that binds specifically to a membrane protein enriched fraction derived from chapter 3, by several rounds of biopanning (polyclonal selection).
2. Clone and purify individual scFv variants from within the scFv library that bind to the membrane protein fraction (monoclonal selection).
3. Define optimal conditions for mite protein binding to plates and scFv/phage binding to mite proteins.

4.2 Methodology

4.2.1 Quantifying protein binding conditions

i) The effect of detergent on protein binding to a Maxisorp® ELISA plate

The protein fraction purified from Chapter 3 contained a maximum of 0.1% (v/v) Tween 20 and 1% (v/v) Triton X-100, both of which can affect protein binding to ELISA plates during biopanning. The effect of detergent on protein binding in this system was therefore evaluated using purified, maltose binding protein.

0.2g Maltose Binding protein (MBP), containing a C-terminal six histidine (his) tag (expressed in plasmid pMal, a gift from Dr Rob Noad), was purified from bacteria using a His-tag purification column following manufacturer's instructions (Sigma, #6781535001) and solubilised in 10ml of 1xPBS (final 20mg/ml). 100µl of this was further diluted in 10ml 1XPBS (final 200µg/ml). This was labelled as the 'MBP/his stock solution'. 200µl Triton X-100 detergent (Thermo scientific) was added to 5ml 1xPBS (final 4% v/v Triton X-100). This solution was further diluted in 1xPBS at varying ratios until $1x10^{-4}$% (v/v) was reached. 500µl of each dilution was then mixed with 500µl of 200µg/ml MBP to create a final dilution series of 2%-$5x10^{-5}$% Triton X-100 (v/v) samples, each containing 100µg/ml MBP.

100µl/well of each dilution was pipetted in triplicate into a flat-bottomed Maxisorp® ELISA plate (NUNC, UK), including a 100µl triplicate repeat of MBP stock solution without Triton X-100. The plate was incubated for 2 hours, non-bound protein was poured away and the plate was rinsed 3 times with 1xPBS. All wells were filled with blocking solution (see Appendix) and left for a further 2 hours. Blocking solution was removed and the plate was rinsed three times in wash buffer (see Appendix).

100µl/well of a 1:5000 anti-his antibody (Sigma #H1029-.2ML) in 1x PBS solution was incubated for 1 hour, the plate was rinsed three times and 100µl/well of a 1:10,000 goat anti-mouse HRP (Sigma # AP127P) in 1x PBS was incubated for 1 hour. The plate was again rinsed three times with wash buffer and further rinsed a final three times with 1x PBS. 100µl of 1:5 TMBeasy/dH_20 (Acros Organics) was added to each well to quantify protein–antibody binding by colorimetric reaction of horse radish peroxidase substrate interacting with bound antibodies. The reaction was stopped after 10 minutes by addition of 50µl 1M Sulphuric acid. The absorbances of each reaction were measured at 450nm by spectrophotometry.

The experiment was repeated using the same MBP/his tag stock solution but with addition of 0.1% Tween 20 rather than Triton X-100. A dilution series of Tween 20 from 0.1% (v/v) to $7.4x10^{-5}$% (v/v) was established from addition of 500µl 0.2%-$1.48x10^{-4}$% (v/v) Tween 20 to 500µl of 200µg/ml MBP. Effect of Tween 20 on protein binding was also analysed using anti-his tag antibodies and absorbance at 450nm in the same way as above.

ii) The effect of aqueous and dry conditions on protein binding to a Maxisorb® plate

Protein can bind to a plate through precipitation, i.e. removal of water during evaporation in a dry environment, or through hydrophobic interactions with plastic in an aqueous environment. These methods of protein binding were examined using MBP/his protein plates and anti-his antibodies in ELISA as before.

A two fold dilution series of 1mg/ml to 1.9ug/ml MBP/his was set up in a 1:1 dilution (500µl:500µl 1x PBS) from the MBP/his stock. 100µl of each dilution was incubated in

a Maxisorb® plate well in triplicate overnight at 4°C covered by Parafilm (Bemis NA) or at 37°C, allowing buffer to evaporate and protein to dry onto the plate. All wells were washed, blocked, introduced to anti-his and anti-mouse antibodies and absorbances read at 450nm as before to determine amount of protein bound to each well.

iii) Effect of buffer on protein binding

The buffer in which a protein is suspended and the pH of that buffer can affect the behaviour of a protein during binding due to addition or loss of ions affecting protein structure and exposure of hydrophobic regions. To test the effect of pH on protein binding to a Maxisorp® plate, MBP-his was suspended in three separate buffers with a range of pH. Protein binding was measured by ELISA as before using anti-his antibodies. The same experimental protocol at 4°C and 37°C as described in section 4.2.1 ii) was repeated however MBP protein in the 'MBP/his stock solution' was solubulised in 1x PBS (pH 7.0), in 50mM Sodium Bicarbonate (pH 9.5) or in 1M Tris /HCl (pH 8.5). The binding of proteins at 37°C dry conditions or at 4°C in aqueous conditions for these buffers was analysed by anti-his antibody binding and reading absorbances at 450nm as before.

4.2.2 Selection of a scFv library specific to a membrane protein enriched mite fraction

4ml of membrane protein-enriched mite fraction (see Chapter 3) at 10μg/ml was incubated in a Maxisorp® immunotube (NUNC, UK) for 2 hours at room temperature on a roller (Stuat science, UK). Unbound homogenate was tipped out and the tube was rinsed three times with 2ml 1xPBS. The tube was then filled with 4ml blocking buffer

(1% Bio-rad blocking buffer, 0.1% Tween 20, 1x PBS), incubated for a further 2 hours then rinsed ten times with 1xPBS. 2ml of Tomlinson scFv library J ($1x10^{12}$ cfu) was added to the tube and incubated on a roller at room temperature for 2 hours. Unbound scFv/phage was discarded into 2% Virkon/dH_2O and the tube was rinsed ten times with 1x PBS. Bound scFv/phage was eluted by addition of 500µl 4mg/ml trypsin solution (40mg Trypsin, 137mM NaCl, 2mM CaCl2, 10mM Tris-HCl, up to 10ml dH_20) and incubated on a roller for 30 minutes. A further 500µl trypsin solution was added and the 1ml solution was incubated for a further 30 minutes.

4.2.3 Quantification and amplification of recovered phage

The 1ml eluted phage/trypsin solution was added to 1ml of mid-log TG1 E. coli cells and incubated at 37°C to allow phage to infect cells. Phage recovery was quantified by using 20µl of the culture to set up a neat-10^{-8} dilution series (10µl culture / 90µl Luria Broth) and spotting 10µl of each dilution onto one plate of Luria-Agar, 50µg/ml Ampicillin, 1% (w/v) Glucose, and one plate Luria-Agar only. Plates were allowed to dry, were inverted and were incubated overnight at 37°C. The remaining phage/E. coli mixture was centrifuged at 12,000rpm for 10 minutes and, apart from 100µl, the supernatant was poured into 2% Virkon. The pellet was resuspended in the remaining 100µl supernatant and spread over two plates of Luria-Agar, 50µg/ml Ampicillin, 1% (w/v) Glucose. These plates were allowed to dry, inverted and incubated overnight at 37°C. Only cells infected with phage would have the ampicillin resistance gene and thus all bacteria grown overnight would in theory be phage-infected.

Colonies from incubated plates were scraped into 5ml of Luria Broth and 150µl of this suspension was used to inoculate 50ml Luria Broth, 100µg/ml Ampicillin, 1% (w/v)

Glucose. This solution was shaken at 200rpm/37°C until the OD600, detected by spectrophotometry, reached 0.4. Glycerol stocks of the remaining scrapped scFv library were created by adding 138ml sterile 80% (v/v) glycerol to 962ml of resuspended scrapped cell aliquots. Stocks were gently mixed by inversion, incubated at room temperature for 10 minutes and stored at -80°C. Once 0D600 of the incubated cell culture reached 0.4 then $5x10^{10}$ pfu KM13 helper phage was added to 10ml of the culture and incubated at 37°C for a further 30 minutes, allowing infection of helper phage into cells. Infected cells were centrifuged at 3000g for 10 minutes, the supernatant was removed into 2% Virkon and the pellet was resuspended in 50ml Luria Broth, 0.1% (w/v) glucose, 100μg/ml Ampicillin, 50μg/ml kanamycin. The resuspended pellet mixture was incubated in a 500ml flask in a shaker at 32°C overnight. KM13 helper phage are required by M13 phage to package ssDNA during replication. KM13 included a kanamycin resistance gene so only bacteria infected with the M13 phage and KM13 helper phage would grow.

4.2.4 scFv/Phage purification

Phage infected cells grown up overnight were sedimented by centrifugation at 4000rpm for 10 minutes and 40ml supernatant was mixed by inversion with 10ml ice cold 20% (w/v) PEG6000 solution (see Appendix). Phage were left to precipitate within the PEG solution on ice for 1 hour then sedimented by centrifugation at 4000rpm for 30 minutes at 4°C. Supernatant was removed into 2% Virkon, the pellet containing the precipitated phage was resuspended into 2ml 1x PBS and spun at 11,600g for 10 minutes to sediment insoluble material. This supernatant containing purified scFv/phage molecules was placed in a new tube, labelled as 'round one phage stock'

and either stored short term at 4°C or long term (more than a few weeks) at -80°C as aliquots in 40% glycerol. The scFv/phage library was quantified by using a 100 fold dilution series (1µl phage sample, 97µl Luria Broth and 2µl mid-log TG1 cells) and quantification of infected bacterial cell colony numbers from each dilution. 1µl phage stock was diluted across a range of dilutions from $1x10^{-2}$ – $1x10^{-14}$ using 97µl Luria broth and 2µl for each dilution. All dilutions were incubated at 37°C for 30 minutes to allow phage to infect cells and infected cells to proliferate. 10µl of each dilution sample was spotted onto a Luria-Agar, 1% w/v glucose, 50µg/ml Ampicillin plate. The plate was dried, inverted and incubated at 37°C overnight. Total number of phage equalled the number of colonies formed x dilution factor x 200 due to the original volume of phage stock being 2000µl and from this 10µl being taken for bacterial growth.

4.2.5 Repeat rounds of biopanning

A further two rounds of biopanning were repeated using the exact same method as above, however using $1x10^{-12}$cfu of the phage library selected in the previous panning round instead of an unselected library. Over several rounds of biopanning the scFv/phage library should have gained progressively higher binding affinity to fractionated mite proteins as phage that did not bind were removed by elution during each round. Biopanning was also carried out over three rounds against unfractionated mite homogenate (4ml at 10µg/µl). This allowed evidence for validation of the biopanning technique if the scFv/phage library was able to show increased binding capacity to an alternative protein mixture.

4.2.6 ELISA to determine phage library binding capacity

ELISA was carried out to determine if there was an increase in binding capacity of each scFv/phage 'sub-library' which was purified after each round against either fractionated or unfractionated mite protein mixture. All phage sub-libraries were also analysed in triplicate against blocking solution (see Appendix), to identify if 'non-specific' binding had decreased throughout the selection process. 100µl of 100µg/ml 'membrane enriched' mite homogenate was added to 12 wells of a 96 well Maxisorb® plate. 100µl of 100µg/ml unfractionated mite homogenate was also added to an additional 12 wells. 100µg/µl blocking buffer (see Appendix) was added to a further 24 wells at 100µl/well. The plate was at room temperature for 2 hours covered in Parafilm to bind protein onto the plate. The plate was rinsed three times with wash buffer (see Appendix) and blocked for 1 hour at room temperature with 500µl/well blocking buffer. The blocking buffer was removed and the plate was washed a further three times. 70µl of each phage library, adjusted to $1x10^{12}$ cfu/ml, plus 30µl blocking buffer, was added to three wells that had been incubated with the corresponding protein mixture (i.e. fractionated or unfractionated mite homogenate) and three wells containing just blocking buffer. An unselected 'Round zero or R0' scFv/phage library (positive control) was also incubated with 3 wells of each protein mixture as was 100µl blocking buffer (negative control).

The plate was incubated for 2 hours at room temperature to allow scFv binding. Unbound scFv/blocking solution was removed into 2% Virkon and the plate was washed 10 times with wash buffer, ensuring all solution was removed from every well each time. 100µl of rabbit anti-Fd phage polyclonal antibody (Sigma #B7786) at 1:5000

dilution in wash buffer was added to each well and incubated for one hour at room temperature. Anti-Fd antibody was removed, the plate washed further five times and 100µl goat anti-Rabbit peroxidise conjugate polyclonal antibody (Sigma-Aldrich) in a 1:10,000 dilution was added to each well. This was incubated for 1 hour at room temperature. The goat anti-rabbit antibody was removed, the plate was washed five times in wash buffer and then five times in 1x PBS. ScFv/phage binding capacity was detected by adding 100µl per well of TMBeasy solution (Fisher) diluted 1:5 in dH_2O. Colour was allowed to develop up to 30 minutes then reaction was stopped by addition of 50µl 1M H_2SO_4 per well. Absorbance was read on a spectrophotometer at a wavelength of 450nm.

4.2.7 Selection of individual scFv/phage variants

Round 3 phage infected TG-1 cells selected in biopanning against the fractionated mite protein mixture were streaked onto a plate of Luria-Agar 50µg/ml Ampicillin, 1% (w/v) glucose and grown over night at 37°C. 95 individual colonies were picked and used to inoculate a sterile 96 well plate (Fisher), one colony per well, including 100µl LB, 100µg/ml Ampicillin, 0.1% (w/v) glucose per well. The plate was covered and placed overnight at 37°C and labelled the 'master plate'. A new sterile 96 well plate was set up with 50µl LB, 100µg/ml Ampicillin, 0.1 % (w/v) glucose, 10^9pfu/ml KM13 helper phage (Source Biosciences) in each well. Each well was inoculated using a multi-channel pipette which had been rinsed in the corresponding wells of the master plate. This allowed growth of each individual colony in a separate well whilst all been infected with KM13 helper phage to ensure phage production.

The plate was incubated at 37°C for 2-4 hours until growth was visible in all wells. A further 100µl LB, 100µg/ml Ampicillin, 65µg/ml Kanamycin, 0.1% (w/v) glucose was added to each well and further incubated at 37°C overnight to allow growth of KM13 infected cells only. 50µl of sterile 30% (v/v) glycerol in 1x PBS was added to each well of the master plate, incubated for 10 minutes at room temperature and the plate was stored at -80°C as a stock for each scFv/phage variant. A final volume of 10% of glycerol penetrated cells stocks would allow maintenance of cell membrane and internal protein structure during freezing.

4.2.8 ELISA of monoclonal phage binding capacity

This was carried out in a similar manner to the mixed phage library ELISA, however covering all wells in a 96 well plate with 100µl of 100µg/ml fractionated homogenate over several hours at room temperature. In place of replicate phage libraries, 70µl of individual phage colonies previously grown up overnight was added per individual well. Again, TMBeasy was added and absorbance at 450nm was measured on a spectrophotometer to assess the binding capacity of each scFv/phage variant to fractionated mite proteins. This overall process was repeated for 760 colonies from streaked out plate – growing up 760 individual colonies in 8 plates.

4.2.9 Statistics

Statistical analysis of arithmetic mean, standard error of the mean and ANOVA with associated post hoc Bonferroni tests were performed using SPSS version 23 (IBM Corp).

4.3 Results

4.3.1 Analysis of protein binding conditions

i) The effect of detergent on protein binding to a Maxisorp® ELISA plate

A dilution series of 100μg/μl Maltose binding protein with variable concentrations of Triton X-100 or Tween 20 was bound to a Maxisorb® plate. The amount of bound protein after washing was determined by ELISA, using anti-his antibodies. Absorbance readings taken at 450nm were used as a quantifiable value for how much protein was bound. Addition of $1.55x10^{-2}$% - 1.00% Triton X-100 significantly reduced the amount of protein binding compared to addition of no detergent (p = 0.00-0.01 between groups, one way ANOVA) (Figure 4.4). At 0.01% (v/v) Triton X-100 and below however, protein binding was not significantly affected by detergent (p values all >0.05, one way ANOVA, apart from 0.0005% Triton X-100) (Figure 4.4 - red column). Absorbance readings, apart from at readings at 0.0005% (v/v) Triton X-100, were clustered between 0.39 and 0.49.

Similarly, Tween 20 significantly reduced protein binding at concentrations from 0.1%-$7.4x10^{-4}$% (p values = 0.00, one way ANOVA), however absorbance readings from $1.5x10^{-4}$% to $7.4x10^{-5}$% Tween 20 compared to no detergent were not significantly different (p values = 0.15 – 1.00, one way ANOVA). Both the Triton X-100 and Tween 20 'unaffected protein binding' detergent concentrations (red columns) were below the critical micelle concentration (CMC) for each respective detergent (yellow columns, Figures 4.4 and 4.5).

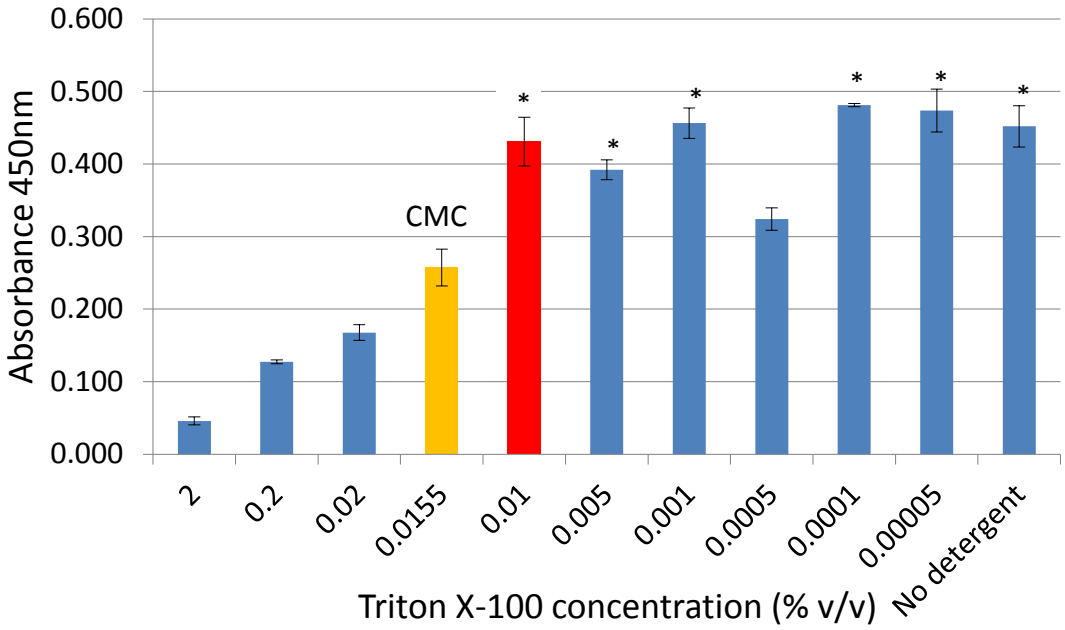


Figure 4.4: The binding capacity of MBP to an ELISA plate in the presence of varying concentrations of Triton X-100. Absorbance values at 450nm were relative to amount of MBP bound. Triton X-100 appeared to decrease protein binding with increase in detergent concentration. At 0.01% (v/v) and lower, apart from at $5x10^{-4}$%, Triton X-100 did not significantly affect protein binding (p values >0.05, one way ANOVA). Absorbance values (0.39-0.49) were similar to protein absorbance values in the absence of detergent (0.45). The critical micelle concentration (CMC) is shown in yellow and the highest concentration of Triton X-100 that can be present in biopanning whilst not effecting protein binding is marked in red. Error bars indicated are standard error from triplicate repeats of each dilution. * indicates all results that are not statistically different from 'no detergent' absorbance value (p value >0.05, one way ANOVA).

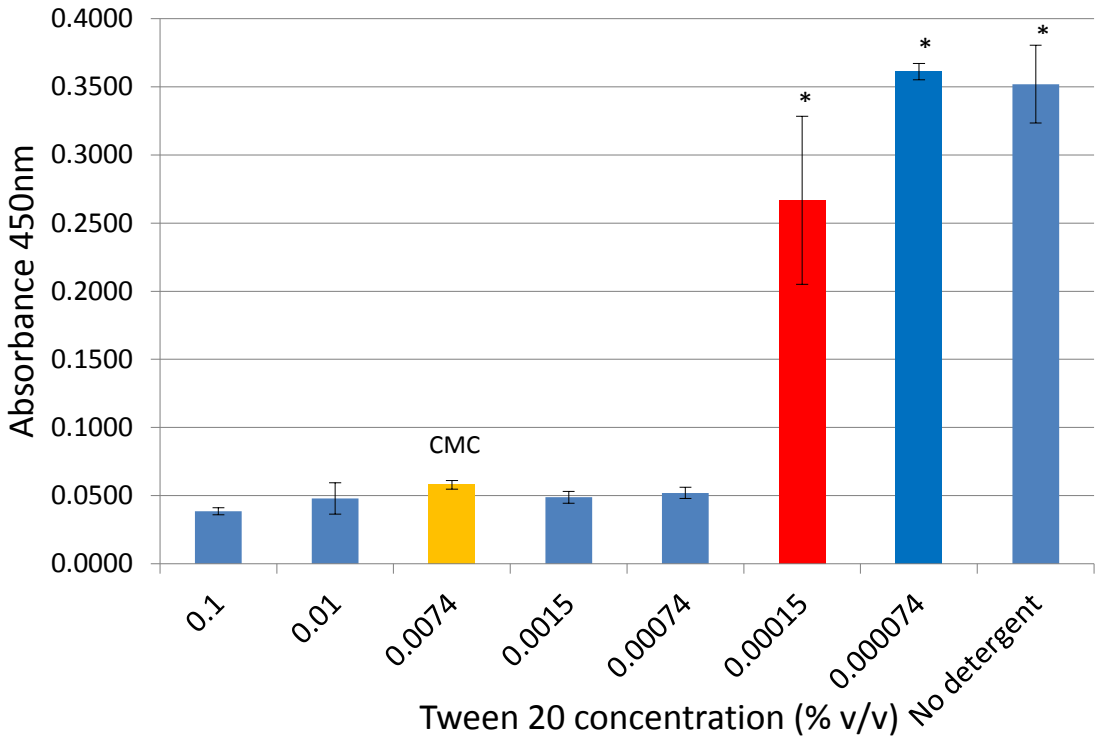


Figure 4.5: The binding capacity of MBP to an ELISA plate in the presence of varying concentrations of Tween 20. Absorbance values at 450nm were relative to amount of MBP bound. Tween 20 decreased protein binding with an increase in detergent concentration. At $1.5x10^{-4}$% protein binding values (0.27) were similar to no detergent control values (0.35), indicating Tween 20 did not significantly decrease protein binding at this concentration and below (p values = 0.15-1.00, one way ANOVA). The critical micelle concentration (CMC) is shown in yellow and the highest concentration of Tween 20 that can be present in panning whilst not significantly effecting protein binding is marked in red. Error bars indicated are standard error from triplicate repeats of each dilution. * indicates all results that are not statistically different from 'no detergent' absorbance value (p values all =0.00, one way ANOVA). Standard errors from triplicate repeats are shown.

ii/iii) Effect of pH or binding conditions to protein binding

ELISA was carried out on MBP protein solubilised in different buffers (1x PBS pH 7.0, 1M Tris-Hcl pH 8.5, 50mM Sodium Bicarbonate pH 9.5) and bound overnight to a Maxisorb® plate either at 37°C in dry conditions or at 4°C in aqueous conditions. Solubilisation of MBP between all three different buffers had no significant effect on protein binding (p value>0.05, two way ANOVA and Bonferroni post-hoc test). Non-significant differences could however be seen, for example at 125μg/μl MBP in dry conditions the Tris/HCl sample absorbance = 0.25, whereas the comparable PBS buffer sample was 0.3 (see Figure 4.6).

More notable was the significant difference in protein binding absorbance values between aqueous conditions and drying conditions (p value = 0.006, two way ANOVA, Figure 4.6). At 125μg/μl MBP, a protein concentration similar to the 100μg/μl of mite homogenate used in the biopanning experiments, absorbance values for dry and aqueous conditions were between 0.25-0.30 and 0.35-0.40 respectively. Absorbance values were relative to the amount of protein bound to the Maxisorp® plate, thus it was beneficial to use aqueous conditions at 4°C, or at room temperature, instead of 37°C to bind protein in biopanning experiments.

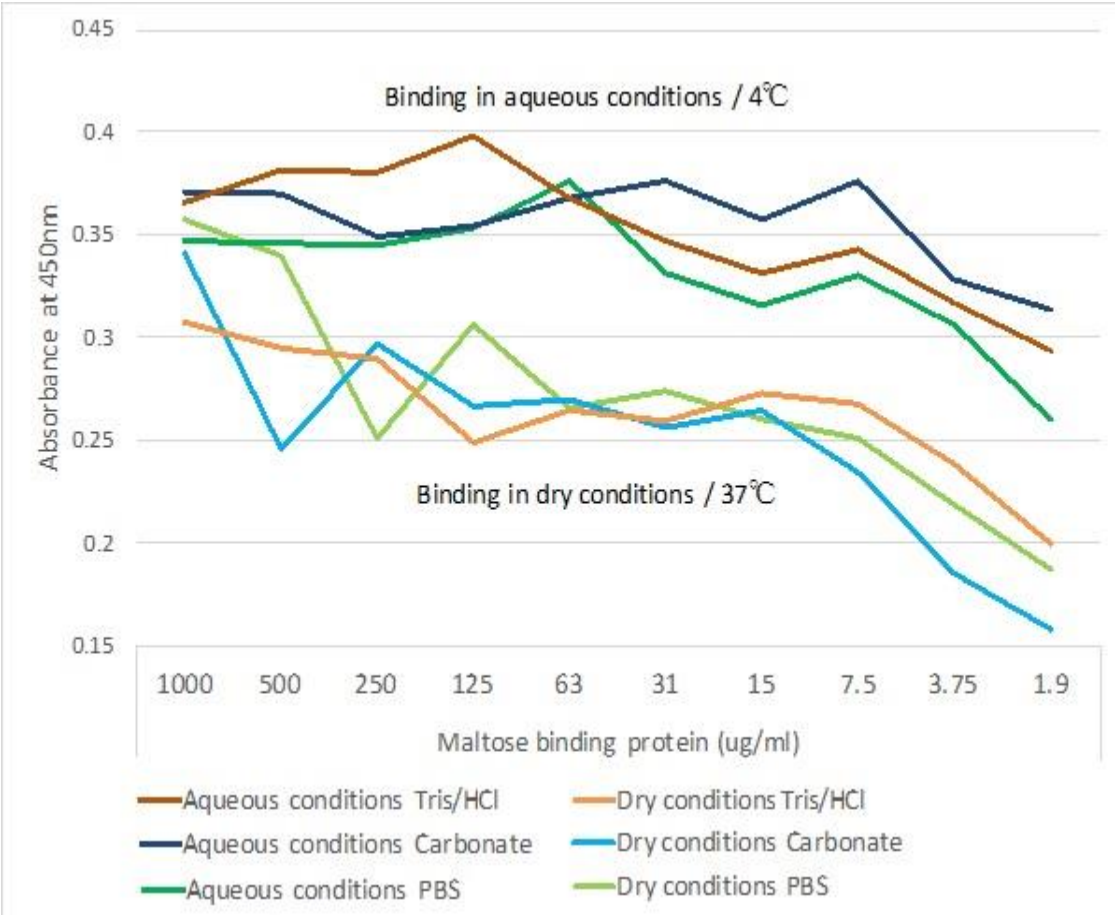


Figure 4.6: The binding capacity of MBP protein to a Maxisorb® plate in various binding conditions. Absorbance values at 450nm were relative to amount of bound protein. No significant difference in protein binding occurred between MBP solubilised in PBS (pH 7.0), 1M Tris/HCl (pH 8.5) or 50mM Sodium Bicarbonate (pH 9.5) (p value>0.05, two way ANOVA). Protein binding did however significantly increase if bound in aqueous conditions at 4°C rather than in dry conditions at 37°C (p value = 0.006, two way ANOVA). As expected, absorbances decreased overall in all conditions when less protein was added.

4.3.2 Polyclonal selection

Changes in scFv-phage binding capacity after subsequent rounds of panning to fractionated and unfractionated mite protein samples were assessed by ELISA. Binding of the scFv/phage library was also analysed against blocking buffer to determine non-specific binding efficiencies. Binding capacity of unselected phage library (round 0 or R0) was analysed, as were triplicate wells without a phage library, used as a negative control.

ScFv-protein binding capacity increased between round 0 (the unselected library) and purified scFv/phage sub-libraries at the end of round 3 (R3), however not significantly (p values between 0.22-1.00, one way ANOVA) (see Figure 4.7). Surprisingly, both the unfractionated and fractionated homogenate bound the unselected (R0) scFv/phage library in already high amounts (Figure 4.7, absorbance values 1.17 and 1.61 respectively). Sub-libraries selected for against each protein mixture did increase in binding capacity after selection in Round 1 (absorbances = 1.53 and 2.07 respectively) though not from Round 1 to Round 3 (absorbances = 1.53-1.51 and 2.06-2.07 respectively). This indicated the majority of selection occurred in both libraries during R1 or there was potentially excess phage and that all protein binding sites were saturated. If bound scFv/phage were already present in large concentrations, then selection ability of each round may have had limited impact at isolating the large numbers of high binding scFv/phage.

Initial round 1 purified scFv/phage mixtures also showed high specificity to blocking solution as well as mite protein (Figure 4.7 absorbances = 1.40 and 1.34), though this significantly reduced over consecutive round selections (Figure 4.7, absorbances = 1.40-1.08 and 1.34-0.72, p values = 0.05 and 0.00, one way ANOVA). Blocking solution

was overall shown to be bound to significantly less than mite proteins (F value = 49.4, p value = 0.00, two way ANOVA), presumably due to increase in scFv preference to bind mite protein instead of blocking solution over the three rounds. Negative control - the absence of a phage library, appeared to show only small amounts of secondary or tertiary antibody binding without the presence of a phage library (Figure 4.7 negative control = 0.16).

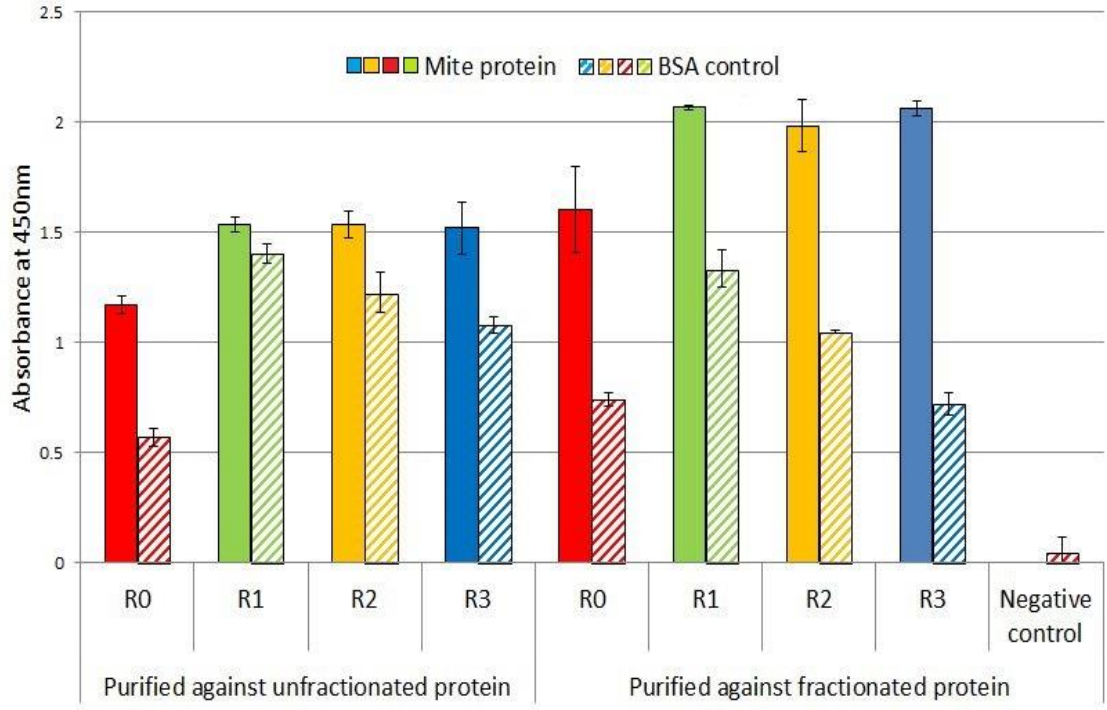


Figure 4.7: Analysis of the binding capacity of the Tomlinson J scFv-phage library over three rounds of biopanning exposed to either fractionated mite homogenate, unfractionated mite homogenate or blocking solution. The capacity of a scFv/phage library (Tomlinson j – MRC) (R0) and the purified scFv/phage sub-libraries after three rounds of biopanning (R1-R3) against either unfractionated or fractionated homogenate was examined by ELISA. Absorbance values at 450nm shown are relative to amount of scFv/phage bound to protein. Both libraries between R0 and R3 showed increased binding to mite proteins (1.17→1.53 and 1.61→2.07), though not significantly (p values > 0.05). No further increase was seen between R1 and R3. After an initial increase in binding from R0 to R1 against blocking solution control solution (0.57→1.41 and 0.74→1.34), both libraries showed consecutive decreases in binding from R1 to R3 (1.41→1.08, p value = 0.05, and 1.34→0.72, p value = 0.00). Negative controls without the presence of a phage library (labelled 'negative') showed comparatively little binding (0.16).

4.3.3 Monoclonal selection

Single scFv variants from the R3 'membrane-enriched fraction' phage library were expressed individually and their binding capacities were analysed via ELISA. A total of 760 'monoclonas' variants were analysed from 8 x 96 well plates (minus one well/plate for negative controls). Each scFv was also tested against a 100μg/ml blocking solution to test binding capacity to a non-specific protein. Figure 4.9 shows example ELISA results of plate two (of the plates one to eight – see supplementary S1-7 for other plate results).

From the 760 scFv/phage analysed by ELISA, 730 (96%) showed a higher binding to red mite proteins than to blocking solution. The average absorbance value of scFv/phage binding mite protein (0.55) was more than double the average of scFv/phage binding to blocking solution (0.18). The top 10 highest binding scFv/phage from each plate (expect plate 2 where 8 were chosen) were selected for further triplicate ELISA analysis. 63 of the 68 total showed consistently high binding absorbance rates when compared to other population of 692 scFv/phage variants tested (see Figure 4.10, p value = 0.00, two way ANOVA). Plate 1 high binders were not replicated as overall absorbances of scFv-phage in plate one were very low (av. 0.13) compared to plates 2-8 (av. 0.61), likely due to reduced amounts of protein incubated, miscalculated by human error. The absorbance values for plates 2-8 high binders are shown as graphs in supplementary S8 and S9. Significant difference was seen between absorbances from different plates as expected (p value = 0.00), due to variation in time required for colorimetric change in Horse radish peroxidase.

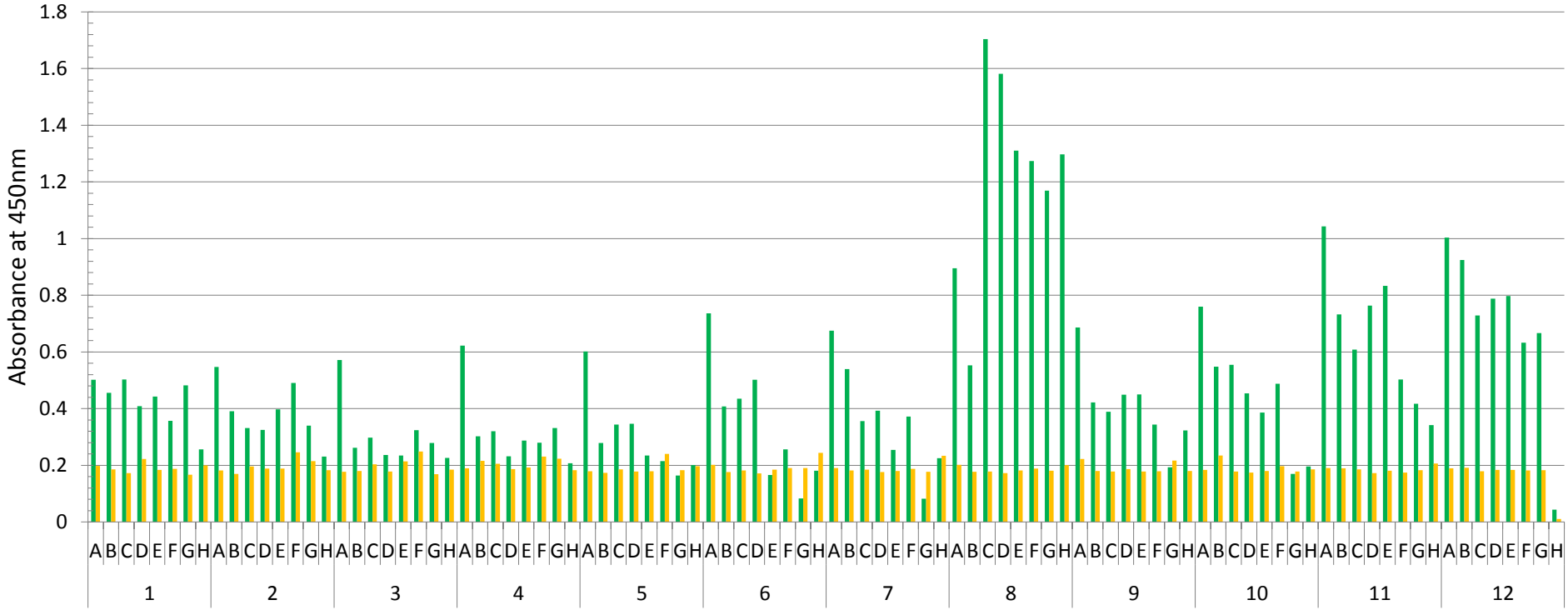


Figure 4.9: The binding capacity of 95 monoclonal scFv/phage variants from biopanning plate 2 measured by ELISA. Absorbance values were relative to the amount of phage binding to either fractionated mite proteins (green) or blocking solution (yellow). ScFv/phage binding to mite fraction absorbances varied considerably (0.08-1.70 average 0.49) though were much higher in 88/95 scFv/phage when compared to non-specific binding to absorbance values of scFv/phage blocking solution (0.01-0.24 average 0.19). Phage binding was shown to be higher in row 8 (average 1.22) than the rest of the plate (average 0.42 without row 8). Negative controls (12H; mite – 0.04 blocking – 0.01) showed low binding.

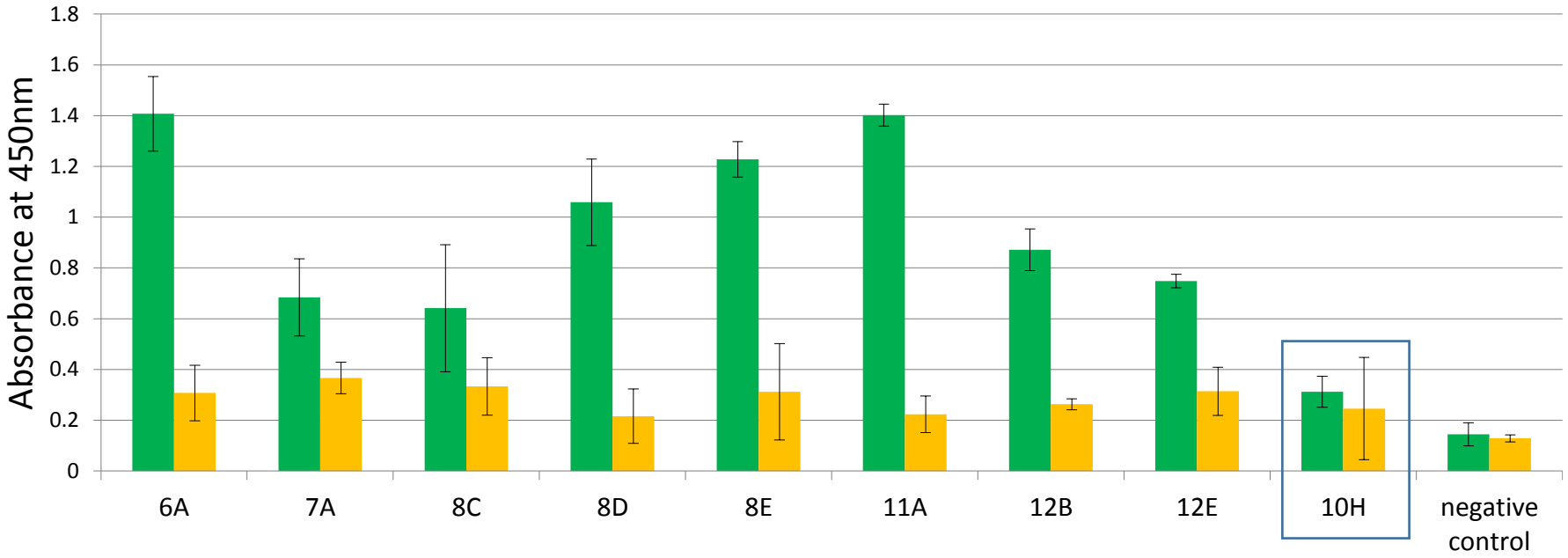


Figure 4.10: Triplicate repeat ELISA analysis of high binding monoclonal scFv/phage variants chosen from Figure 4.9. Absorbance values were relative to protein/phage binding. All eight high binding scFv/phage showed high binding values to mite protein (green - 0.64-1.41 av. 0.85) when compared to low binding scFv/phage controls (10H, blue box; 0.31). Binding to blocking solution was significantly lower than mite protein (yellow - av. 0.29, p value = 0.00, one ANOVA), as was the negative control value (0.14). Error bars are standard deviations of the triplicate repeats for each phage (average s.d. = 0.11 for mite and = 0.09 for blocking solution.

4.3.4 Summary of results

- Both Triton X-100 and Tween 20 detergents had a strong negative effect on protein binding to ELISA plates. This effect was negligible at concentrations of 0.01% (v/v) Triton X-100 and $7.4x10^{-5}$% (v/v) Tween 20. Both of these concentrations were below the CMC of the detergents (Figures 4.4. and 4.5).
- Protein binding to an ELISA plate was not influenced significantly by buffer/pH alteration, however binding in aqueous conditions at 4°C resulted in more protein binding than at 37°C (Figure 4.6).
- There was an increase in specificity of scFv/phage libraries to bind to mite proteins from both unfractionated and fractionated samples between R0 and R3, though most increase in binding happened after R1. Specificity to blocking solution controls reduced over the three rounds of biopanning.
- From a library of $5.4x10^{13}$ scFv/phage, 760 individual phage colonies were grown up and tested for binding capacity. scFv/phage 63 of 68 high binding scFv showed comparatively high binding in repeat triplicate experiments. These scFv/phage were progressed to further selection studies using immunohistochemistry (Chapter 5).

4.4 Discussion

4.4.1 Analysis of protein binding conditions

Biopanning requires successful binding of a protein mixture to an immobile surface, such as an ELISA plate or tube. Biopanning also requires an environment in which phage-protein binding can occur. Variability in temperature, pH or hydration can influence protein structure and the capacity to create protein-protein bonds.

i) Change in temperature and hydration

Results indicate that binding of mite protein was significantly higher in aqueous conditions than by precipitation of the protein caused by buffer evaporation at 37°C (p value = 0.00). Protein binding to an immobile ELISA plate surface occurs through hydrophobic interactions forming between plastic and the hydrophobic side chains in a protein structure. Dehydration of water can significantly alter the conformational change in a protein due to change in protein structure caused by loss of protein charge or bonding. Epitopes binding the anti-his antibody may have not been in a functional state to interact and thus binding in dehydrated conditions appeared less than in aqueous conditions. This same breakdown in protein structure may also have presented the hydrophobic plastic to hydrophobic regions of protein due to the removal of hydrophilic water molecule 'shells'. Increased hydrophobic interactions would therefore increase protein binding in dry, dehydrated conditions, however this was not seen.

Use of an anti-his antibody would also test the capacity of an antibody to interact with bound proteins in aqueous and dehydrated conditions using the Maxisorp® plate

system. This was relevant to scFv/phage-protein interactions as scFv and antibodies bind epitopes in a similar manner through non-covalent bonding. Whether dehydration would interrupt the various epitopes that scFv/phage bound to during panning however is not clear.

ii) Change in buffer/pH

Overall change in buffer generally did not affect protein binding to the plate (p values >0.05, one way ANOVA and Bonferroni test) (Figure 4.6). 1x PBS pH7.4 however did produce better binding absorbance values at 4°C at 125µg/µl which is close to the 100µg/µl used for biopanning. Other buffers tested included 50mM sodium bicarbonate (pH 9.5) and 1M Tris/HCl (pH 8.5). These buffers were chosen based on having a slightly more alkali pH and both being widely used in ELISA experiments. Salt bridging and hydrogen bonding form part of a protein's structure and can be influenced by change in pH due to addition or removal of ions. Loss of bonding may therefore induce loss of protein structure and expose hydrophobic regions of proteins that would bind to the hydrophobic plastic.

Different proteins are specialised to exist and function at a range of particular but different pH, though intracellular proteins are often stable at pH 6.8-7.4 (Deutsch, Taylor et al. 1982). Antibodies that function in poultry blood also function with a comparably neutral pH of 7.3 (Bogin and Hadani 1973; Kenyon and Knox 2002). 1x PBS was therefore seen as a suitable buffer for scFv/phage binding after protein had bound to the plate. Whether selected mite proteins would display the same epitopes during blood feeding is uncertain. Anterior midgut epithelial cells in Acari contain large vacuoles and are involved in low pH intracellular digestion between pH4-5 (Brody,

McGrath et al. 1972; Coons 1978). In storage food mites the midgut has a pH gradient from an acidic pH 5.4 anterior end to a more neutral pH 7.0 posterior (Erban and Hubert 2010). pH in a haematophagous PRM midgut may differ though, as digestion in Acari of a blood meal requires function of an array of different proteolytic enzymes and the presence of poultry blood pH 7.3.

Overall, protein binding was significantly higher in aqueous conditions. Protein binding was therefore decided to be carried out in 1xPBS and at room temperature to ensure aqueous conditions were kept but at a higher temperature than 4°C to increase binding timing.

iii) Change in detergent

Presence of detergents at levels above 0.01% (v/v) Triton X-100 or $7.4x10^{-5}$% (v/v) Tween 20 significantly reduced protein binding to the plate (p values = 0.00-0.01). At these concentrations and below however, there appeared to be insufficient detergent to significantly negatively affect protein binding (p values = 0.15 – 1.00). Membrane fractions purified by differential centrifugation would have had a maximum of 1% (v/v) Triton X-100 or 0.1% (v/v) Tween 20, however were diluted by 1:100 to be used as 100μg/μl protein in biopanning. Protein used for biopanning would therefore contain a maximum concentration of 0.01% Triton X-100 and thus Triton X-100 would not affect protein binding. Conversely, Tween 20 may have been present at concentrations of up to $1x10^{-3}$% during biopanning, one log higher than the $1.5x10^{-4}$% limit that was required for Tween 20 not to effect protein binding. Though counter-intuitive, this was seen as an acceptable dilution as excess Tween 20 may have been largely removed during protein purification (see chapter 3). Tween 20 addition was followed by a

25,000g spin for 20 minutes at 4°C where soluble detergent monomers should have been largely removed in the 15ml supernatant. Whether this would remove sufficient Tween 20 to concentrations below $1.5x10^{-4}$% is unknown. Maxisorp® plates have been shown to work optimally with both Tween 20 and Triton X-100 at concentrations up to 1% (v/v) over other detergents (Opitz and Cyr 1986; Esser 1990).

4.4.2 Selecting scFv-phage

A starting scFv/phage library of 1.37×10^8 genetically variable scFv/phage underwent three rounds of biopanning to select scFv/phage that bound fractionated red mite proteins. 760 scFv variants from the selected scFv/phage library were analysed individually for mite protein binding capacity. The purified library from round one to round three of biopanning showed similar binding values (absorbance values = 2.06-2.07, Figure 4.7). Individual scFv/phage were purified from the round three sub-library as, though mite protein specific binding didn't increase, non-specific binding (i.e. binding to blocking solution) did decrease over the three rounds (1.34→0.72, Figure 4.7). Reduction in unspecific binding suggested there was still a large amount of scFv/phage binding to mite protein, however much less phage binding in an unspecific manner e.g. through scFv interaction with the hydrophobic plate or to other already bound phage molecules. This justification of using the round three sub-library for individual scFv/phage selection is also demonstrated in other studies where several rounds of biopanning are required for sufficient purification of phage (Felici, Castagnoli et al. 1991; Scott, Loganathan et al. 1992). The sudden increase in phage specificity to mite protein after round one suggested scFv binding sites were saturated, thus many potentially high binding scFv variants may have been washed away in the initial wash steps. Another limitation of the study may be that some scFv that were more

numerous, yet may not have had such a high binding capacity, saturated scFv binding sites. The Tomlinson J library was derived from a bacterial culture where phage production is limited by the addition of 1% glucose that promotes cyclic AMP production and suppression of the lac operon inserted in the phage plasmid. Phage infected cells all produce phage from a set point for a set amount of time so scFv/phage variants with a comparably efficient production rate are not produced in much larger numbers than other scFv/phage.

63 of the 760 individually analysed scFv/phage showed significantly high binding absorbance values when compared to absorbance values of the other 697 scFv (p value = 0.00). Whether these scFv were all genetically distinct in the hypervariable regions was unknown at this stage. All plates, including replicate plates, showed negative controls to be consistently and significantly lower in absorbance values than scFv/phage samples (p values = 0.00). This gave indirect indication scFv were present and secondary antibody cross-reaction was limited. Replicate plates of high binding scFv also contained a low binding phage that would often replicate low binding qualities. Interestingly, the low binding phage selected for plate 3 (phage 3-4D) actually had a higher replicate absorbance value (av. 0.407 s.e. 0.232) than that of five 'high binding' phage (av. 0.271-0.460). These results indicated that, in general, the majority of scFv/phage repetitively bound at similar values, however not all scFv/phage did. It was therefore important to validate true high binding phage by use of repeated triplicate ELISAs.

Some specific lanes or rows of phage in the ELISA plates appeared to have high binding values concentrated at specific regions of the plate. For example, plate 2 row 8 showed very high absorbance rates (av. 1.22) compared to the rest of the plate (av.

without row 8 = 0.42) (Figure 4.9). Due to the repetitive nature and numerous wells incorporated in the biopanning, high binding in these 'clustered' areas may have been due to human error. Over-pipetting of protein/antibody or perhaps insufficient washing could all have led to higher binding values. These apparent high binding phage were therefore not further tested in replicate studies.

Also of note was the variability between average absorbance values of phage from each plate to mite proteins, e.g. plate 3 average absorbance = 0.59, plate 6 = 0.77 and plate 8 = 0.73. Colorimetric change between plates was variable and ranged from 1-30 minutes before adequate reaction could be seen. Though this variability between plate average absorbances was not significant (t value = 0.0002, p value <0.005), the plate 1 average absorbance in particular (plate 1 av. = 0.13) was distinctly lower than the rest (overall plates av. = 0.55). Phage from plate 1 therefore were not comparatively high binding when compared to phage on other plates. As no reliable comparison between plate 1 absorbance values and absorbance values from other plates could be established, plate 1 phage were not further tested by replicate ELISA.

Though scFv/phage binding capacity was tested, it was assumed that scFv were binding to epitopes of immunogenic proteins. Whilst this may be true in part, antibodies and scFv can also bind carbohydrate targets that are expressed on cell surfaces such as glycoproteins and glycoproteins (Kosma and Müller-Loennies 2011). Furthermore, scFv are smaller molecules than whole antibodies present in a chicken and so may bind epitopes that are hidden to antibodies in an *in vivo* setting. These limitations were acknowledged and challenged by biopanning for multiple scFv/phage variants to increase chance of binding an immunogenic protein.

4.5 Conclusions

The experiments in this chapter set out to select a group of monoclonal scFv/phage variants, initially taken from a complex phage library, which would show strong binding to proteins from the membrane-enriched fraction purified in Chapter 3. Aims from the beginning of the chapter in purifying a small library of mite-binding scFv variants were successfully fulfilled. 63 of the most highly binding scFv/phage were selected for downstream immunohistochemistry and immunoprecipitation experiments (Chapter 5) to identify bound mite proteins and show their expression patterns in mite anatomy.

Chapter 5: Identification of gut antigens by immunoscreening with scFv

5.1 Introduction

The membrane protein enriched fraction of PRM homogenate described in Chapter 3 was used in biopanning experiments in Chapter 4 to isolate 63 scFv/phage variants that bound to the homogenate. In theory, each variant would have a good possibility of binding to a separate epitope expressed by PRM (the Tomlinson J library contains 1.37×10^8 scFv variants). This chapter details experiments that identified PRM gut tissue binding scFv/phage. Antigens that bound to such scFv would be gut expressed, potentially membrane proteins and could efficiently bind antibodies. These characteristics are what is required of a protein to be accepted as a vaccine candidate in this project.

To identify gut-binding scFv, sections of mite tissue were exposed to each scFv/phage variant in immunohistochemistry (IHC) experiments. 3-Amino-9-ethylcarbazole (AEC) staining from bound scFv/phage would determine which scFv/phage were binding to midgut tissues specifically. Immunohistochemistry of PRM required understanding of PRM internal anatomy, optimisation of mite specific sectioning and staining protocols and sequencing of scFv regions to validate that every scFv/phage was genetically unique.

5.1.1. Understanding the PRM digestive system

The general internal biology of PRM was described in Chapter 1 (1.1.4 Morphology & Anatomy 2: Internal morphology). What follows is a more detailed description specifically of the PRM digestive system. The majority of blood meal digestion in Acari occurs in the midgut and the six connected caeca (Akov 1982; Sojka, Franta et al. 2013) (for PRM see Figure 5.1). In PRM, the midgut and caecae occupy >70% of the body cavity when a mite is fully engorged, and decreases to approximately one quarter the size during periods of starvation (Figure 1.2, Chapter 1). To identify specific proteins involved in digestion, it is important to consider the different cell types and biological interactions that occur within the mite digestive tract. Acari digestive cells are generally classified into three types, based on their function and location (anterior midgut cells, caecal cells and posterior midgut/hindgut cells). Anterior midgut epithelial cells (Figure 5.1 – orange area) contain large vacuoles and go through a state of cytoplasmic degeneration whilst digesting food (Brody, McGrath et al. 1972; Coons 1978). In engorged mites, these cells detach from the gut mucosa and engulf ingested material within the gut lumen, becoming swollen and highly vacuolated. The presence of large intracellular vacuoles that contain material of a similar density to that seen in the gut lumen suggests that food digestion is carried out intracellularly, at least in part, in the midgut (Mathieson and Lehane 2002). The autophagic-lysosomal pathway is the most likely way that intracellular digestion occurs and is thought to be initiated by the action of parasite endopeptidases such as Cathepsin D and Cathepsin L (Nisbet and Billingsley 2000). Vaccination of poultry with recombinant PRM Cathepsin D or Cathepsin L induces anti-Cathepsin D- or anti-Cathepsin L-specific IgY immunoglobulins and when these are ingested by PRM in an *in vitro* feeding system, the IgY causes

increases in mite mortality (Bartley, Huntley et al. 2012). Most likely these IgY antibodies bind directly to secreted Cathepsins D and L in the lumen of the mite gut blocking digestive break-down processes. Vaccine-induced immunity in PRM midguts is also believed to cause damage to the gut barrier through direct binding of immunoglobins to membrane-bound proteins (Kemp, Pearson et al. 1989; Bartley, Huntley et al. 2012), though complement-induced antibody upregulation may be required. The posterior midgut (Figure 5.1 – blue area) appears to have a similar function to the anterior midgut in several mite species (Wright and Newell 1964; Filimonova 2013), however in *Psorptes ovis* the posterior cells are described as being more squamous with extended microvilli (Mathieson and Lehane 2002).

Caecal epithelial cells in various mite species (Figure 5.1 – yellow area) are densely packed with lysosomes, smooth endoplasmic reticulum and mitochondria, all indicative of high metabolic activity related to digestive enzyme activity. Brody, McGrath et al. (1972) proposed that the lack of visible particulate material in the caeca of the house dust mite *D ermatophagoides farinae* indicates that caecal cells secrete enzymes that are used for digestion in the anterior midgut. Erban and Hubert (2011) however demonstrated that midgut and caecal-wide hydrolysis of fluorescent substrates by several proteolytic enzymes occurred in the storage mite *Lepidoglyphus destructor*. Given the significant expansion in size and large volume of blood found in the caeca in engorged PRM, we suggested that caeca are also actively involved in food digestion (Pritchard, Kuster et al. 2015).

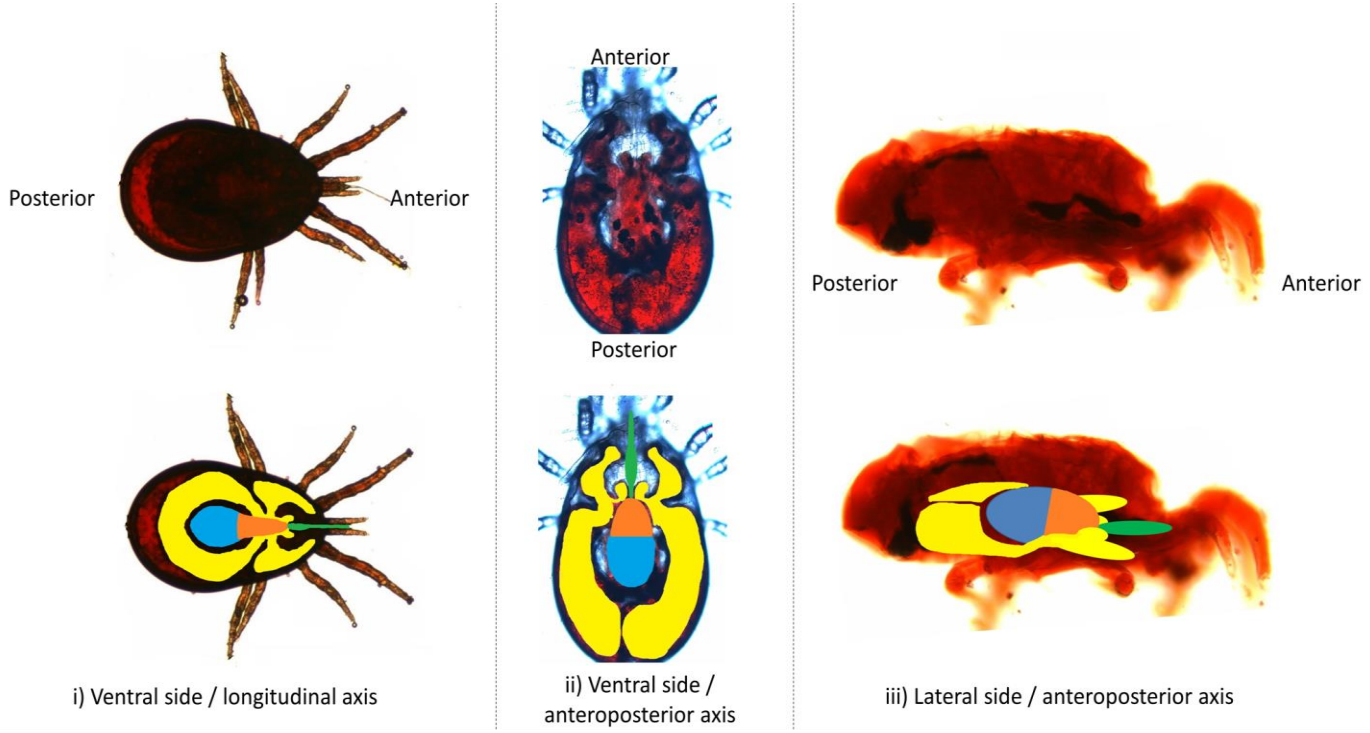


Figure 5.1: The digestive system of *Dermanyssus gallinae*. Images of PRM taken by light microscopy (x100-x200) and superimposed by coloured shapes indicating sections of the mite digestive system. This includes the oesophagus (green), the anterior midgut (orange), the posterior midgut (blue), three pairs of caecae (yellow) and a hindgut (not shown). Food travels from anterior to posterior.

5.1.2 Immunohistochemistry in mites

Immunohistochemistry (IHC) is the visualisation of specific antigens in a tissue section by binding of antibodies and the use of a chemical or fluorescent dye to stain bound antibodies. IHC is used in many areas of biological research, including disease diagnosis and drug development. IHC was first demonstrated by Coons, Creech et al. (1942) using fluorescein isothiocyanate labelled antibodies to identify *Pneumococcal* antigens. Since then there have been many advances in tissue fixation, sectioning, antigen retrieval and staining, reviewed by Chu and Weiss (2014). IHC often requires optimisation from 'general' protocols, depending on the tissue or organism under study. IHC has not been widely applied to species of Acari, but some examples do exist (Liu, Li et al. 2005; Willis, Fischer et al. 2006; Santillan-Galicia, Carzaniga et al. 2008). In studies that aim to characterise proteins where little is known of protein location and level of expression, there is wide variability in success or failure. For example, successful IHC localisation of digestive protease enzymes was carried out for the scabies mite *Sarcoptes scabiei* (Willis, Fischer et al. 2006) and the house dust mite *Dermatophagoides farinae* (Liu, Li et al. 2005), however the use of IHC in the honey bee mite *Varroa destructor* mite failed to identify deformed wing virus in mite tissue (Santillan-Galicia, Carzaniga et al. (2008). Similarly, Zhang, Ongus et al. (2007) failed to detect viral antigen with this technique even though antigen was clearly visible in mites using electron microscopy.

Poultry red mites are not well studied and, to the best of our knowledge, only a single publication exists that includes IHC. Bartley, Nisbet et al. (2009) demonstrated expression of histamine release factor (HRF) protein in the PRM digestive and reproductive tissues using a polyclonal anti-HRF rabbit serum. The serum from this

study, along with an anti-Cathepsin D IgY antibody (Bartley, Huntley et al. 2012), was kindly donated as a gift from Dr Alasdair Nisbet (Moredun, Scotland) to use in IHC studies in this project as a positive control.

5.1.3 Aims & Hypothesis

From a library that displays $1.37x10^8$ variations of a single chain variable fragment, 63 potentially genetically variable scFv were shown to bind membrane enriched PRM protein. This sub-library of 63 scFv variants in theory should have bound distinct epitopes on individual immunogenic proteins. This project aimed at identifying such immunogenic proteins expressed specifically in the digestive tract of the PRM. This chapter details experiments that iintroduced each individual scFv/phage in immunohistochemistry to identify which scFv-binding proteins were gut expressed. Using midgut binding scFv in future immunoprecipitation experiments could then be used to purify and identify immunogenic gut proteins. The specific aims of this chapter are listed below:

1. Develop and optimise methods for sectioning, histochemical staining and IHC staining of PRM using both polyclonal and scFv antibodies.

This project aimed to identify proteins expressed in the digestive tract of the PRM. To identify protein expression localisation, immunohistochemical staining was carried out. An in-house protocol was designed to align mites in a single plane in order to cut multiple longitudinal PRM sections at once. An IHC protocol was also developed and verified by the use of PRM specific polyclonal antibodies.

2. Define localisation of target protein(s) bound to scFv variants selected in Chapter 4.

ScFv/phage variants selected from biopanning (chapter 4) were used in immunohistochemistry to identify target proteins from the PRM gut. This required understanding where the mite gut was located (see Pritchard, Kuster et al. (2015)) and comparing staining intensity and location of each scFv/phage. Understanding of what proteins may be bound to the scFv/phage is also discussed (see 5.4.2: immunohistochemistry results).

3. Sequence the scFv region of each scFv/phage clone and compare genetic sequences to reveal if each variant is genetically distinct.

ScFv/phage variants chosen come from a library of 1.37 x 108 potential variants and so are unlikely to be similar. Some scFv/phage may grow on plates or replicate in LB more efficiently than others, or may simply bind to a common epitope. To investigate scFv/phage variant genetic variability the scFv region of each phage was sequenced. Sequences produced and the importance of the results is discussed (see 5.4.3 sequencing of individual phage).

Methods

5.2.1 Slide preparation for immunohistochemistry

i) Fixing

1g of live mites were captured and stored at room temperature for 7 days to allow complete blood digestion (see '2.1.1 Mite collection'). Mites were placed in a 100ml bottle (Schott, USA) containing 50ml Carnoy's fixative (see Appendix). The bottle top was closed tightly and further sealed with Parafilm®. The bottle was placed on a rocker (Grant-bio #PMR-30) for 5 days at 4°C to fix mite tissue, ensuring all mites were covered by Carnoy's fixative. In a fume cupboard Carnoy's fixative was slowly pipetted out whilst mites settled at the bottom of the flask. 100ml of 150mM NaCl in dH2O was added and mites were resuspended by gentle agitation. Mites were stored at 4°C in the 150mM NaCl for up to 6 months.

ii) Mite alignment

20ml of 4% agarose (Invitrogen) in dH_2O and 2ml of 5% gelatin (Sigma) in dH_2O were microwaved separately (~30-60 second) until in a liquid state. 1ml of each was added to a single 2ml Eppendorf tube, gently mixed together and placed in a dry heat block at 40°C to remain liquid.

Two metal slides, originally designed for moulding agarose gels before DNA electrophoresis, were placed on the 40°C dry heat block to heat up. Between 100 and 500 mites were placed on one slide and 200µl of gelatin-agarose solution was pipetted on top, submerging all mites. The second metal slide was placed on top and the whole 'sandwich' was placed in a box at -20°C for 20 minutes (see Figure 5.2). The sandwich was placed at room temperature and gently opened with a scalpel. The now flattened

gel-mite mix was removed with tweezers and placed in a foam-insulated embedding cassette.

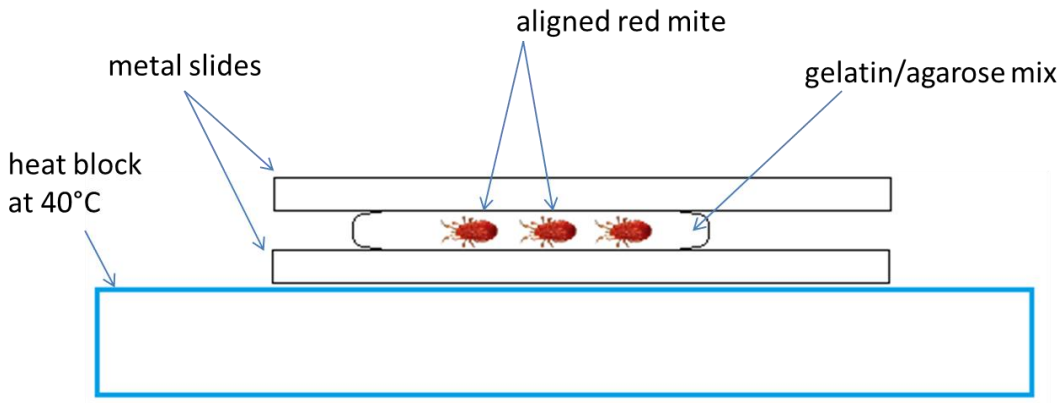


Figure 5.2: The 'sandwich' created before embedding of mites which allowed alignment of mites in a single planar formation on a wax block.

iii) Embedding

Cassettes were placed in a tissue processer (Tissue-Tek VIP 6, Sakura) then into a mold where molten paraffin wax at 60°C was poured over the samples. Tissue/gelatin samples were held at the base of the mold by heated forceps during pouring and the cassette was placed on top of the wax. The mold-wax was placed on a cold plate at -25°C allowing wax to harden. This created a cassette with an attached wax block containing the mite sample. This was stored at room temperature until required.

5.2.2 Immunohistochemistry

iv) Sectioning

Embedding cassettes including the wax block were placed at -20°C for 30 minutes then secured to a microtome with an attached blade (MX35, Thermo scientific). The face of the block was damped with a wet sponge to hydrate the mite tissue, a 10µm section

was cut and the section was placed in a water bath at 30°C. This was repeated until no mites were left to cut from. Sections were mounted on slides (Superfrost Plus, Thermo Scientific), placed overnight at 37°C then stored at room temperature until required.

v) Rehydration and addition of antibodies

Slides were placed on a 60°C dry heat block for 5 minutes to melt the wax. Slides were then placed in a succession of solutions to completely rehydrate tissue, allowing 5 minutes in each solution in a coplin jar placed under a fume cupboard. This included incubation in histoclear clearing solution (Sigma #H2779-1L) followed by → histoclear again → 100% ethanol →90% ethanol → 70% ethanol → dH_2O → dH_2O and finally into 3% hydrogen peroxide for 20 minutes. Slides were briefly washed in wash buffer (see appendix) then a circle was drawn around the tissue using a hydrophobic pen (Vector labs) and 200ul of blocking buffer (see appendix) was used to cover all sectioned tissue. Slides were incubated for 1 hour at room temperature. Blocking buffer was removed and slides were washed 3 x 5 minutes in wash buffer. 200ul of primary antibody (in this case supernatant of scFv/phage infected cells grown up overnight and spun at 3000g, 10mins) was added to each slide and the slides were stored overnight in a sealed humid chamber at 4°C. Slides again were washed 3 x 5 minutes and the process was repeated for secondary (1:5,000 rabbit anti-his, Sigma #H1029) and tertiary antibodies (1:5,000 goat anti-rabbit HRP, Abcam #ab6721) diluted in wash buffer. A final 3 washes with 1xPBS were carried out and 3-Amino-9-ethylcarbazole substrate colour change due to presence of bound peroxidase was shown using an AEC staining kit (Sigma #AEC101), following the manufacturer's instructions. Staining was monitored using x100 microscopy and stopped by placing

slides in dH_20. Unbound stain was removed by placing slides under running water for 10 minutes. Slides were kept hydrated in water until photography of staining by x100 microscopy was carried out.

5.2.3 Sequencing individual phage

To validate presence of scFv/phage and that each scFv/phage was a unique variant, each scFv/phage was amplified overnight and the DNA was extracted (see 2.3.1 'Phage DNA extraction') and quantified (see 2.3.2 'DNA quantification'). 20µl of 30-100ng/µl DNA from each phage sample was sent to GATC Biotech (UK) for sequencing, also sending 20µl of 10pg/µl of LMB3 forward primer (CAG GAA ACA GCT ATG AC) and pHEN reverse primer (CTA TGC GGC CCC ATT CA) to amplify to scFv region. Received sequences were analysed using CLC Main Workbench 5.7.1. Phage scFv regions without a scFv insert were an expected size of 329 base pairs, and with insert an expected size of 935 base pairs.

5.3 Results

5.3.1 Immunohistochemistry

From the 62 scFv/phage variants that produced repeatedly high binding in ELISA, 31 showed binding to specific anatomical locations in mite tissue in repeat experiments. Of these, 19 scFv/phage isolates bound to the midgut, hindgut and/or caecae (See table 5.1).

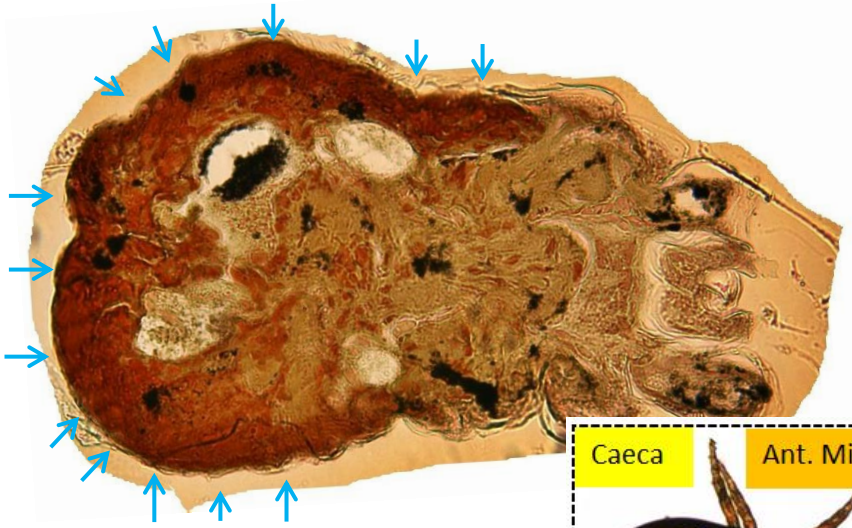
	Grouping	Number of phage variants displaying pattern	Number of phage variants that also bind to the digestive tract
Frequency of staining pattern	Once (1 mite per slide)	2	2
	Occasionally (2-3 mites per slide)	7	4
	Often (>3 mites per slide)	22	13
Comparative intensity of staining	Faint	2	2
	Strong	23	13
	Very Strong	6	4
Total number		31	19

Table 5.1: Results of the staining patterns produced by IHC using selected scFv/phage variants. A total of 31 from 62 scFv/phage tested showed reproducible binding patterns in triplicate IHC experiments. Each scFv/phage stained varying numbers of mites per slide and at varying intensities of staining. Of note, 19 scFv/phage bound to the digestive tract and 8 of these stained 3-10 mites per slide and 5 scFv/phage bound 11-20 mites per slide from a total of 10-50 mites per slide total. No scFv/phage were

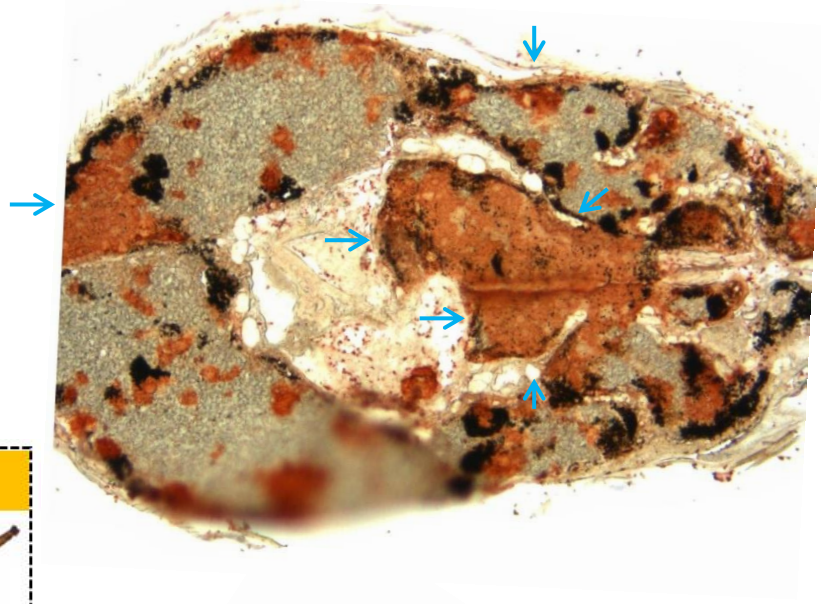
seen to bind a majority of mites per slide as mites were of mixed life cycle stages and digestion stages (see 5.4.2 IHC – results).

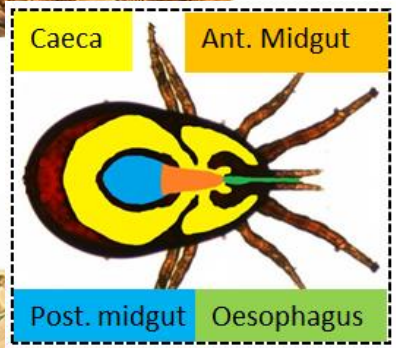
Staining intensity was analysed as well as mite numbers stained per slide. 17 of the 19 scFv/phage showed strong or very strong staining (see Figure 5.3). The colorimetric scale of the staining was either faint (5.3i - pink), strong (5.3ii - red) or very strong staining (5.3iii – dark red) as is highlighted by blue arrows. Some stained mites indicated organ/tissue wide staining, such as 5.3i, that displays heavy staining of the 3rd caecal pairing. Other mites indicated a 'spotting' of smaller regions, such as 5.3ii, indicating only localised expression of certain proteins. More extensive detail of each specific scFv/phage binding localisation and amount of staining is shown in the supplementary S10.

i) Caecae (phage 4-2E)

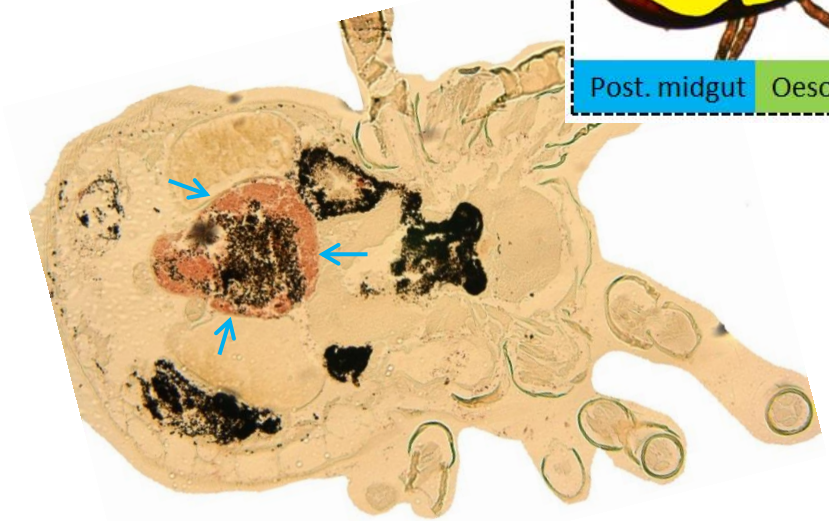


ii) Midgut and localised spots (phage 8-6G)

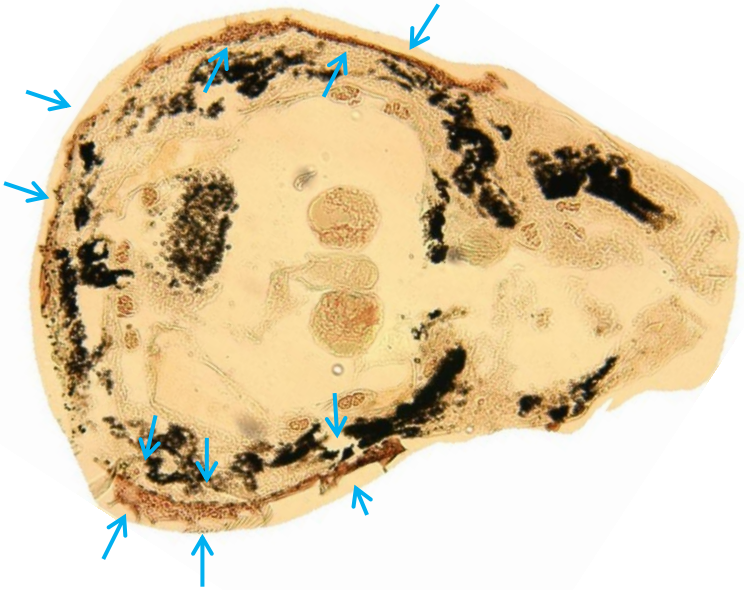




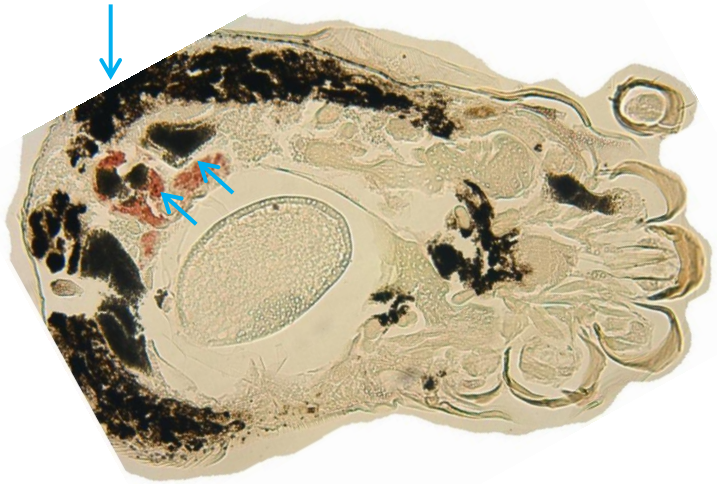
Iii) Posterior midgut (phage 4-3E)



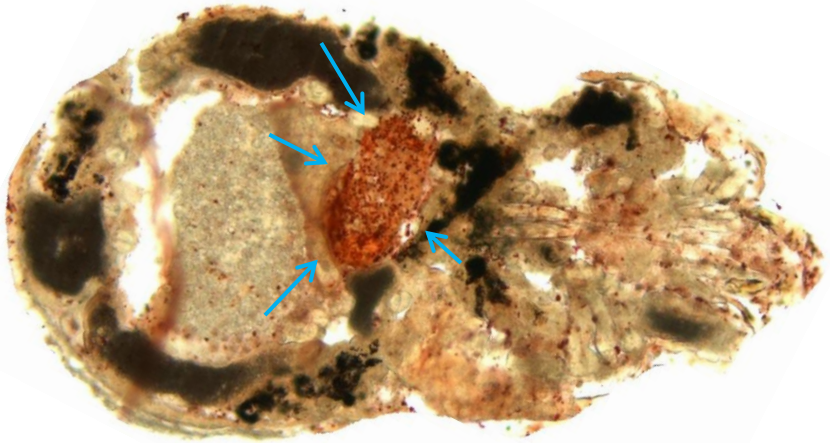
iv) Exoskeleton (phage 4-7C)



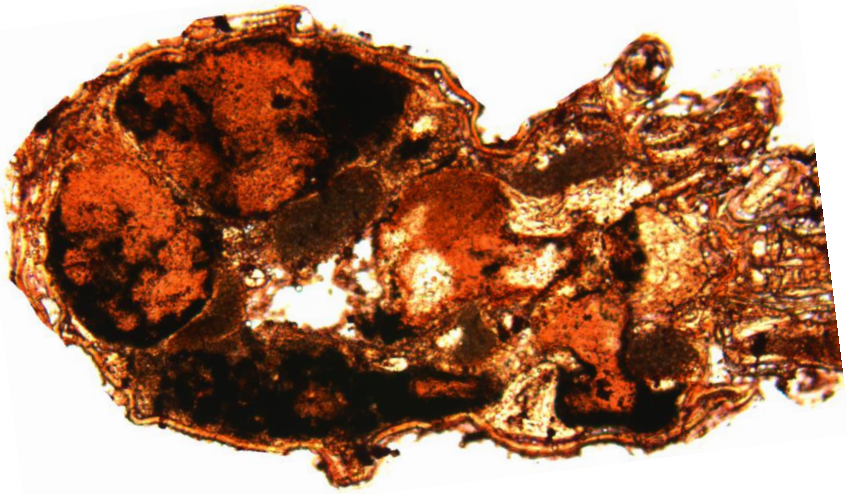
v) Tissue near egg (phage 211E)



vi) Egg (phage 5-6F)



vii) All mite (anti-mite antibody)



viii) No binding (phage 8-5G)

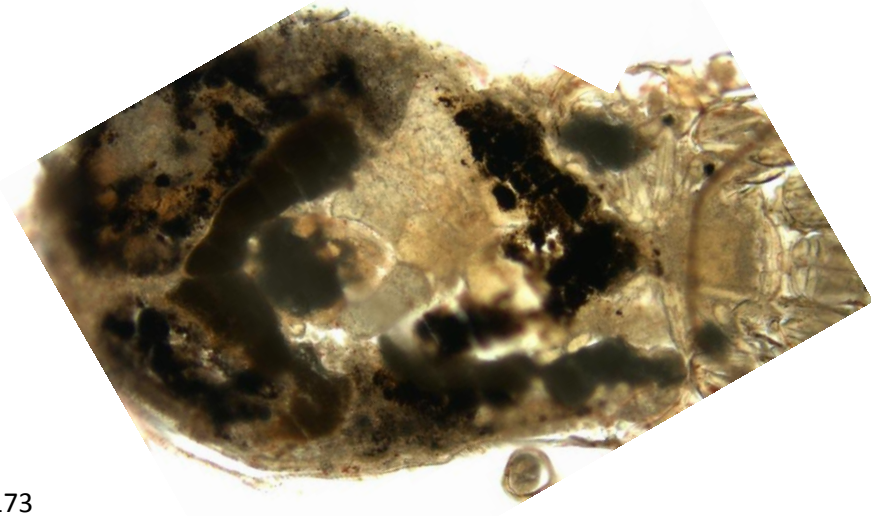


Figure 5.3: Staining of scFv/phage variants bound to mite tissue during IHC. All sections faced posterior (left) to anterior (right) in a longitudinal axis viewed from the ventral side at x100 microscopy. Images i-iii showed binding (in pink/red/brown) of scFv/phage to proteins expressed in the PRM digestive system, noting some were localised (ii and iii) whereas other phage patterns were more general (i). A descriptive image of the PRM digestive system is presented in the middle of the figure as an anatomical guide (originally taken from Figure 5.1). Other 'non-gut' organs also displayed specific staining (images iv-vi), see blue arrows. Staining intensity was classed as very strong (I – dark red), strong (ii -red) or faint (iii – pink). A rabbit anti-red mite antibody bound strongly to all the mite body (image vii) whilst many phage we classed as 'negative' as they showed no binding (image viii).

5.3.2 Sequencing of scFv/phage variants

Of the 32 scFv, only 12 scFv/phage variants were sequenced. Of this 10 were individual sequences. These results was translated into amino acid sequences *in silico* using CLC workbench and compared against a reference scFv/phage (see Figure 5.4). 6 scFv regions were completely sequenced and 6 only sequenced one of either the light or heavy variable chain (e.g. just the light chain in phage 7, Figure 5.4).

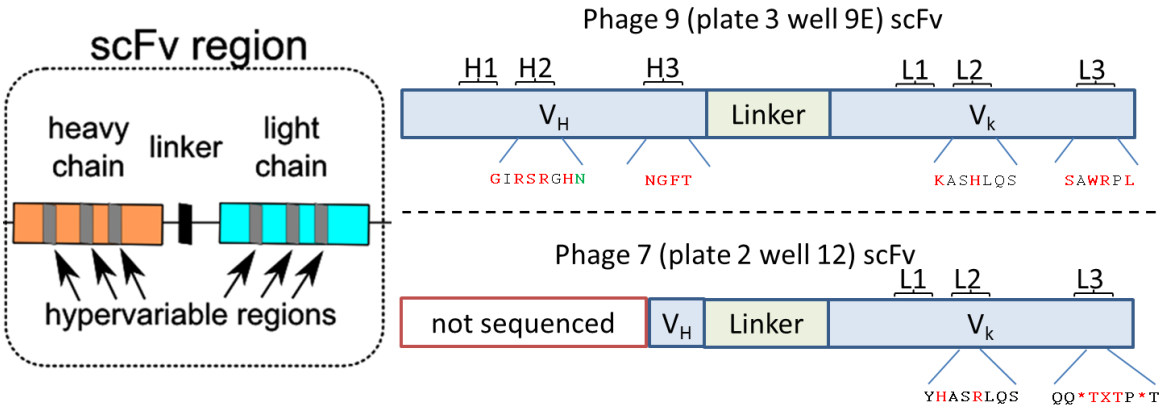


Figure 5.4: Sequencing of the scFv region of two scFv/phage variants showing difference in hypervariable region amino acid sequences. The single chain variable fragment consists of a heavy chain and light chain joined by a linker sequence (left of figure). Both the heavy and light chains interact with epitopes through six hypervariable regions which contain different amino acid sequences between different scFv variants. Sequencing of selected scFv/phage produced both fully sequenced (light and heavy chains) scFv regions such as phage 9 (top) or partially sequenced (either light or heavy chain) scFv regions such as phage 7 (bottom). ScFv hypervariable region amino acid sequences are compared to a reference scFv (not shown) where amino acids are shown to be substituted (red), added (green) or conserved (black) from the reference scFv sequence.

5.3.3 Summary of results

- In the absence of a clear scientific report describing the internal anatomy of PRM in the scientific literature, we published a report from our learnt understanding using microscopy and reviews of other mite species' anatomy. This knowledge was utilised during IHC experiments.
- From immunohistochemistry of 62 scFv/phage variants, 31 bound repeatedly to a particular tissue or organ. Of these, 19 bound specifically to the tissue of the PRM digestive tract.
- From 31 scFv/phage 12 were sequenced. 6 Showed full sequence of the heavy and light chain whilst 6 only showed sequence for one of either the heavy or light chain. 19 failed to be sequenced.

5.4 Discussion

5.4.1 Immunohistochemistry - optimisation

IHC using the selected scFv/phage was previously thought of as a difficult and potentially very challenging technique to optimise. The major problems faced in experiments were with sectioning of sufficient PRM at once in a comparable angle. Initially, many sections were torn due to the cuticle of the mite that remained after fixing. This is common with invertebrate histology (Carlisle 1960; Barbosa, Berry et al. 2014) as exoskeletons are both tough due to chitin proteins and hydrophobic due to a waxy outer layer (Krantz and Walter 2009). To circumvent this, a sectioning technique was developed involving addition of a slight layer of moisture to the wax block before each section was cut. This allowed partial hydration of the mite tissue and made sectioning mite tissue into 5-10µm sections with intact mite morphology.

A second problem faced was regarding the lack of mites that were obtained from a single section, whilst all being in a similar orientation. Ideally, as shown in Figure 5.3, mites would be orientated in a lateral axis, all viewed from the ventral side. To align mites in the correct position mites were added to liquid agarose then sandwiched the sample in hot gel combs and quickly froze the gel to a solid phase at -20°C (Figure 5.2). This not only aligned sufficient numbers of mites in a similar plane but also helped align the orientation of each mite. Mites otherwise would either rise to the top or sink to the bottom of the sandwich. This may have been due to the waxy hydrophobic cuticle of the mite exoskeleton and the aqueous conditions of the gel.

A final problem of the IHC was regarding staining. As is obvious to see from Figure 5.3 viii (no binding), PRM sections were often brown and this interfered with red/brown staining of the substrate of the AEC kit (Sigma, UK). An eosin counterstain was

attempted and, although pink staining was detected against the blue background in some cases (see supplementary S11), these sections required thinner 5µm sections that did not reliably show detailed mite anatomy. Instead, the original protocol using 10µm and the AEC staining kit (Sigma, UK) was carried out, however keeping slides in aqueous conditions to photograph staining. Dehydration and fixing of stained slides is recommended for long term preservation of tissues (Burry ; Dabbs 2013), dehydration however increased the brown colour of the mite. This is a common occurrence in IHC and dark brown colouring with dehydration has been attributed to artefacts created from chemical pigmentation during processing (Chatterjee 2014) or through loss of water causing tissue to become more rigorous and darker (Krause 2001). Melanin interacts with some fixing solutions causing dark brown pigmentation of tissue sections (Nadji 1986). This is possible in PRM sections, as melanin is common in invertebrates, functioning in scleretization of the exoskeleton and acting as a dark brown pigment (Sugumaran 2002). Various artefacts in histology can be removed by additional treatment of tissues in alcoholic ammonia (Chatterjee 2014). This was tried and with some success (not shown). The process was deemed not required enough for results as photography in aqueous conditions produced clear enough PRM staining differentiation.

To validate the IHC protocol an anti-mite serum created in vaccinated rabbits (Figure 5.3 image vii), an anti-Histamine release factor antibody donated by Dr Alisdair Nisbet (Moredun) and the post-biopanning round 3 scFv/phage library all produced positive binding when presented in IHC (see supplementary S12). A commercially bought drosophila-specific anti-nervana 1 showed up as negative (i.e. no binding) when using

the same goat anti-rabbit secondary antibody as a negative control (see supplementary S12).

5.4.2 Immunohistochemistry - results

Of the 62 scFv/phage tested in immunohistochemistry, 31 scFv/phage displayed repeated binding and of these 19 scFv/phage bound specifically to the mite digestive tract. Each scFv/phage underwent IHC a minimum of three times, using two slides per repeat, to ensure scFv staining seen was genuine. ScFv/phage staining intensity and localisation is discussed below.

i) ScFv/Phage binding intensity

Some scFv/phage variants stained strongly so 3-Amino-9-ethylcarbazole colour appeared red/brown (phage 4-2E, Figure 5.3 part i) whereas other scFv/phage bound in lesser amounts, thus displaying pink staining (phage 4-3E, Figure 5.3 part iii). Whilst theoretically all scFv/phage variants invade bacterial cells in a ubiquitous manner, some scFv/phage may have been expressed in higher titres than others, hence giving a stronger staining. Evidence from collaborator experiments (Rod Noad, RVC, unpublished) suggest scFv/phage variants containing an amber stop mutation in the scFv region are better expressed than scFv/phage without. TG-1 E. coli cells used in biopanning are amber-supressing (amber stop codons are 'read-though', see chapter 4: biopanning). TG-1 cells however, are not 100% efficient, often stopping translation of 50% or more of the amber codon containing sequence creating a shorter, truncated protein. This production of truncated proteins can enhance expression of scFv/phage in two ways. Firstly, less protein is produced so metabolic pathways in the E. coli cell are less stressed and therefore replicate at a higher rate due to increase of metabolic

activity towards cell processes. Secondly, fully expressed proteins (as opposed to truncated forms) also express the fusion coat protein pIII which genetically is located downstream of the scFv region. PIII expression on the surface of E. coli cells inhibits superinfection of KM13 helper phage that is required for scFv/phage packaging from the cell. Counterintuitively therefore more full length proteins expressed means more pIII coat proteins expressed, less helper phage infection and less production of soluble scFv/phage.

It is also likely that, although scFv/phage were initially selected for high binding in ELISA format, the affinity of scFv to fixed tissue on a slide may be variable. Dehydration and sectioning can disrupt protein structure (Ross and Pawlina 2006), however fixative solution creates cross-linking covalent bonds between proteins in tissue to limit protein degradation.

ii) Localisation of scFv/phage binding

Some scFv/phage were found to bind reproducibly in replicate experiments but only to a small number of mites per slide (e.g. phage 3-2C, outlining midgut, supplementary S13) whereas others bound up to one in three mites per slide (e.g. phage 2-8C, midgut, supplementary S14). The PRM used for IHC were left for 7 days to digest blood; however, were a mixed population that included eggs, nymphs and adults. Specific protein expression could have been therefore variable between digestion states and life cycle stages. Determining how long to leave mites before fixation required a trade-off between allowing blood digestion to occur yet simultaneously not losing expression of digestive proteins that may be potential vaccine candidates. Blood meals were largely not visible from mites that had been starved for 7 days after capture (see Figure

1.3 in Introduction) but they remained generally larger and more rounded than at Days 10-14. PRM digestion is similar to that of hard ticks where digestion is completed in between 7-13 days (Akov 1982; Tarnowski and Coons 1989). As previously mentioned (5.1.1 understanding the PRM digestive system) digestive processes are likely to be variable in different sections of the mite gut. ScFv/phage binding to caeca expressed proteins for example (Figure 5.3i, phage 4-2E) perhaps bind to digestive enzymes released and diffused into the gut content. This would explain the staining of the whole third pair caecal structure of phage 4-2E. PRM in late stages of digestion may have only being processing digested blood meal in the later parts of the digestive tract when fixation occurred. Scfv/phage 4-3E (Figure 5.3iii) specifically binds to posterior midgut tissue and little else suggesting the corresponding protein may have a late stage digestive function. Other scFv/phage bind to tissue of unknown function, such as scFv/phage 2-11E (Figure 5.1v). Interestingly, this scFv/phage binding epitope bound to 6 of the 12 scFv/phage that bound to 'non-gut' PRM tissue. Staining was not present in other scFv/phage and was highly specific to that tissue region in the scFv/phage that did bind. All mites that presented binding of that tissue location were carrying a developing egg so it is reasonable to theorise this protein may have some function in egg development, such as PRM vitellogenin targeted by Bartley, Wright et al. (2015).

Overall, 19/31 (61%) of scFv/phage bound to the gut which is in alignment with the capacity of a fully engorged mite gut to represent >70% of the mite body cavity. Though space is taken up by blood meal, the proportion of total protein expression that is required for digestion of blood is likely to be a large amount, as is reported in mosquito and haematophagous tick species (Dinglasan, Devenport et al. 2009; Kongsuwan, Josh et al. 2010).

5.4.3 Sequencing of individual phage

Single scFv/phage variants were sequenced in the scFv region sequence to identify each scFv/phage as individual. Of 31 scFv/phage, 6 produced sequences for heavy and light chain regions, 6 produced sequences for only one of the heavy or light chain and 19 were not successfully sequenced. This was unexpected so PCR was carried out to amplify the scFv region of each scFv/phage variant using the pHEN and LMB3 primers that failed in sequencing. All 31 scFv variants amplified and produced bands in a DNA electrophoresis gel at 800-900bp as expected (not shown). Unsequenced scFv variants also produced a secondary band at 500bp indicating there was potentially also a truncated secondary scFv variant in these samples. The addition of a secondary template would interfere with sequencing and therefore re-streaking of colonies and sequencing several individual colonies of each variant would remove infection of secondary scFv/phage variants. Alternatively, cutting out of a single band from PCR amplification DNA electrophoresis and sequencing would also remove contaminating secondary scFv.

Six of the sequenced scFv only sequenced one chain. Binding of single chain fragments to target epitopes through binding of only one variable chain (heavy or light) is possible (Holliger and Hudson 2005). Even including both heavy and light chains, extracellular conditions or disruption of protein structure may hide parts of an epitope region that prevents binding of both regions. Insertion of the of scFv regions in the Tomlinson J library was carried out by two step cloning, so a likely explanation for scFv fragments missing one chain is due to one of the cloning steps failing. Alternatively, M13 inserts are not completely stable, it is possible for recombination during the

ssDNA replication which leads to partial deletion of the insert. This would explain the scFv clones where there was only part of the domain missing rather than the complete loss of one of the chains.

5.5 Conclusions

This chapter has described the use of immunohistochemistry to identify scFv/phage that bind specifically to gut tissue of PRM. Protocols were established for sectioning and staining of mites using scFv/phage as a primary antibody followed by anti-phage antibodies and a 3-Amino-9-ethylcarbazole stain substrate. Staining localisation and intensity were varied between scFv/phage demonstrating the variability of protein expression levels in a eukaryotic organism with many cell types and metabolic processes. Gut staining scFv/phage and immunoprecipitation of PRM homogenate could be used in future experiments to purify gut expressed, antibody binding proteins. Such proteins match the criteria of vaccine candidates targeted by this project.

Chapter 6: Overall Discussion

6.1 Summary of the project results

This project aimed to identify proteins that could potentially be used in a vaccine against the poultry red mite (PRM). Following success of the Cattle tick TickGARD vaccine, using the membrane gut protein BM86, this project targeted mite proteins that were similarly a) membrane bound b) expressed in the digestive tract and c) immunogenic. Differential centrifugation was used to separate a subcellular mite homogenate into a fraction enriched for membrane proteins. Mass spectrometry of this fraction aligned peptides to 11,186 RNA transcripts from PRM. Biopanning of the Tomlinson J scFv/phage library ($5.4x10^{13}$ variants) against this membrane protein enriched fraction over three rounds followed by individual binding analysis of 760 individual scFv/phage variants resulted in 68 highly binding scFv/phage. Localisation of where in the mite the target proteins of these scFv/phage were expressed was determined by immunohistochemistry. Of the 68 scFv/phage variants, 31 scFv/phage bound specifically and repetitively to mite tissues, of which 19 bound to the PRM digestive tract. In future studies these 19 scFv/phage will provide a tool to purify protein targets associated with the gut. Such proteins are likely to satisfy vaccine target criteria of being membrane proteins, expressed in the PRM gut and being able to bind strongly to antibodies (i.e. being immunogenic).

6.2 Limitations of project aims and methodology, including potential alternatives

6.2.1 Purification of integral membrane proteins

The use of Triton X-100 and differential centrifugation to enrich for integral membrane proteins is a common technique and has been well documented for use in arthropods, including mites (Wright, Bartley et al. 2009), ticks (Opdebeeck, Wong et al. 1989) and mosquitoes (Barreau, Conrad et al. 1999). The protocol selected in Chapter 3 using differential centrifugation to enrich for membrane proteins (see section 3.2.3) was derived from an earlier publication (Wright, Bartley et al. 2009) that aimed at fractionating PRM proteins in a similar manner. Fractions separated by differential centrifugation were not expected to be 100% 'pure' fractions of nuclei, mitochondria or plasma membrane proteins, as was demonstrated by multiple bands in every fraction in SDS-PAGE (Figure 3.8). Some bands in SDS-PAGE were specific to a single fraction however, suggesting separation of proteins did occur at some capacity.

It is generally accepted there is no single leading technique for isolation of membrane proteins (von Jagow 1994; Smith 2011), rather investigators use a series of methods, such as homogenisation then centrifugation, that should be tested and optimised - as was carried out in chapter 3. Without a full understanding of the PRM transcriptome (see 6.3.3 Use of PRM genomic and transcriptomic data) it is difficult to develop a clear picture of the amount of membrane proteins in the 'membrane-enriched' fraction. Alternative techniques for membrane protein purification, such as chromatography or ultrafiltration, are successfully used in membrane protein enrichment (Nakao, Osada et al. 1988; Ghosh 2002). These techniques however hold no relevant advantage over centrifugation when the amount, functions or sizes of targeted proteins are unknown. This methodology is well exemplified by early vaccine studies into the cattle tick that first targeted whole tick homogenate, then

fractionated membrane enriched fractions, and finally individual proteins were discovered (explained in 1.4.8 A Vaccine against *D. gallinae*) (Willadsen, McKenna et al. 1988; Willadsen, Riding et al. 1989; Willadsen, Bird et al. 1995). Unknown biology of the cattle tick led to the use of fractionation via centrifugation to separate protein fractions for testing, ultimately leading to the discovery of BM86 and the TickGARD vaccine.

The addition of Tween 20 in Chapter 3 differential centrifugation protocols was to remove peripheral proteins. Whilst peripheral proteins may be included as 'membrane proteins', along with integral membrane proteins, many are displayed intracellularly or have a transient reversible attachment to the membrane (Ahmed 2004). Many have vital functions (e.g. cytochrome C transporting electrons to cytochrome oxidase), however hypothesis of antibody-mediated success in the PRM midgut is based on expression of proteins that are consistently exposed to immunoglobulin molecules during blood digestion. Though integral membrane proteins can laterally transverse a plasma membrane (i.e. the fluid mosaic model (Singer and Nicolson 1972)), they are permanently bound to the membrane. Integral proteins of midgut cell epithelium therefore will consistently be present during a blood meal. Comparatively, a reason for many PRM vaccine studies producing IgY immunoglobulins, but resulting in lack of mite mortality, may be due to the inability of antibodies to reach target proteins such as peripheral membrane proteins in the host (see '6.2.2 alternative target proteins' below).

6.2.2 Alternative target proteins

Membrane proteins expressed in the PRM midgut were targeted due to there being a comparatively high chance of protein exposure to immune molecules during blood meal digestion. Whilst this strategy has been previously successful in tick vaccines (Willadsen 2004), there was no guarantee such a strategy would work for PRM. Successful vaccine studies have targeted so-called 'hard ticks' (family 'Ixodidae') that feed for hours/days at a time on hosts, unlike 'soft' ticks (family 'Argasidae') and mites that feed for seconds/minutes. Comparatively

shortened biting time means less blood volume, and hence less antibody titre, is consumed by mites thus denoting antibody-mediated immunology may be less prominent in the PRM compared to hard tick species. This is also relevant to why salivary proteins would not be effective vaccine candidates in PRM, but are reported as potential targets against hard tick species (Rand, Moore et al. 1989; Schoeler and Wikel 2001). Saliva is introduced by hard ticks into host blood as an anti-coagulant, as well as having immuno-modulatory properties. The extended length of feeding time allows immune recognition of salivary proteins, hence there is potential for saliva-specific antibodies to enter tick blood feed. Whilst there is potential for PRM to have similar function for saliva, the comparatively faster feeding time and therefore lower amounts of saliva would likely not induce immune recognition of salivary proteins.

It is hypothesised that successful immunoglobulin binding and disruption of digestion would detrimentally effect PRM either directly through starvation or rather at a population level by reduced egg development and fecundity. It was shown by Bartley, Huntley et al. (2012) that targeting digestive proteases such as Cathepsins can reduce mite weight and egg development. Further to this, targeting of proteins expressed outside of the digestive tract (e.g. haemocyte proteins or neural excretory proteins) may also be possible as antibody molecules can pass through the midgut epithelium in ticks (Brossard and Rais 1984). Alternative vaccine targets of particular interest include neural secretory proteins, such as acetylcholine, that are already targeted by chemical acaricides (Committee 2012) and natural oils (George, Smith et al. 2009). Also, proteins involved in egg development that have already been targeted in mites (Bartley, Wright et al. 2015) and ticks (Tellam, Kemp et al. 2002) may reduce PRM numbers at a population level. Immunohistochemistry results from this study identified scFv/phage that bound exclusively in pregnant female mites, at tissue localised next to developing eggs (Figure 5.3v). Various scFv/phage also targeted protein expressed in both midgut and the synganglion tissue (scFv/phage 6-8F, not shown). It is important to recognise that though this project has targeted midgut proteins, scFv/phage binding proteins from other

tissues may also be valid tools used to identify vaccine targets to test in downstream experiments. What is clear is whilst many proteins have been implicated as vaccine candidates against poultry red mite, none have resulted in development of protective immunity or a significant increase in mite mortality rates (Arkle, Harrington et al. 2008; Harrington, Canales et al. 2009; Bartley, Huntley et al. 2012). A key balance in vaccine studies is to identify immunogenic proteins, but not to isolate too few proteins that trials fail if a single target does not perform as expected. Mutations in the Cattle *tick R. microplus* have already resulted in resistant populations of tick to BM86 (García-García, Montero et al. 2000), promoting the addition of a secondary antigen, Bm95, and the creation of the second generation vaccine TickGARD plus (Jonsson, Matschoss et al. 2000).

6.2.3 Developing a PRM-specific scFv/phage library

ScFv/phage library biopanning allows purification of proteins that are able to bind to a scFv molecule through the antigen binding site (derived from antibody molecules). In this respect, the scFv can select specific target proteins, whereas other previously failed PRM vaccine attempts have used complex fractions of many protein types without success (Arkle, Harrington et al. 2008; Wright, Nisbet et al. 2011). ScFv are particularly useful in biopanning as the whole molecule can be expressed as a single peptide in a prokaryotic system. A small sized scFv molecule (25kDa) can also access epitopes in small molecular spaces where it may not be possible for a larger chicken immunoglobulin (80-120kDa) to bind. This is useful in binding proteins during biopanning or IHC, however if such epitopes are in areas where larger antibody fragments cannot reach in an *in vivo* setting, such target proteins maybe limited in their protective capacity.

Previous studies in PRM have indicated successful production of PRM protein specific immunoglobulins, but without a resultant protective, behaviour exhibited by increased mite mortality rates (Harrington, Canales et al. 2009; Bartley, Huntley et al. 2012). This may suggest

biopanning for proteins that can bind antibody molecules may not be an efficient method for selecting suitable vaccine candidates. Alternative methods for identifying immunogenic proteins include direct immunisation of poultry in large scale trials, use of camelid antibodies or development of hybridoma cells. Large scale trials are entirely possible, as was seen in the development of TickGARD (Willadsen, McKenna et al. 1988). Fractions from whole parasite homogenate are separated and inoculated into test subjects and the fraction found to be the most immunogenic is separated further and vaccination is repeated. This 'fractionate and vaccinate' method was neither suitable in time scale nor financially plausible for this project. Animal husbandry and staff support are logistically challenging and expensive when large scale trials are carried out. ScFv/phage technology alternatively targets immunogenic proteins based on *in vitro* identification therefore negating requirement for animal testing. Production of a monoclonal antibody library would also require injection of many animals (usually rodent) for extraction of hybridoma cells. Investigators rely on the immune system of mice to find the combination of v, j and d segments that recombine to form functional antibodies that recognize the target antigen. Camelid and shark antibodies are unique in that they have two identical heavy chains rather than a heavy and light chain. Similar to scFv molecules, camelid antibodies have a small size and can be implemented into phage as a single DNA strand. Initial ideas for this project involved the vaccination of a llama and extraction of B cells that could have been immortalised with hybridoma cells. Use of scFv/phage libraries however not only provides a broader starting repertoire of antigen binding sites than hybridoma cells but also avoids time spent selecting hybridoma cells. A secondary plan during the project involved injection of a rabbit with PRM membrane enriched homogenate and extraction of the rabbit serum. This was to be used as a secondary option if scFv/phage biopanning had failed, however was not required. This serum was successfully used as a positive control in western blots and IHC experiments though (see Figure 5.3 vii).

6.3 Future studies

6.3.1 Identification of protein targets

This project aimed to identify vaccine targets to be analysed in trials and, whilst the aim of identifying immunogenic proteins specifically was not reached, a tool was developed for the purification of target proteins. Several scFv/phage can now be used in protein purification techniques, such as immunoprecipitation or chromatography. Initial purification studies demonstrated insufficient scFv/phage are produced by TG-1 *E. coli* cells for pull down of a mite protein via phage binding to either protein A sepharose (Sigma #P3391) or a his-tag column. This was unexpected as amplified scFv/phage titres of 2.7x1013 cfu/ml were of similar concentrations to other publications using the Tomlinson J library (Garet, Cabado et al. 2010; El-Magd, Vozza et al. 2016) and that isolated scFv using His-bond Nickel affinity chromatography (Yan, Ko et al. 2004).

Prokaryotic cells such as TG-1 *E. coli* are commonly used as recombinant protein expression systems as such systems can produce large amounts of protein via culturing large volumes. If insufficient phage numbers were produced, then more TG-1 cells could have been grown in increased volumes. Even a 1 log increase from $2.7x10^{12}$ to $2.7x10^{13}$ cfu/ml however would have required growing 50 litres of TG-1 cells in LB broth. A more feasible approach would have been to transfect scFv from TG-1 cells into a eukaryotic system. Prokaryotic systems often fail to produce non-functional eukaryotic proteins (such as the human-derived scFv) due to lack of appropriate intracellular environment for folding during post translational processing (Merten, Mattanovich et al. 2013). Furthermore, proteins may become insoluble as inclusion bodies and can be difficult to extract without harsh denaturation protocols and requirement for protein refolding procedures. This problem using the Tomlinson J library was also reported by other collaborators (Dr Rob Noad, RVC). Initial experiments transfecting scFv from TG-1 cells to Fall army worm *Spodoptera frugiperda* cells has visually shown scFv to bind target proteins on a

nitrocellulose membrane, whereas previous *E. coli* expressed scFv showed no binding. Transfection occurred through PCR amplification and electroporation of the scFv region into the *Autographa californica multiple nuclear polyhedrosis* virus (AcMNPV). In nature this barculovirus is eaten by the Fall ant worm and infects larvae systemically. Several hundred copies of the virus genome are present in each worm cell and are driven by a very strong promoter. In a normal infection 30% of the total protein of each cell is viral crystal-protein that the virus uses to prevent degradation when in the outside environment. This is the gene that was replaced with the scFv gene and hence why scFv expression is high in this system. Once scFv immunoprecipitation pull down of proteins is successfully shown by western blot and SDS-PAGE, then protein identification can be carried out by mass spectrometry. To test sufficient amounts of protein as a vaccine candidate, recombinant versions would most likely be expressed in *E. coli* for each vaccine candidate.

6.3.2 Vaccine trials

As is evident from the Willadsen group (CSIRO, Australia) publications developing the TickGARD vaccine (1988-2006), the commercialisation of a successful vaccine requires both the initial identification of a protective antigen and the capacity to evaluate antigens in large-scale trials. Whilst several attempts have been made to test PRM target proteins in small scale trials (Arkle, Harrington et al. 2008; Wright, Nisbet et al. 2011), only one project has tested PRM vaccine candidates in large scale trials (Dr Alasdair Nisbet, Morden, personal communication). Capture and analysis of fed mites makes evaluation complicated, though this may be overcome with the introduction of better *in vivo* feeding chambers attached to birds or the introduction of alternative rodent models (see 1.5.7 *in vivo* feeding).

In vitro feeding columns do exist (Bruneau, Dernburg et al. 2001; Harrington, Canales et al. 2009), though suffer from evaluating only small numbers of mites per trial as well as inefficiencies in mite feeding rates. Furthermore, introduction of IgY immunoglobulins from

vaccinated hen eggs, inoculated into heparinised blood, may not accurately represent true vaccinated bird blood. Alternatively, rodent feeding cages could offer an environment where mites could be easily contained and collected without the complication and animal welfare implications of binding multiple mite feeding chambers to mobile birds. Rodent feeding assays could also be followed up by isolation of single mites, or groups of mites, into ELISA plate wells or 1.5ml tubes, to analyse vaccine effect on PRM egg laying and behaviour. In the absence of a functional protocol for *in vivo* trials, we have demonstrated experimentally that PRM can feed on MF1 strain mice and be successfully contained, collected and analysed (not published). Initial results showed 331 of 1272 (26%) PRM fed over a 24 hour period in the presence of 4 mice. Whilst mite feeding rates are similar to that of *in vitro* feeding assays (McDevitt, Nisbet et al. 2006), the overall numbers of mites collected was x3 higher. Mites are collected unharmed by the use of a small keyboard cleaning vacuum and contained by modifications to a standard mouse transport cage (Innocage) by addition of 0.026m mesh that allows ventilation without mite escape. Future optimisation of this system will require improvement of animal welfare as PRM feeding time of 24 hours may be unnecessarily stressful for host mice. The addition of a nude-strain of mice may allow quicker feeding times, however many nude mice strains (e.g. Crl:NU(NCr)-Foxn1, Charles River, US) are also immunocompromised and so may develop unwarranted pathology during mite feeding. Future studies will also investigate the possibility of multiple feedings of PRM in a rodent model. While most studies only focus on the mortality of a single generation of PRM (Wright, Bartley et al. 2009; Bartley, Huntley et al. 2012), vaccine studies that monitor several feeding times or even several generations, would be a lot more accurate to the true nature of PRM. Repeat feeding is common in PRM every three days and it is well accepted that a future vaccine is likely to be effective only over an extended period of time through reduction in oviposition and mite behaviour. This is exemplified by initial mortality rates of the BM86 tick vaccine only showing

28% mortality, however measuring tick population reduction over time showed effective tick reduction at levels above 90% (Willadsen, Riding et al. 1989).

Introduction of any vaccination on a large scale would likely be applied alongside currently used chemical controls or desiccant sprays, as vaccines are seldom 100% effective. For example, the Gavac® vaccine (a BM86 subunit vaccine) was implemented in Cuba over an 8 year period, but only in conjunction with acaricidal use, which overall fell by 82% (Valle, Mèndez et al. 2004). A vaccine against PRM would likely be in a recombinant subunit form due to no protective immunity seen from whole parasite vaccination (Arkle, Harrington et al. 2008) and a comparative lack of DNA vaccine progress in the veterinary field (see 1.4 Vaccines). Poultry layer units can be small back yard (1-100 hens) or commercial wholesale (10,000s – 100,000s hens). Introduction of a new anti-PRM vaccine would therefore best suit an already established, easy to use and wide-spread system. Introduction of a vaccine into spraying systems and the respiratory tract is an option (currently used for Newcastle disease and Infectious Bursal disease vaccines), as is application into food or water drinking systems. Recombinant vaccine protein expression in yeast, virus, bacteria and parasite vectors have already all been demonstrated in poultry vaccination studies (Boyle and Coupar 1988; Macreadie, Vaughan et al. 1990; Layton, Morgan et al. 2011; Clark, Oakes et al. 2012).

6.3.3 Use of PRM genomic and transcriptomic data

PRM 'omics research is still in its infancy. Schicht, Qi et al. (2013) have published a transcriptome for PRM that contains 35,751 isotigs (splice variants of a single transcript or isogroup), and grants from the British Egg Marketing Board to the Moredun institute in Edinburgh (2009) and the Royal Veterinary College in London (2012) have generated PRM transcriptome sequences and EST libraries. The transcriptome used in alignment with this project assembled 214,263 contiguous RNA transcripts. Many of these contig sequences overlap and the overall number is much higher than the 10,000-20,000 genes expected from

an Acari species (Holt, Subramanian et al. 2002; Gulia-Nuss, Nuss et al. 2016). Membrane proteins targeted by this project may be especially under-represented in the PRM transcriptome. Signal peptides and transmembrane regions are commonly placed at the 5' and 3' ends of genes and these regions are the most likely to be absent during sequencing.

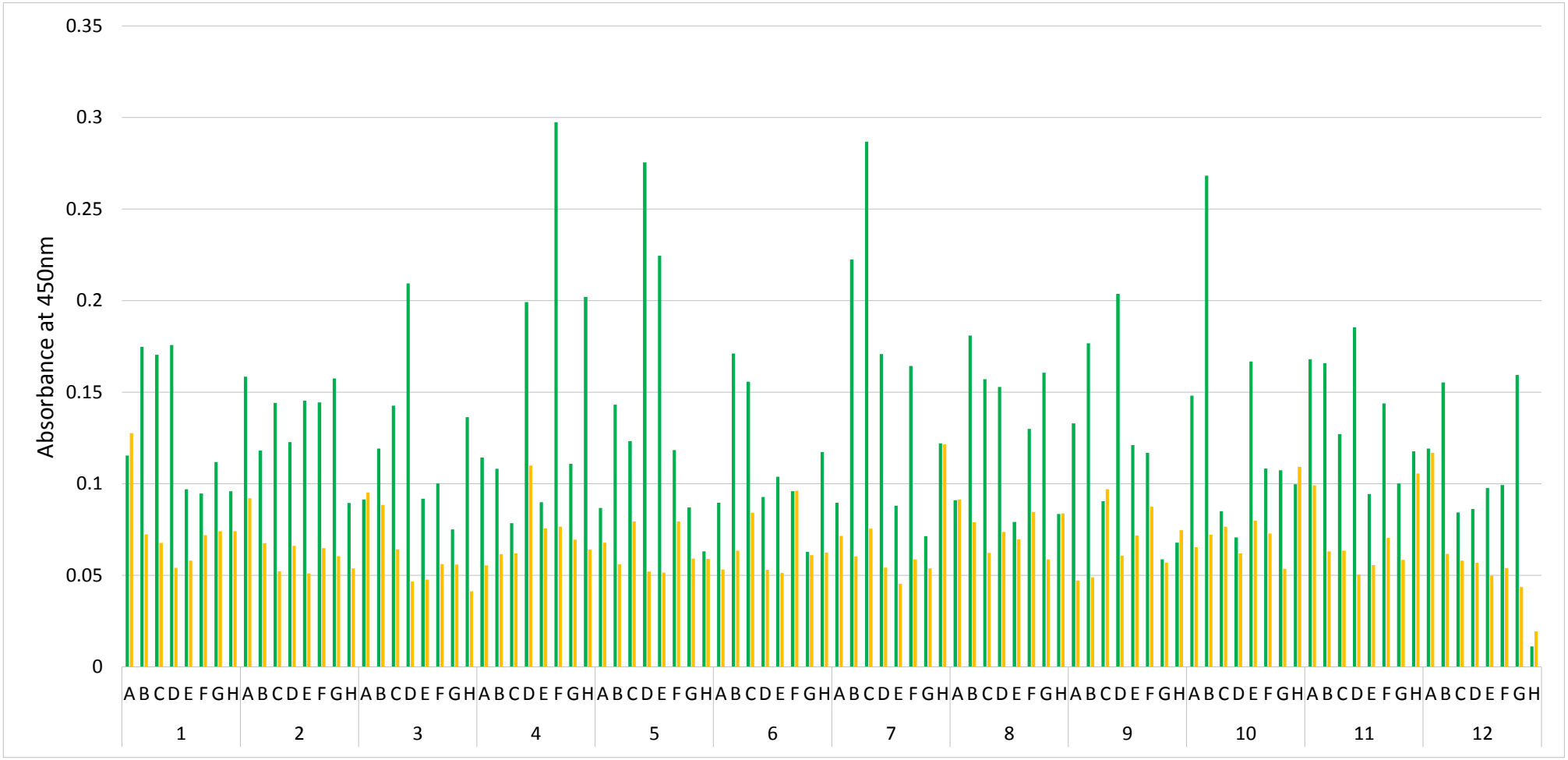
Whole genome analysis, i.e. reverse vaccinology, would be an alternative method to identifying PRM vaccine targets instead of construction of transcriptome data. Genome-wide sequencing allows downstream screening of all genes to match criteria for a vaccine target. For example, genes that encode for membrane proteins can be selected, followed by ability of recombinant proteins to be expressed in bacteria and evidence for membrane expression by ELISA experiments. Such methods found more vaccine candidates in 18 months than had been in 40 years for a vaccine against meningitis when compared to the 'fractionate and vaccinate' method (Pizza, Scarlato et al. 2000).

Transcriptomic and proteomic data offer a more focused method of identification of vaccine proteins compared to a whole genome approach. In PRM the digestive system is targeted, however as was shown in chapter 3, dissection of PRM digestive tissue is not feasible. Another approach to aid target identification could be through assessing expression of mRNA during PRM blood digestion. Indeed, suppressive subtractive hybridization of PRM mRNA during digestion has already identified the proteases Cathepsin D and L (Bartley, Huntley et al. 2012). Alternative life stages may also be targeted specifically by transcriptomic or proteomic studies. For example, targeting of PRM blood feeding stages (the protonymph, deutonymph and adult) would be suitable for studying digestion proteins. If targeting egg development, mRNA sequencing of just fertilized female adults would also be reasonable. For extraction of mRNA or proteins to create a library, life cycle stages of PRM would have to be separated. Whilst blood feeding stages may be drawn towards a blood meal, non-haematophagous larvae are also mobile and may be collected simultaneously. Mite separation is possible, though clear

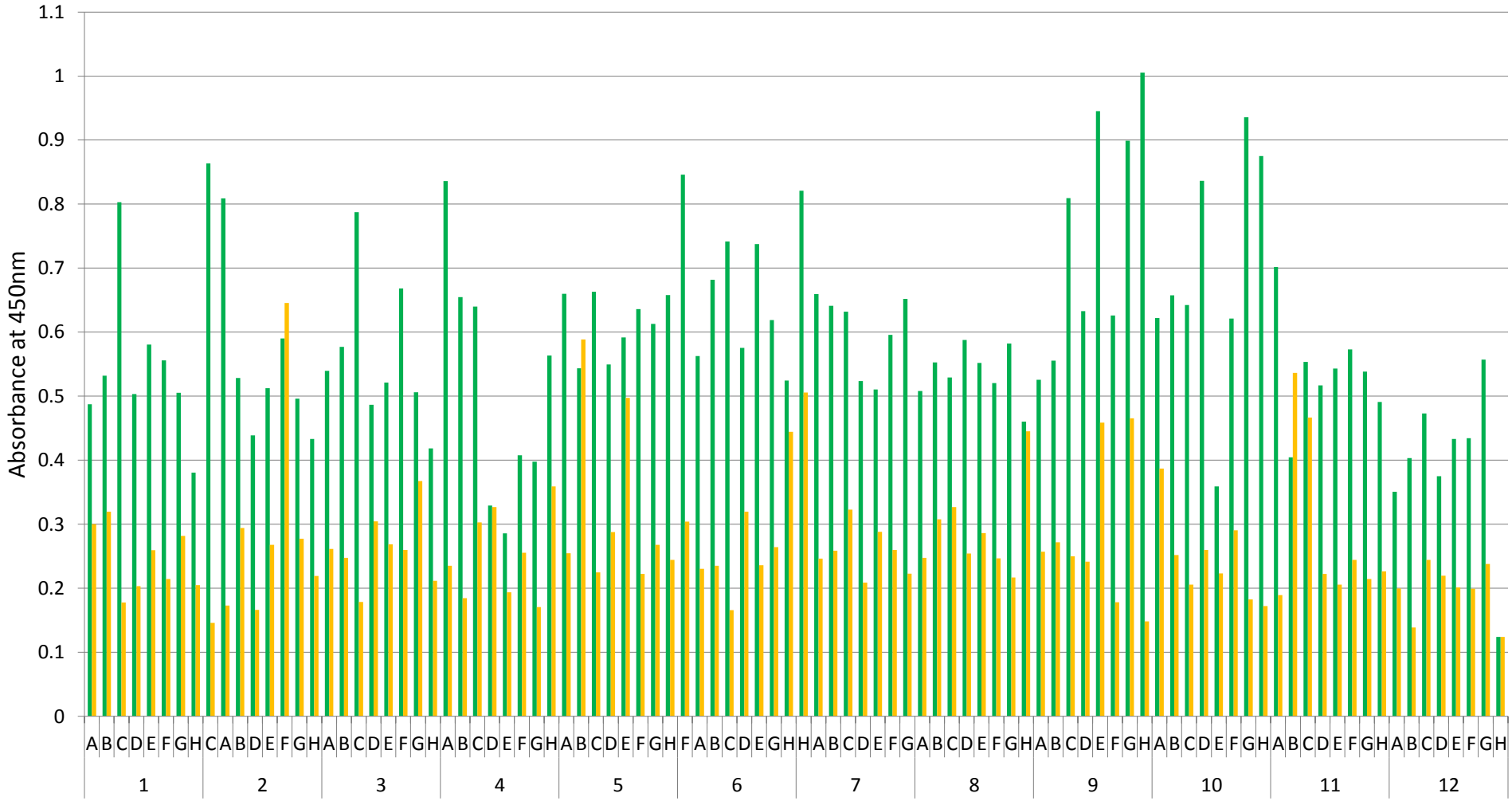
guidelines have not yet been presented in the literature of how this would be done. Differentiation between male and female is also possible; however this requires PRM to be static and monitored by the ventral shield by microscopy (Di Palma, Giangaspero et al. 2012).

The PRM is a threat to the welfare of layer hens and a significant economic problem for the egg laying industry in many parts of the world. Chemical acaricidal controls are failing and, whilst research into alternative controls is ongoing, there is presently no obvious effective method for PRM control. Research into vaccine candidates against PRM has increased in the past decade due to the necessity for alternative options and the advent of affordable generation of genomic databanks. This project, and others like it, seek to investigate proteins involved in PRM blood digestion in order to identify targets for vaccine induced antibody-mediated immunity. Future work will look to expand on understanding the basic biology of the PRM, whilst simultaneously further developing genomic, transcriptomic and proteomic resources. Increase in knowledge into PRM protein function, expression, localization and ability to create a protective immunity offers a valid route into discovering effective vaccine candidates and developing a viable control option against PRM.

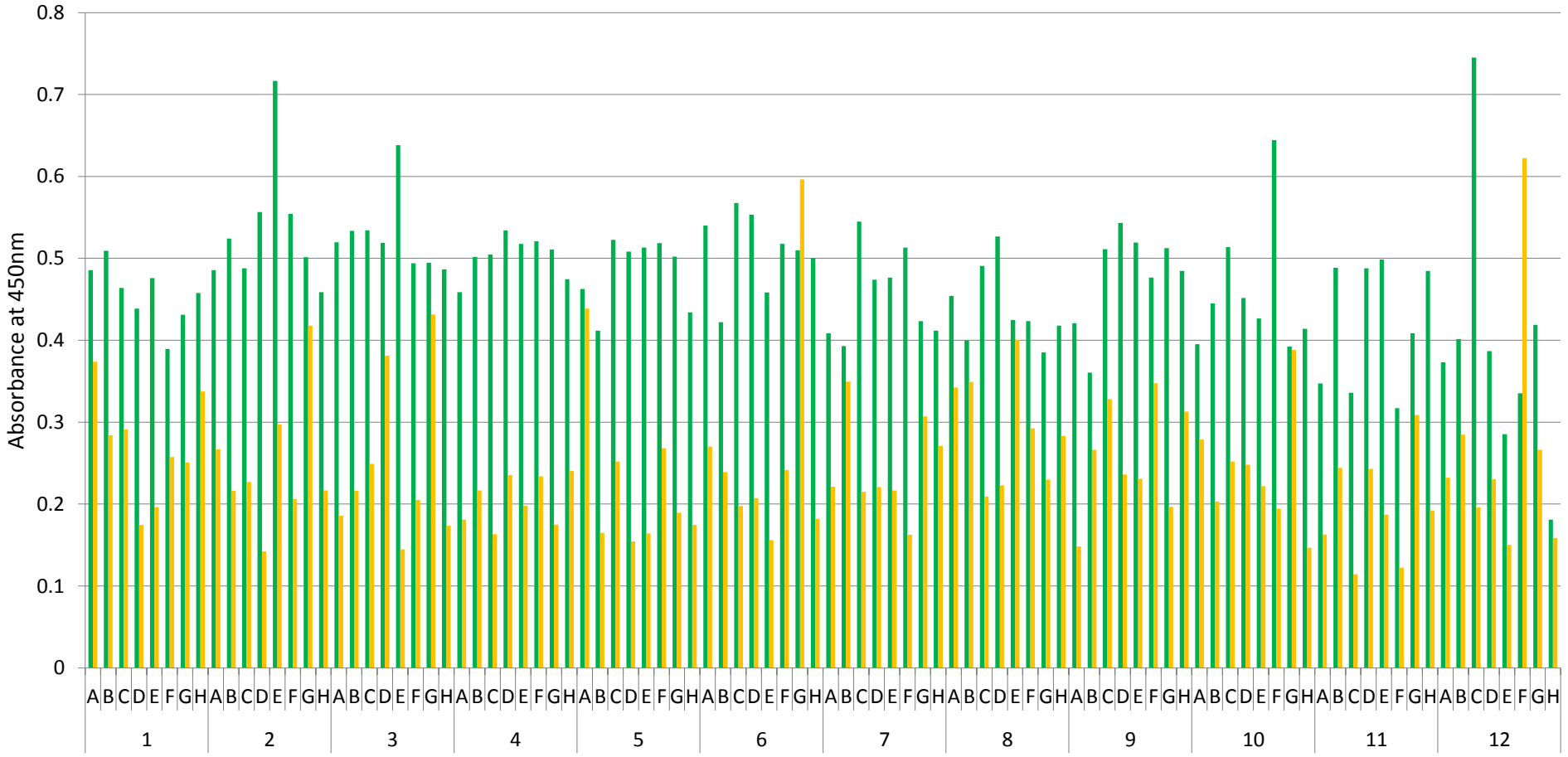
Supplementary



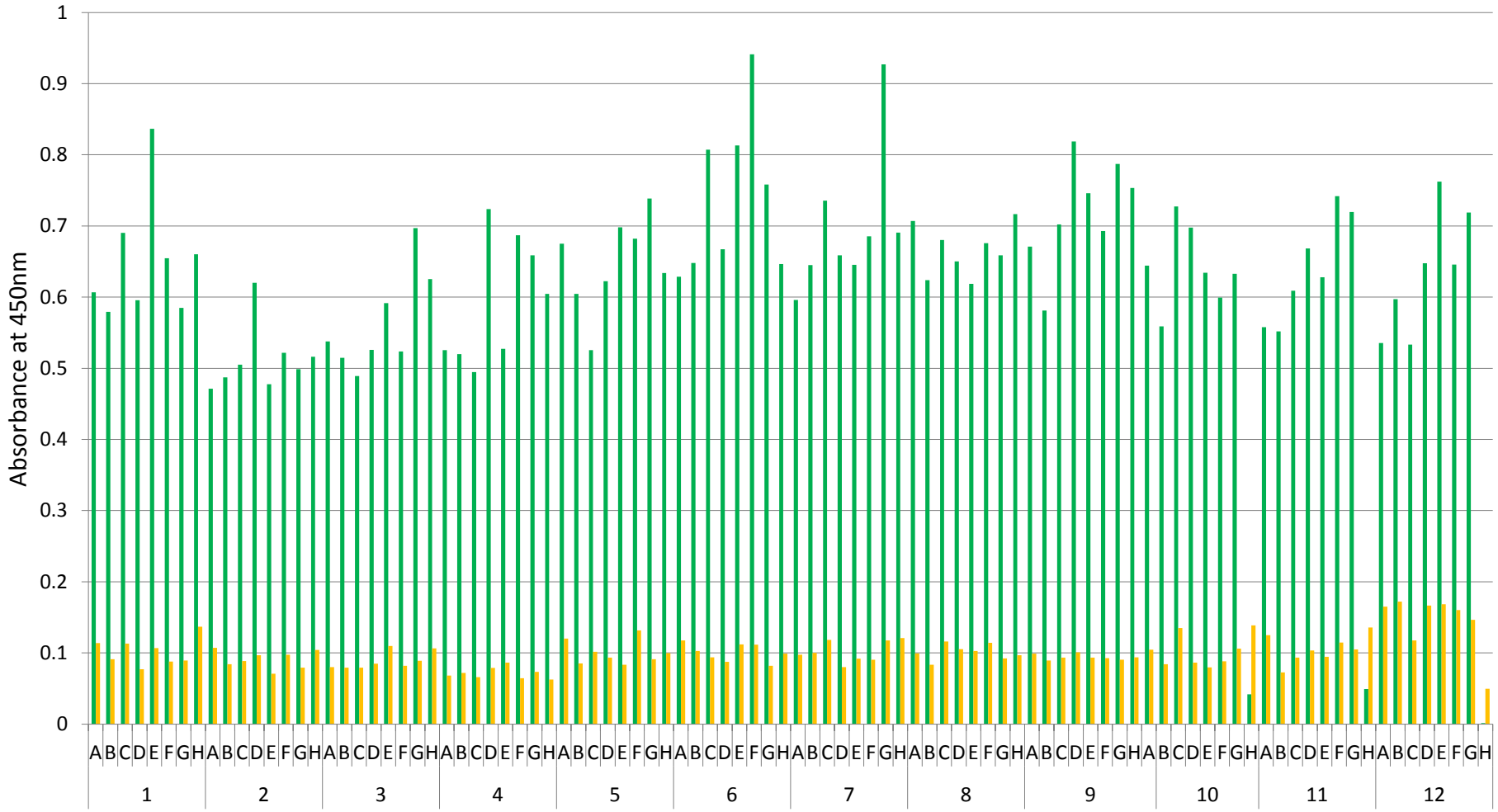
S1: Phage binding results of plate 1



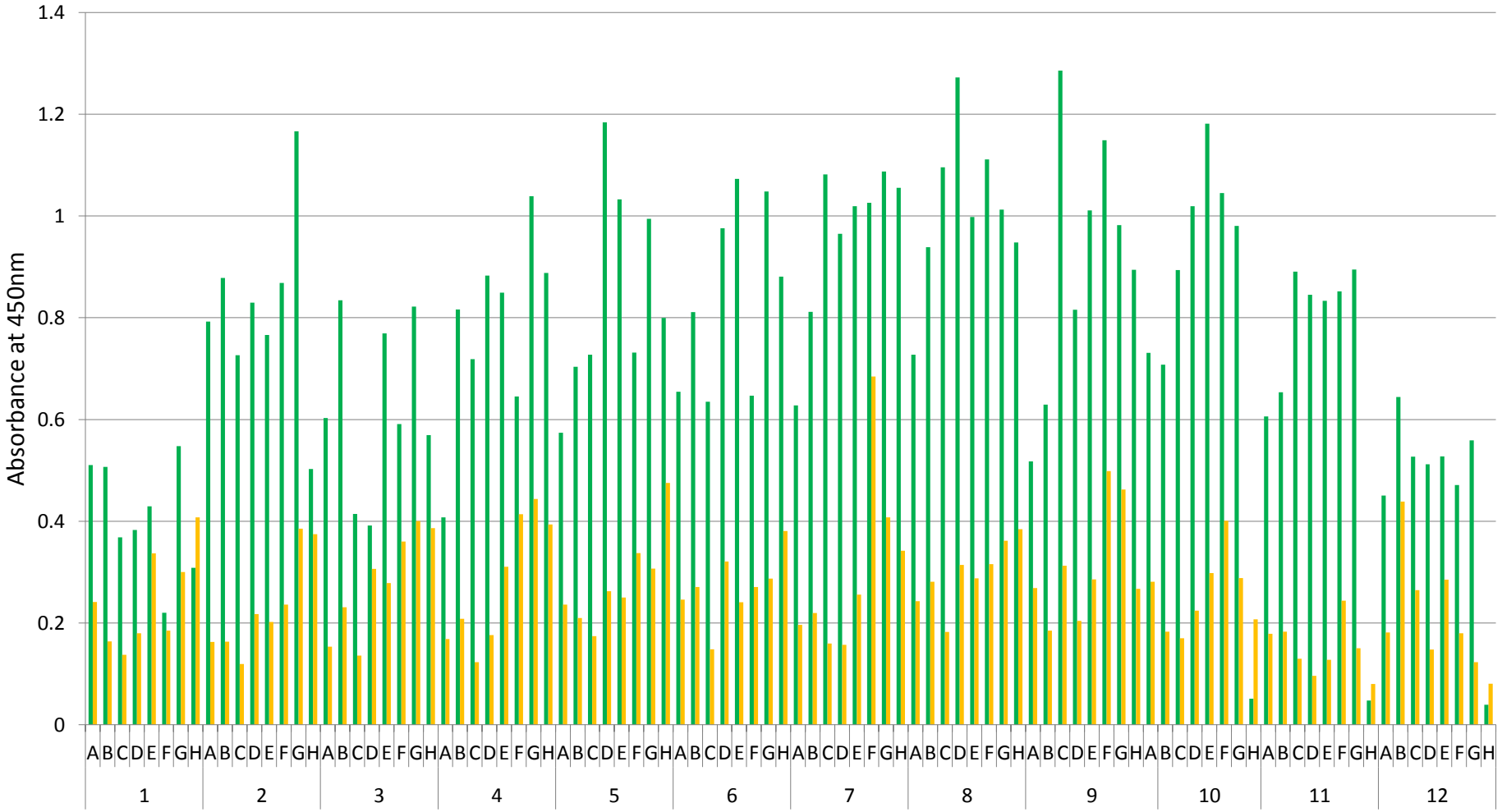
S2: Phage binding results of plate 3



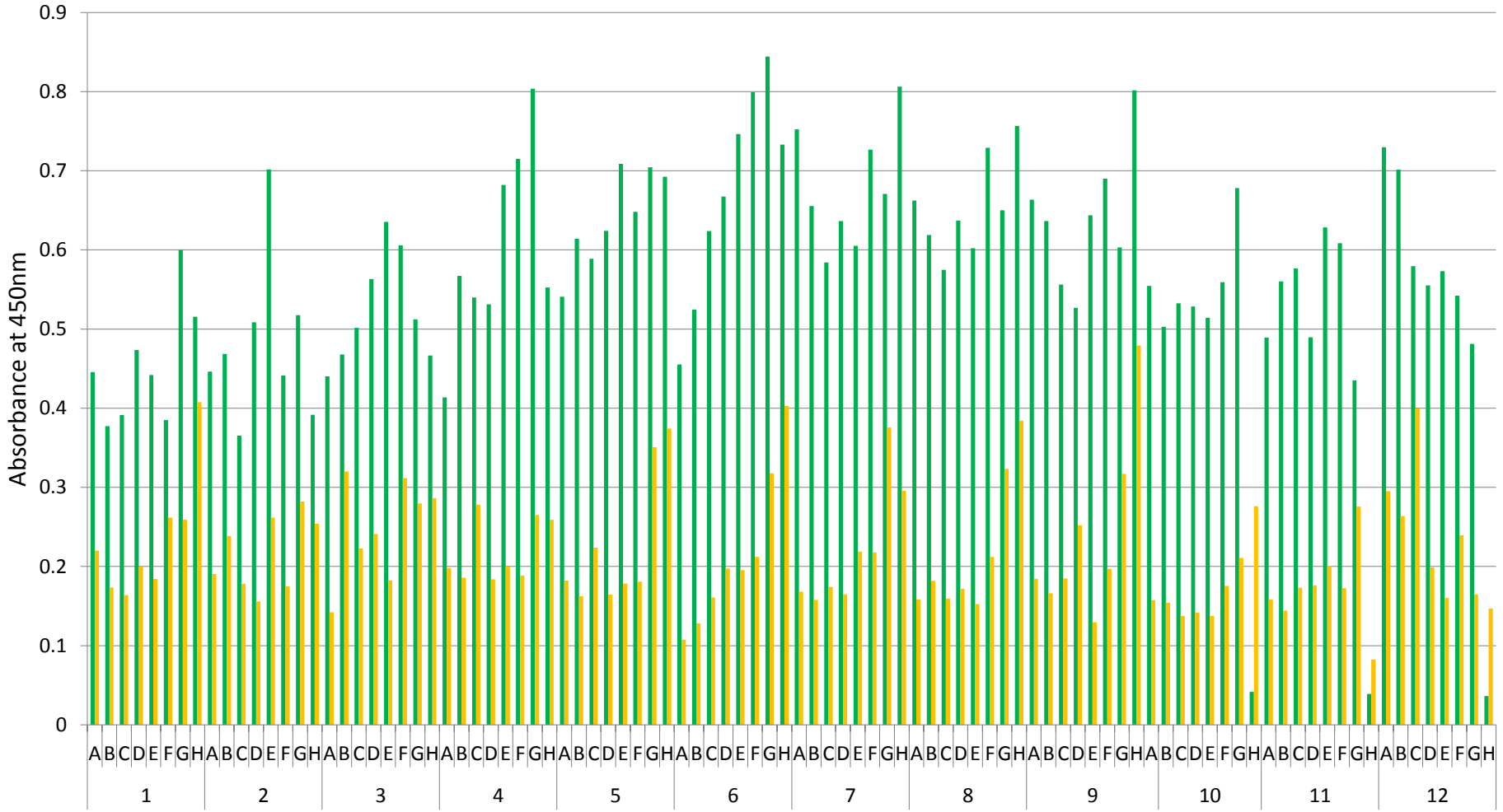
S3: Phage binding results of plate 4



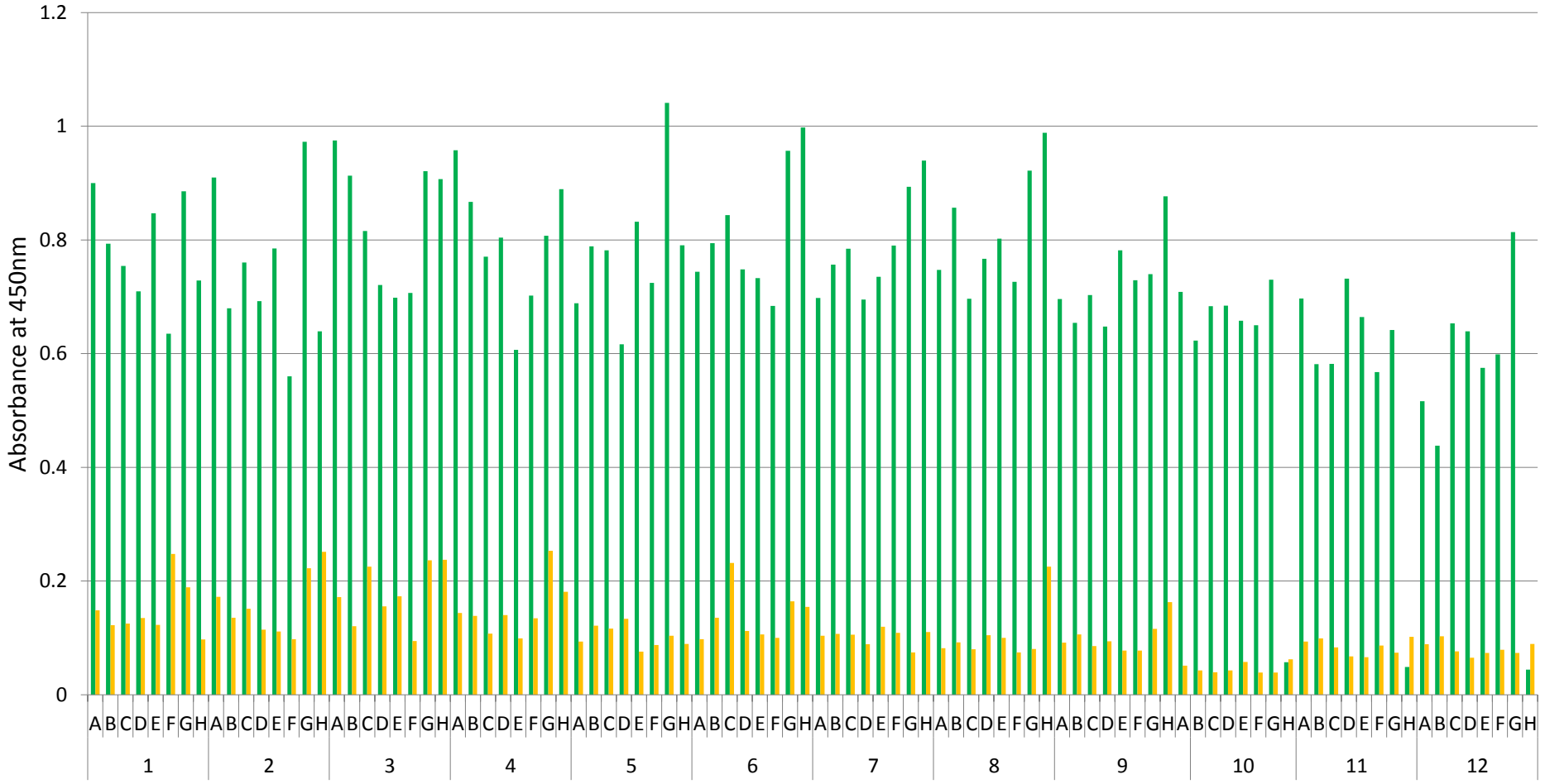
S4: Phage binding results of plate 5



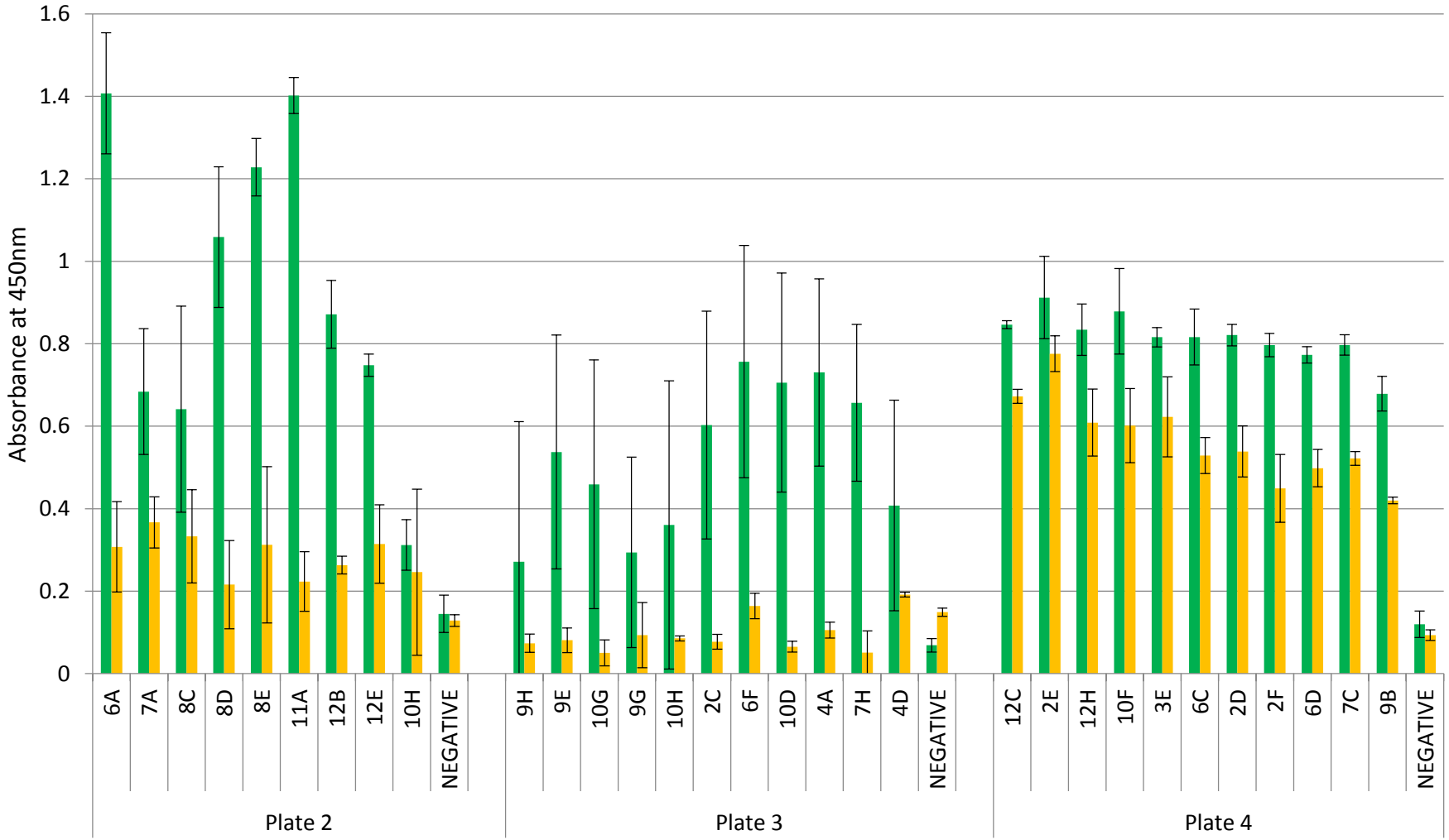
S5: Phage binding results of plate 6



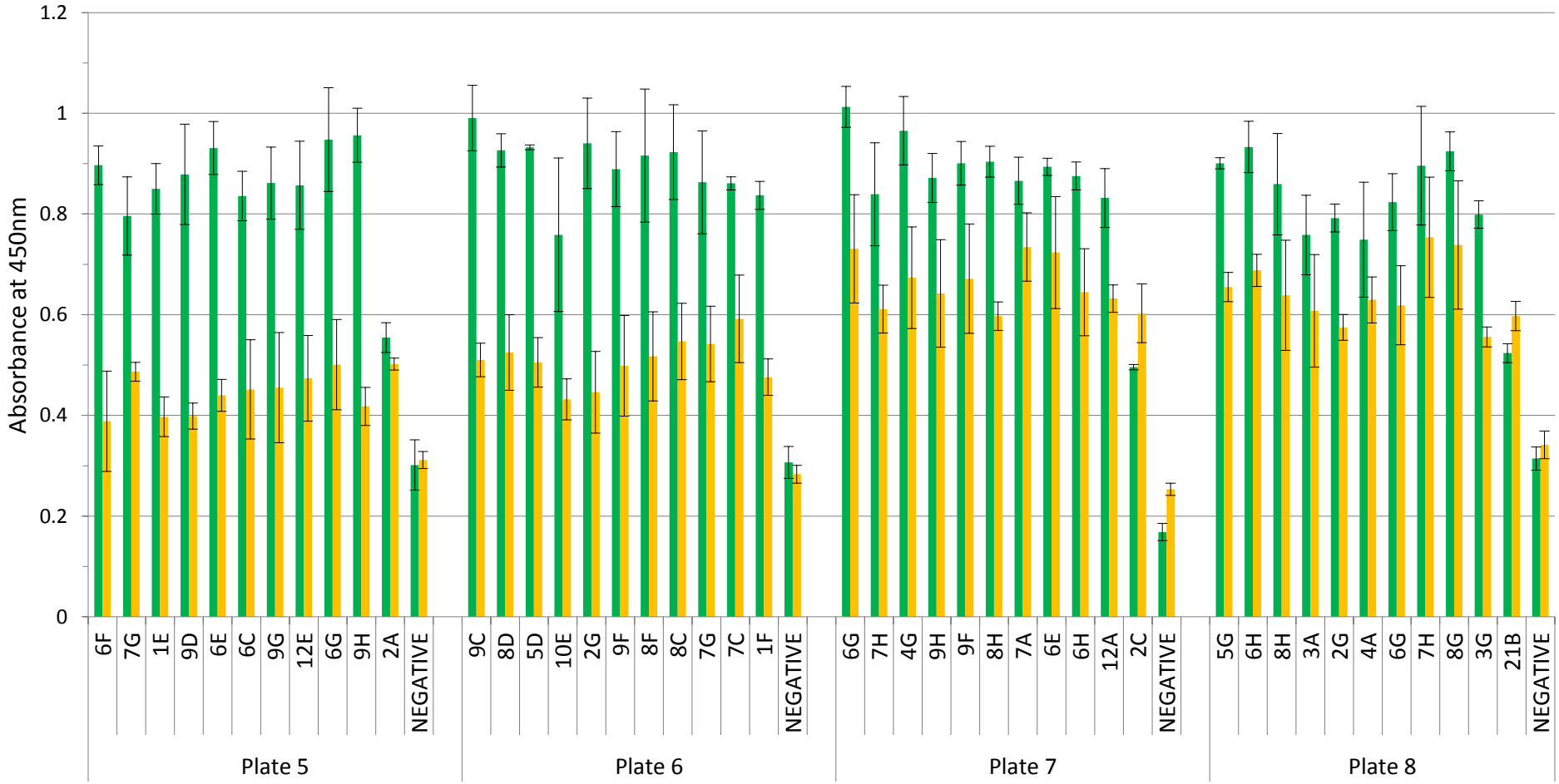
S6: Phage binding results of plate 7



S7: Phage binding results of plate 8



S8: Top binding candidate phage from ELISA plates 2-4.

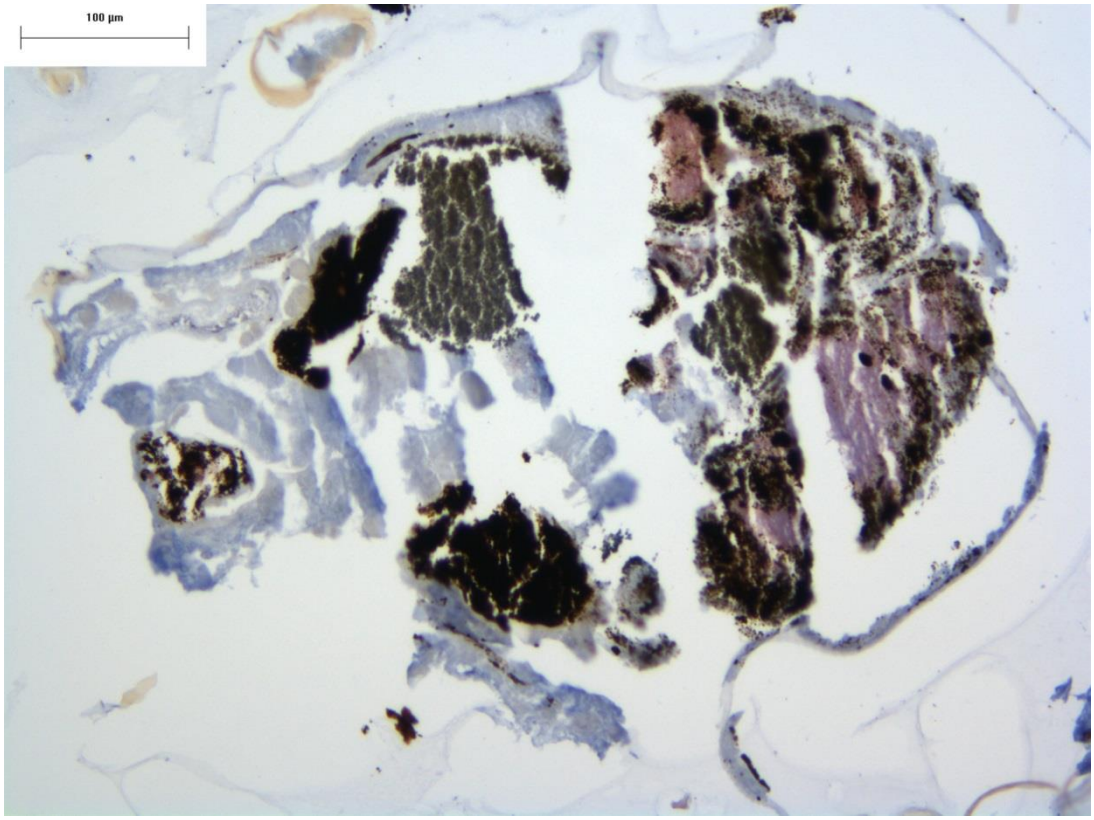


S9: Top binding candidate phage from ELISA plates 5-8.

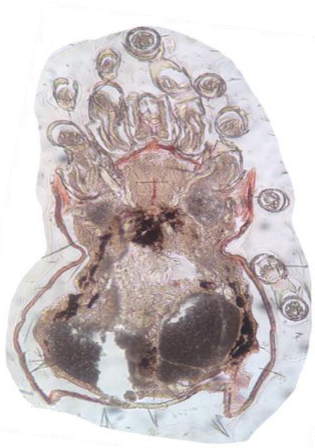
Plate	Well number	Location	Consistency	Staining
Plate 2	6A	Caecae	Often	Strong
	7A	All gut	Often	Faint
	8C	Midgut	Often	Strong
	8D	Negative	-	-
	8E	All gut	Often	Strong
	11A	Tissue surrounding egg	Occasionally	Strong
	12B	Midgut	Often	Strong
	12E	Localised spots in gut	Often	Very Strong
Plate 3	2C	Outline of midgut	Once	Strong
	4A	Negative	-	-
	6F	Negative	-	-
	7H	Negative	-	-
	9E	Tissue surrounding egg	Often	Strong
	10D	Tissue surrounding egg	Often	Strong
	10G	Tissue surrounding egg	Often	Strong
Plate 4	2D	Leg joints	Often	Strong
	2E	All gut	Often	Very Strong
	2F	Negative	-	-
	3E	Unknown structure	Often	Strong
	6C	Negative	-	-
	6D	Negative	-	-
	7C	Exoskeleton	Often	Very Strong
	10F	Localised spots in hindgut	Occasionally	Strong
	12C	Unknown structure	Often	Very Strong
	12H	Negative	-	-
Plate 5	1E	Negative	-	-
	6C	Negative	-	-
	6E	Negative	-	-
	6F	Egg	Often	Strong

	6G	Negative	-	-
	7G	Negative	-	-
	9D	Negative	-	-
	9G	Negative	-	-
	9H	Tissue surrounding egg	Occasionally	Strong
	12E	Negative	-	-
Plate 6	2G	Negative	-	-
	5D	Hindgut	Occasionally	Strong
	8C	Negative	-	-
	8D	Negative	-	-
	8F	All gut	Often	Very Strong
	9C	Cuticle	Often	Strong
	9F	Negative	-	-
Plate 7	4G	Caecae	Occasionally	Strong
	6E	Negative	-	-
	6G	Negative	-	-
	6H	Negative	-	-
	7A	Negative	-	-
	7H	Midgut / caeca	Often	Strong
	8H	Negative	-	-
	9F	Localised spots in guts	Once	Strong
	9H	All gut	Often	Very Strong
	12A	Negative	-	-
Plate 8	2G	All gut	Often	Very Strong
	3A	Palps	Often	Strong
	3G	Negative	-	-
	4A	Negative	-	-
	5G	All gut	Often	Strong
	6G	Localised spots in guts	Occasionally	Strong
	6H	Negative	-	-
	7H	Palps	serveral times	Strong
	8G	Negative	-	-
	8H	Negative	-	-

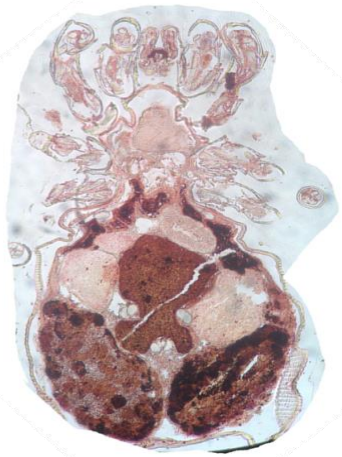
S10: Description of the immunohistochemical staining of each phage on mite sections.



S11: A mite sectioned at 5µm using an eosin counterstain during IHC. Binding of the phage via HRP staining can be seen (pink) however the 5µm section created a fractioned section.



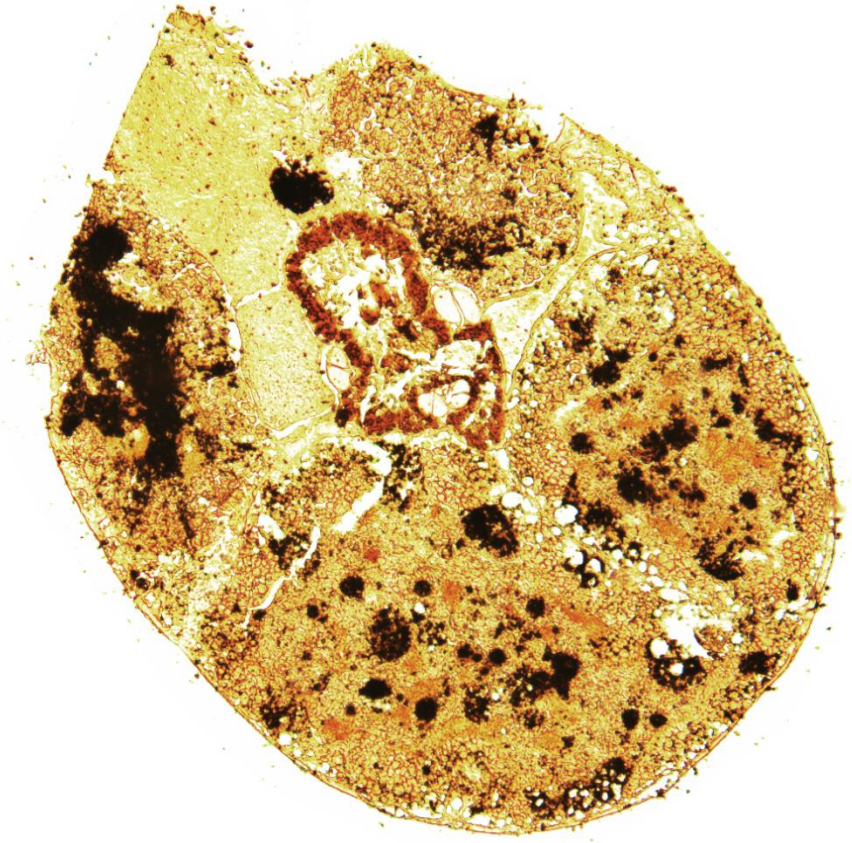
i) Rabbit anti-mite serum



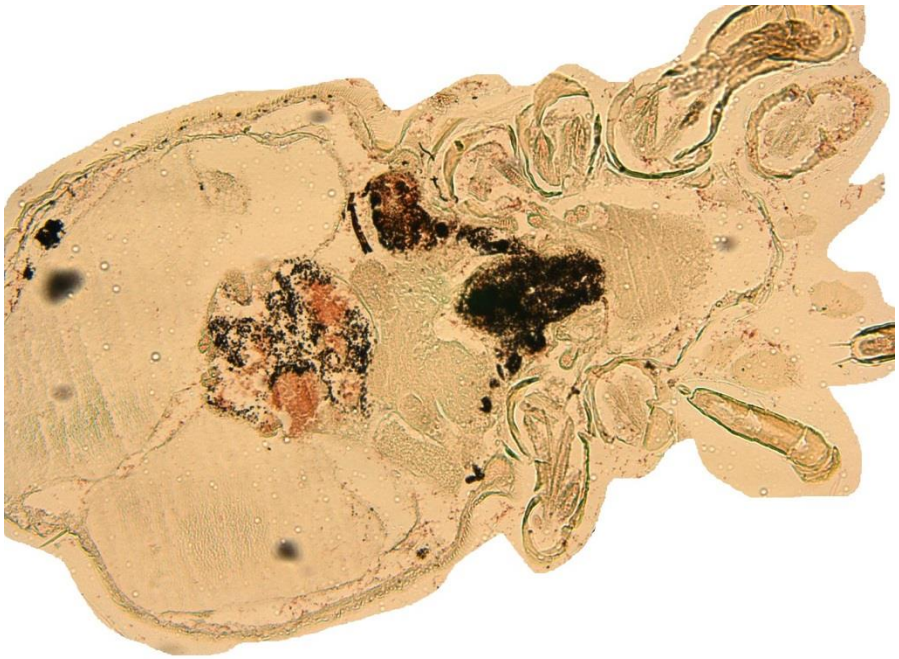
ii) Round 3 phage library

iii) Drosophila anti-nervana 1

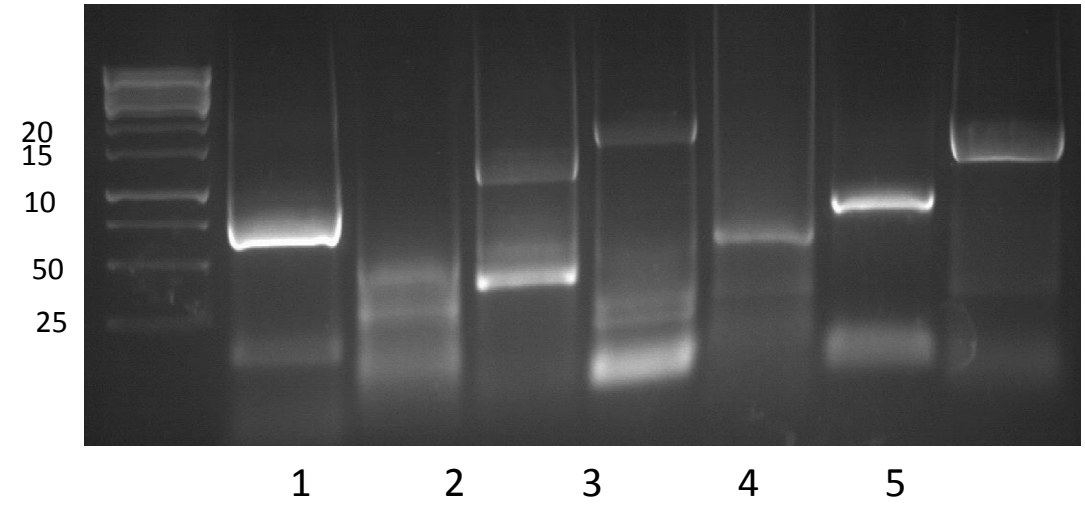
S12 Positive validation of the immunohistochemistry technique: Both rabbit anti-mite and the round 3 phage library (proceeded by a secondary anti-phage antibody) showed to bind strongly to many parts of the mite as expected. A rabbit anti-nervana specific to drosophila was shown to be negative. Both results indicate that IHC was a valid test in our experiments. Mites photographed in aqueous conditions using light microscopy at x100.



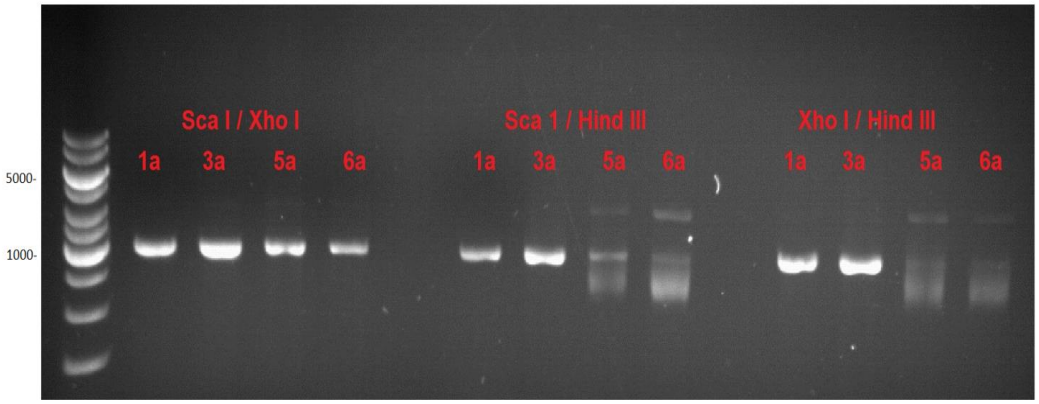
S13: ScFv/phage 2-8C binding to the outline of the midgut during IHC.



S14: ScFv/phage 2-8C binding to the midgut of a PRM during IHC.



S12: DNA banding of PCR products created from sequences of transcriptome. 1 – zinc transporter zip 9, 2 – voltage-gated ion channel, 3 – voltage dependant p q type calcium channel, 4 – transmembrane anterior/posterior domain, 5 – t family of potassium channels protein 18, 6 – alkaline ceramidase 3, 7 – beta-mannosyltransferase. Only bands in lanes 3, 4, 5 and 6 showed bands of correct sizes.



S13: Example of failed restriction digest of mini-prep samples of bacteria induced with pGEM plasmid + PCR products. Various combinations of restriction enzymes failed to digest plasmids and PCR products suggesting at either failed ligation or restriction enzymes do not work.

References

Abcam (2016).

Aflitto, N. C. and R. W. Hofstetter (2014). "Use of acoustics to deter bark beetles from entering tree material." Pest Management Science.

Ahmed, H. (2004). Principles and reactions of protein extraction, purification, and characterization, CRC Press.

Akov, S. (1982). "Blood digestion in ticks." Physiology of ticks: 197-211.

Al-Sheikhly, F. and R. Truscott (1977). "The interaction of Clostridium perfringens and its toxins in the production of necrotic enteritis of chickens." Avian diseases: 256-263.

Alasaad, S., L. Rossi, et al. (2009). "Sarcoptes mite from collection to DNA extraction: the lost realm of the neglected parasite." Parasitology research **104**(4): 723-732.

Aldovini, A. and R. A. Young (1991). "Humoral and cell-mediated immune responses to live recombinant BCG-HIV vaccines." Nature **351**(6326): 479-482.

Ali, W., D. R. George, et al. (2012). "Laboratory screening of potential predators of the poultry red mite (*Dermanyssus gallinae*) and assessment of *Hypoaspis miles* performance under varying biotic and abiotic conditions." Veterinary Parasitology **187**(1): 341-344.

Allingham, P., I. East, et al. (1998). "Digestion of host immunoglobulin and activity of midgut proteases in the buffalo fly *Haematobia irritans exigua*." Journal of insect physiology **44**(5): 445-450.

Almazan, C., K. M. Kocan, et al. (2005). "Vaccination with recombinant tick antigens for the control of *Ixodes scapularis* adult infestations." Vaccine **23**(46-47): 5294-5298.

Alsharifi, M. and A. Müllbacher (2010). "The [gamma]-irradiated influenza vaccine and the prospect of producing safe vaccines in general." Immunology and cell biology **88**(2): 103.

Álvarez-Padilla, F. and G. Hormiga (2007). "A protocol for digesting internal soft tissues and mounting spiders for scanning electron microscopy." Journal of Arachnology **35**(3): 538-542.

Anatrace (2015). "Detergents and Their Uses in Membrane Protein Science."

Appleby, M. C. (2003). "The European Union ban on conventional cages for laying hens: history and prospects." Journal of applied animal welfare science **6**(2): 103-121.

Arkle, S., D. Harrington, et al. (2008). "Immunological Control of the Poultry Red Mite." Annals of the New York Academy of Sciences **1149**(1): 36-40.

Arkle, S., D. Harrington, et al. (2008). "The Use of Whole Mite Antigens as a Vaccine Candidate." Annals of the New York Academy of Sciences **1149**: 36-40.

Arlian, L., C. Rapp, et al. (1990). "Development of Dermatophagoides pteronyssinus (Acari: Pyroglyphidae)." Journal of medical entomology **27**(6): 1035-1040.

Arzey, G. (1990). "Mechanisms of spread of Newcastle disease." Technical Bulletin-NSW Agriculture and Fisheries(42).

Auger, P., J. Nantel, et al. (1979). "Skin acariasis caused by Dermanyssus gallinae (de Geer): an in-hospital outbreak." Canadian Medical Association journal **120**(6): 700-703.

Axtell, R. C. (1999). "Poultry integrated pest management: status and future." Integrated Pest Management Reviews **4**(1): 53-73.

Bande, F., S. S. Arshad, et al. (2015). "Progress and challenges toward the development of vaccines against avian infectious bronchitis." Journal of immunology research **2015**.

Bar-Shira, E. and A. Friedman (2006). "Development and adaptations of innate immunity in the gastrointestinal tract of the newly hatched chick." Developmental & Comparative Immunology **30**(10): 930-941.

Barbosa, P. (1974). "Manual of basic techniques in insect histology."

Barbosa, P., D. Berry, et al. (2014). Insect Histology: Practical Laboratory Techniques, John Wiley & Sons.

Barreau, C., J. Conrad, et al. (1999). "Identification of surface molecules on salivary glands of the mosquito, Aedes aegypti, by a panel of monoclonal antibodies." Insect biochemistry and molecular biology **29**(6): 515-526.

Bartley, K., J. F. Huntley, et al. (2012). "Assessment of cathepsin D and L-like proteinases of poultry red mite, *Dermanyssus gallinae* (De Geer), as potential vaccine antigens." Parasitology **139**(6): 755-765.

Bartley, K., A. J. Nisbet, et al. (2009). "Histamine release factor from *Dermanyssus gallinae* (De Geer): characterization and in vitro assessment as a protective antigen." International Journal for Parasitology **39**(4): 447-456.

Bartley, K., H. W. Wright, et al. (2015). "Identification and evaluation of vaccine candidate antigens from the poultry red mite (Dermanyssus gallinae)." International Journal for Parasitology **45**(13): 819-830.

BEIC (2016). "The British Egg Industry Council."

Berge, A., A. Briese, et al. (2009). Salmonella-a modern European perspective. Sustainable animal husbandry: prevention is better than cure, Volume 2. Proceedings of the 14th International Congress of the International Society for Animal Hygiene (ISAH), Tribun EU.

Bertrand, J. Y., N. C. Chi, et al. (2010). "Haematopoietic stem cells derive directly from aortic endothelium during development." Nature **464**(7285): 108-111.

Beugnet, F., C. Chauve, et al. (1997). "Resistance of the red poultry mite to pyrethroids in France." Veterinary research **140**(22): 577-579.

Bhairi, S. M. (2001). "Detergents A guide to the properties and uses of detergents in biological systems."

Biocompare (2008).

Bogin, E. and A. Hadani (1973). "Digestive enzymes in “hard ticks”(ixodoidea, ixodidae)." Zeitschrift für Parasitenkunde **41**(2): 139-146.

Bornstein, S., T. Mörner, et al. (2001). "Sarcoptes scabiei and sarcoptic mange." Parasitic diseases of wild mammals: 107-119.

Bosch, D. E. and D. P. Siderovski (2013). "G protein signaling in the parasite *Entamoeba histolytica*." Experimental & molecular medicine **45**(3): e15.

Boss, M. A., J. H. Kenten, et al. (1984). "Assembly of functional antibodies from immunoglobulin heavy and light chains synthesised in E. coli." Nucleic Acids Research **12**(9): 3791-3806.

Bouchon, D., T. Rigaud, et al. (1998). "Evidence for widespread Wolbachia infection in isopod crustaceans: molecular identification and host feminization." Proceedings of the Royal Society of London B: Biological Sciences **265**(1401): 1081-1090.

Boyle, D. B. and B. E. Coupar (1988). "Construction of recombinant fowlpox viruses as vectors for poultry vaccines." Virus Research **10**(4): 343-356.

Brannstrom, S., D. A. Morrison, et al. (2008). "Genetic differences in internal transcribed spacer 1 between *Dermanyssus gallinae* from wild birds and domestic chickens." Medical and Veterinary Entomology **22**(2): 152-155.

Brochier, B., M. Kieny, et al. (1990). "Large-scale eradication of rabies using recombinant vaccinia-rabies vaccine." Nature **354**(6354): 520-522.

Brockis, D. (1980). "Mite infestations." Veterinary Record **107**(13): 315-316.

Brody, A. R., J. C. McGrath, et al. (1972). "*Dermatophagoides farinae*: the digestive system." Journal of the New York Entomological Society: 152-177.

Brossard, M. and O. Rais (1984). "Passage of hemolysins through the midgut epithelium of femaleIxodes ricinus L. fed on rabbits infested or reinfested with ticks." Experientia **40**(6): 561-563.

Brossard, M. and S. Wikel (2004). "Tick immunobiology." Parasitology **129**(S1): S161-S176.

Brown, R. E., K. L. Jarvis, et al. (1989). "Protein measurement using bicinchoninic acid: elimination of interfering substances." Analytical biochemistry **180**(1): 136-139.

Bruneau, A., A. Dernburg, et al. (2001). "First in vitro cycle of the chicken mite, *Dermanyssus gallinae* (DeGeer 1778), utilizing an artificial feeding device." Parasitology **123**(Pt 6): 583-589.

Burden, D. W. (2008). "Guide to the homogenization of biological samples." Random Primers **7**: 1-14.

Burry, R. Immunocytochemistry: a practical guide for biomedical research. 2010, Springer, New York Google Scholar.

Byreddy, A. R., A. Gupta, et al. (2015). "Comparison of cell disruption methods for improving lipid extraction from Thraustochytrid strains." Marine drugs **13**(8): 5111-5127.

Cabilly, S., A. D. Riggs, et al. (1984). "Generation of antibody activity from immunoglobulin polypeptide chains produced in Escherichia coli." Proceedings of the National Academy of Sciences **81**(11): 3273-3277.

Cafiero, M. A., A. Camarda, et al. (2008). "Pseudoscabies caused by *Dermanyssus gallinae* in Italian city dwellers: a new setting for an old dermatitis." Journal of the European Academy of Dermatology and Venereology **22**(11): 1382-1383.

Cafiero, M. A., D. Galante, et al. (2011). "Why dermanyssosis should be listed as an occupational hazard." Occupational and Environmental Medicine **68**(8): 628-628.

Calderón, R., J. Van Veen, et al. (2010). "Reproductive biology of *Varroa destructor* in Africanized honey bees (Apis mellifera)." Experimental and Applied Acarology **50**(4): 281-297.

Campbell, E. M., M. Burdin, et al. (2010). "Role of an aquaporin in the sheep tick Ixodes ricinus: assessment as a potential control target." International Journal for Parasitology **40**(1): 15-23.

Capua, I. (2007). "Vaccination for notifiable avian influenza in poultry." Revue scientifique et technique (International Office of Epizootics) **26**(1): 217-227.

Carlisle, D. (1960). "Softening chitin for histology."

Carmen, S. and L. Jermutus (2002). "Concepts in antibody phage display." Briefings in functional genomics & proteomics **1**(2): 189-203.

Carpenter, J. F. and J. H. Crowe (1988). "The mechanism of cryoprotection of proteins by solutes." Cryobiology **25**(3): 244-255.

Chamberlain, R., R. Sikes, et al. (1957). "Attempted laboratory infection of bird mites with the virus of St. Louis encephalitis." Am J Trop Med Hyg **6**(6): 1047-1053.

Chamberlain, R. W. and R. K. Sikes (1955). "Laboratory investigations on the role of bird mites in the transmission of eastern and western equine encephalitis." American Journal of Tropical Medicine and Hygiene **4**(1): 106-118.

Chandler, D., G. Davidson, et al. (2000). "Fungal biocontrol of Acari." Biocontrol Science and Technology **10**(4): 357-384.

Chatterjee, S. (2014). "Artefacts in histopathology." Journal of oral and maxillofacial pathology: JOMFP **18**(Suppl 1): S111.

Chauve, C. (1998). "The poultry red mite *Dermanyssus gallinae* (De Geer, 1778): current situation and future prospects for control." Veterinary Parasitology **79**(3): 239-245.

Chirico, J., H. Eriksson, et al. (2003). "The poultry red mite, *Dermanyssus gallinae*, a potential vector of *Erysipelothrix rhusiopathiae* causing erysipelas in hens." Medical and veterinary entomology **17**(2): 232-234.

Chirico, J. and R. Tauson (2002). "Traps containing acaricides for the control of *Dermanyssus gallinae*." Veterinary Parasitology **110**(1–2): 109-116.

Chu, P. and L. Weiss (2014). Modern Immunohistochemistry with DVD-ROM, Cambridge University Press.

Ciolca, A., I. Tanase, et al. (1968). "Role of the poultry red mite, *Dermanyssus gallinae*, in the transmission of spirochaetosis." Arch Vet Pol **5**: 207-215.

Circella, E., N. Pugliese, et al. (2011). "*Chlamydia psittaci* infection in canaries heavily infested by *Dermanyssus gallinae*." Experimental and Applied Acarology **55**(4): 329-338.

Clare, J., F. Rayment, et al. (1991). "High-level expression of tetanus toxin fragment C in Pichia pastoris strains containing multiple tandem integrations of the gene." Bio/technology (Nature Publishing Company) **9**(5): 455-460.

Clark, J. D., R. D. Oakes, et al. (2012). "Eimeria species parasites as novel vaccine delivery vectors: anti-*Campylobacter jejuni* protective immunity induced by Eimeria tenella-delivered CjaA." Vaccine **30**(16): 2683-2688.

Cloudsley-Thompson, J. (1962). "Some aspects of the physiology and behaviour of Dinothrombium (Acari)." Entomologia Experimentalis et Applicata **5**(1): 69-73.

Coleman, J., J. Juhn, et al. (2007). "Dissection of midgut and salivary glands from *Ae. aegypti* mosquitoes." Journal of visualized experiments: JoVE(5).

Committee, I. R. A. (2012). "Acaricide Mode of Action Classification."

Conyers, S. T. and C. H. Bell (2003). "The effect of modified atmospheres on the survival of the eggs of four storage mite species." Experimental & applied acarology **31**(1-2): 115-130.

Coons, A. H., H. Creech, et al. (1942). "The demonstration of pneumococcal antigen in tissues by the use of fluorescent antibody." J. Immunol **45**(3): 159-170.

Coons, L. B. (1978). "Fine structure of the digestive system of *Macrocheles muscaedomesticae*(scopoli)(acarina: Mesostigmata)." International Journal of Insect Morphology and Embryology **7**(2): 137-153.

Corbanie, E., J. P. Remon, et al. (2007). "Spray drying of an attenuated live Newcastle disease vaccine virus intended for respiratory mass vaccination of poultry." Vaccine **25**(49): 8306-8317.

Cosoroaba, I. (2001). "Massive *Dermanyssus gallinae* (De Geer 1778) invasion in battery-husbandry raised fowls in Romania [egg-laying decrease, mortality]." Revue de Medecine Veterinaire (France).

Crotty, S. (2015). "A brief history of T cell help to B cells." Nature Reviews Immunology **15**(3): 185-189.

Cruz, M. D., M. C. Robles, et al. (2005). "Scanning electron microscopy of foreleg tarsal sense organs of the poultry red mite, *Dermanyssus gallinae* (DeGeer) (Acari:Dermanyssidae)." Micron **36**(5): 415-421.

Dabbs, D. J. (2013). Diagnostic immunohistochemistry, Elsevier Health Sciences.

Davis, B. S., G.-J. J. Chang, et al. (2001). "West Nile virus recombinant DNA vaccine protects mouse and horse from virus challenge and expresses in vitro a noninfectious recombinant antigen that can be used in enzyme-linked immunosorbent assays." Journal of Virology **75**(9): 4040-4047.

Davison, F., B. Kaspers, et al. (2011). Avian immunology, Academic Press.

De Boer, J. G. and M. Dicke (2004). "The role of methyl salicylate in prey searching behavior of the predatory mite *Phytoseiulus persimilis*." Journal of chemical ecology **30**(2): 255-271.

de Groot, M., A. Čokl, et al. (2010). "Effects of heterospecific and conspecific vibrational signal overlap and signal-to-noise ratio on male responsiveness in *Nezara viridula* (L.)." The Journal of experimental biology **213**(18): 3213-3222.

De Luna, C. J., S. Arkle, et al. (2008). "The poultry red mite *Dermanyssus gallinae* as a potential carrier of vector-borne diseases." Annals of the New York Academy of Sciences **1149**: 255-258.

De Luna, C. J., C. V. Moro, et al. (2009). "Endosymbiotic bacteria living inside the poultry red mite (*Dermanyssus gallinae*)." Experimental & applied acarology **48**(1-2): 105-113.

Dekeyser, M. A. (2005). "Acaricide mode of action." Pest management science **61**(2): 103-110.

DeLoach, J. R. and J. A. DeVaney (1981). "Northern fowl mite, Ornithonyssus sylviarum (Acari: Macronyssidae), ingests large quantities of blood from white leghorn hens." Journal of medical entomology **18**(5): 374-377.

Desaiah, D., L. K. Cutkomp, et al. (1973). "Inhibition of spider mite ATPases by Plictran and three organochlorine acaricides." Life Sciences **13**(12): 1693-1703.

Desmyter, J., G. De Groote, et al. (1983). "Efficacy of heat-inactivated hepatitis B vaccine in haemodialysis patients and staff: double-blind placebo-controlled trial." The Lancet **322**(8363): 1323-1328.

Deutsch, C., J. Taylor, et al. (1982). "Regulation of intracellular pH by human peripheral blood lymphocytes as measured by 19F NMR." Proceedings of the National Academy of Sciences **79**(24): 7944-7948.

Dhama, K., M. Mahendran, et al. (2008). "DNA vaccines and their applications in veterinary practice: current perspectives." Veterinary Research Communications **32**(5): 341-356.

Di Palma, A., A. Giangaspero, et al. (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)." Parasites & vectors **5**(1): 104.

Dinglasan, R., M. Devenport, et al. (2009). "The Anopheles gambiae adult midgut peritrophic matrix proteome." Insect Biochemistry and Molecular Biology **39**(2): 125-134.

Durden, L. A., K. J. Linthicum, et al. (1993). "Laboratory transmission of eastern equine encephalomyelitis virus to chickens by chicken mites (Acari: Dermanyssidae)." Journal of Medical Entomology **30**(1): 281-285.

Durden, L. A., K. J. Linthicum, et al. (1992). "Mechanical transmission of Venezuelan equine encephalomyelitis virus by hematophagous mites (Acari)." Journal of Medical Entomology **29**(1): 118-121.

East, I., C. Fitzgerald, et al. (1993). "*Lucilia cuprina*: inhibition of larval growth induced by immunization of host sheep with extracts of larval peritrophic membrane." International journal for parasitology **23**(2): 221-229.

Edwards, K., J. Goddard, et al. (2009). "Examination of the internal morphology of the ixodid tick, *Amblyomma maculatum* Koch,(Acari: Ixodidae); a “how-to” pictorial dissection guide." Midsouth Entomol **2**(1): 28-39.

El-Magd, R. M. A., N. F. Vozza, et al. (2016). "Isolation of soluble scFv antibody fragments specific for small biomarker molecule, L-Carnitine, using phage display." Journal of Immunological Methods **428**: 9-19.

Elliott, N. and D. Dent (1995). Integrated pest management, Springer Science & Business Media.

Erban, T. and J. Hubert (2010). "Determination of pH in regions of the midguts of acaridid mites." Journal of Insect science **10**: 42.

Erban, T. and J. Hubert (2011). "Visualization of protein digestion in the midgut of the acarid mite *Lepidoglyphus destructor*." Archives of insect biochemistry and physiology **78**(2): 74-86.

Erf, G. (2004). "Cell-mediated immunity in poultry." Poultry Science **83**(4): 580-590.

Esser, P. (1990). "Detergent in polystyrene ELISA." Thermo Scientific Nunc Bulletin(8): 1-5.

Evans, J. (1962). "Insect neurosecretory material separated by differential centrifugation." Science **136**(3513): 314-315.

Farina, S. F., G.-p. Gao, et al. (2001). "Replication-defective vector based on a chimpanzee adenovirus." Journal of virology **75**(23): 11603-11613.

Felici, F., L. Castagnoli, et al. (1991). "Selection of antibody ligands from a large library of oligopeptides expressed on a multivalent exposition vector." Journal of molecular biology **222**(2): 301-310.

Ferguson MAJ, Kinoshita T, et al. (2009). "Glycosylphosphatidylinositol Anchors." Essentials of Glycobiology. 2nd edition.

Ferrer, M. and S. C. Harrison (1999). "Peptide ligands to human immunodeficiency virus type 1 gp120 identified from phage display libraries." Journal of virology **73**(7): 5795-5802.

Fiddes, M. D., S. Le Gresley, et al. (2005). "Prevalence of the poultry red mite (*Dermanyssus gallinae*) in England." Veterinary research **157**(8): 233-235.

Filimonova, S. (2013). "Morphological aspects of blood digestion in a parasitic mite *Bakericheyla chanayi*." Arthropod Struct Dev **42**(4): 265-276.

Flint, M. L. and R. Van den Bosch (2012). Introduction to integrated pest management, Springer Science & Business Media.

Flower, D. R. and Y. Perrie (2012). Immunomic discovery of adjuvants and candidate subunit vaccines, Springer Science & Business Media.

Folgori, A., R. Tafi, et al. (1994). "A general strategy to identify mimotopes of pathological antigens using only random peptide libraries and human sera." The EMBO journal **13**(9): 2236.

Friedenauer, S. and H. H. Berlet (1989). "Sensitivity and variability of the Bradford protein assay in the presence of detergents." Analytical biochemistry **178**(2): 263-268.

Fujita, Y., H. Shirataki, et al. (1998). "Tomosyn: a syntaxin-1–binding protein that forms a novel complex in the neurotransmitter release process." Neuron **20**(5): 905-915.

Gaaboub, I., A. Donia, et al. (1982). "Ectoparasites of some rodents from the edge of the western desert near Alexandria, Egypt." International Journal of Tropical Insect Science **3**(2-3): 145-150.

Ganapamo, F., B. Rutti, et al. (1996). "Immunosuppression and cytokine production in mice infested with Ixodes ricinus ticks: a possible role of laminin and interleukin-10 on the in vitro responsiveness of lymphocytes to mitogens." Immunology **87**(2): 259-263.

Gao, J., J. Luo, et al. (2009). "Characterization of a concealed antigen Hq05 from the hard tick *Haemaphysalis qinghaiensis* and its effect as a vaccine against tick infestation in sheep." Vaccine **27**(3): 483-490.

García-García, J. C., C. Montero, et al. (2000). "Control of ticks resistant to immunization with Bm86 in cattle vaccinated with the recombinant antigen Bm95 isolated from the cattle tick, Boophilus microplus." Vaccine **18**(21): 2275-2287.

Garet, E., A. G. Cabado, et al. (2010). "Rapid isolation of single-chain antibodies by phage display technology directed against one of the most potent marine toxins: Palytoxin." Toxicon **55**(8): 1519-1526.

George, D., T. Smith, et al. (2009). "Mode of action and variability in efficacy of plant essential oils showing toxicity against the poultry red mite, *Dermanyssus gallinae*." Veterinary Parasitology **161**(3): 276-282.

George, D. R., R. D. Finn, et al. (2014). "Present and future potential of plant-derived products to control arthropods of veterinary and medical significance." Parasites & vectors **7**: 28.

George, D. R., G. Olatunji, et al. (2010). "Effect of plant essential oils as acaricides against the poultry red mite, *Dermanyssus gallinae*, with special focus on exposure time." Veterinary Parasitology **169**(1-2): 222-225.

George, D. R., O. A. E. Sparagano, et al. (2010). "Environmental interactions with the toxicity of plant essential oils to the poultry red mite *Dermanyssus gallinae*." Medical and Veterinary Entomology **24**(1): 1-8.

Ghosh, R. (2002). "Protein separation using membrane chromatography: opportunities and challenges." Journal of Chromatography A **952**(1): 13-27.

Gill, D. M. (1982). "Bacterial toxins: a table of lethal amounts." Microbiological reviews **46**(1): 86.

Goldberg, S. (2008). "Mechanical/physical methods of cell disruption and tissue homogenization." 2D PAGE: Sample Preparation and Fractionation: 3-22.

Graham, J. (2002). "Preparation of crude subcellular fractions by differential centrifugation." The Scientific World Journal **2**: 1638-1642.

Graham, J. M. and J. A. Higgins (1997). Membrane analysis, Bios Scientific Publishers.

Grebenyuk, R., P. Chirov, et al. (1972). "The role of wild animals and blood-sucking arthropods in the epizootiology of infection with Listeria." Rol'Dikikh Zhivotnykh i Krovososushchikh Chlenistonogikh v Epizootologii Listerioza. Frunze, Kirghiz SSR.

Gulia-Nuss, M., A. B. Nuss, et al. (2016). "Genomic insights into the *Ixodes scapularis* tick vector of Lyme disease." Nature communications **7**.

Gurunathan, S., D. M. Klinman, et al. (2000). "DNA vaccines: immunology, application, and optimization*." Annual Review of Immunology **18**(1): 927-974.

Guy, J. H., M. Khajavi, et al. (2004). "Red mite (*Dermanyssus gallinae*) prevalence in laying units in Northern England." British poultry science **45 Suppl 1**: S15-16.

Halstead, S. B. and S. J. Thomas (2011). "New Japanese encephalitis vaccines: alternatives to production in mouse brain." Expert Rev Vaccines **10**(3): 355-364.

Hamilton, K. A., A. J. Nisbet, et al. (2003). "A physiological and biochemical model for digestion in the ectoparasitic mite, *Psoroptes ovis* (Acari: Psoroptidae)." International Journal for Parasitology **33**(8): 773-785.

Hamra, C. (2010). An Assessment of the Potential Profitability of Poultry Farms: A Broiler Farm Feasibility Case Study, The University of Tennessee at Martin.

Hamscher, G., B. Prieß, et al. (2003). "Determination of propoxur residues in eggs by liquid chromatography–diode array detection after treatment of stocked housing facilities for the poultry red mite (Dermanyssus gallinae)." Analytica Chimica Acta **483**(1–2): 19-26.

Harrington, D., M. Canales, et al. (2009). "Immunisation with recombinant proteins subolesin and Bm86 for the control of *Dermanyssus gallinae* in poultry." Vaccine **27**(30): 4056-4063.

Harrington, D., H. M. Din, et al. (2009). "Characterization of the immune response of domestic fowl following immunization with proteins extracted from *Dermanyssus gallinae*." Veterinary Parasitology **160**(3-4): 285-294.

Harrington, D., J. Guy, et al. (2010). "Comparison of synthetic membranes in the development of an in vitro feeding system for *Dermanyssus gallinae*." Bulletin of Entomological Research **100**(02): 127-132.

Harrington, D. W., K. Robinson, et al. (2010). "Immune responses of the domestic fowl to *Dermanyssus gallinae* under laboratory conditions." Parasitology Research **106**(6): 1425-1434.

Harrington, D. W. J., D. R. George, et al. (2011). "Opportunities for integrated pest management to control the poultry red mite, *Dermanyssus gallinae*." World's Poultry Science Journal **67**(01): 83-94.

Hedges, S. B., P. H. Parker, et al. (1996). "Continental breakup and the ordinal diversification of birds and mammals." Nature **381**(6579): 226-229.

Hill, C. A. and S. K. Wikel (2005). "The *Ixodes scapularis* Genome Project: an opportunity for advancing tick research." Trends in Parasitology **21**(4): 151-153.

Hoebe, K., E. Janssen, et al. (2004). "The interface between innate and adaptive immunity." Nature immunology **5**(10): 971-974.

Hoffmann, G. (1988). "Vogelmilben als Lastlinge, Krankheitserzeuger und Vektoren bei Mensch und Nutztier." Deutsche Tieraerztliche Wochenschrift **951**: 7-10.

Höglund, J., H. Nordenfors, et al. (1995). "Prevalence of the poultry red mite, *Dermanyssus gallinae*, in different types of production systems for egg layers in Sweden." Poultry Science **74**(11): 1793-1798.

Hoiseth, S. K. and B. Stocker (1981). "Aromatic-dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines."

Holliger, P. and P. J. Hudson (2005). "Engineered antibody fragments and the rise of single domains." Nature Biotechnology **23**(9): 1126-1136.

Holt, R. A., G. M. Subramanian, et al. (2002). "The genome sequence of the malaria mosquito Anopheles gambiae." Science **298**(5591): 129-149.

Hong, Y. S., S. Kang, et al. (2011). "High quality RNA isolation from *Aedes aegypti* midguts using laser microdissection microscopy." Parasites & vectors **4**(1): 1-9.

Hoogenboom, H. R. (2005). "Selecting and screening recombinant antibody libraries." Nature Biotechnology **23**(9): 1105-1116.

Hoy, M. A., F. Yu, et al. (2013). "Transcriptome sequencing and annotation of the predatory mite *Metaseiulus occidentalis* (Acari: Phytoseiidae): a cautionary tale about possible contamination by prey sequences." Experimental & applied acarology **59**(3): 283-296.

Huang, Z., S. Elankumaran, et al. (2004). "A recombinant Newcastle disease virus (NDV) expressing VP2 protein of infectious bursal disease virus (IBDV) protects against NDV and IBDV." Journal of virology **78**(18): 10054-10063.

Ishaaya, I. and A. Horowitz (1998). Insecticides with novel modes of action: an overview. Insecticides with novel modes of action, Springer**:** 1-24.

Janeway, C. A., P. Travers, et al. (1997). Immunobiology: the immune system in health and disease, Current Biology.

Jespers, L. S., A. Roberts, et al. (1994). "Guiding the selection of human antibodies from phage display repertoires to a single epitope of an antigen." Nature Biotechnology **12**(9): 899-903.

Jones, M. K., L. M. Randall, et al. (2004). "Laser microdissection microscopy in parasitology: microscopes meet thermocyclers." Trends in parasitology **20**(11): 502-506.

Jonsson, N., A. Matschoss, et al. (2000). "Evaluation of TickGARD PLUS, a novel vaccine against Boophilus microplus, in lactating Holstein–Friesian cows." Veterinary parasitology **88**(3): 275-285.

Kadir, H. A. and C. O. Knowles (1991). "Inhibition of ATP dephosphorylation by acaricides with emphasis on the anti-ATPase activity of the carbodiimide metabolite of diafenthiuron." Journal of Economic Entomology **84**(3): 801-805.

Kamau, L., R. Skilton, et al. (2011). "Differential transcription of two highly divergent gut-expressed Bm86 antigen gene homologues in the tick *Rhipicephalus appendiculatus* (Acari: Ixodida)." Insect Molecular Biology **20**(1): 105-114.

Kaoud, H. A. (2010). "Susceptibility of poultry red mites to entomopathogens." International Journal of Poultry Science **9**(3): 259-263.

Kemp, D. H., R. D. Pearson, et al. (1989). "Vaccination against *Boophilus microplus*: Localization of antigens on tick gut cells and their interaction with the host immune system." Experimental and Applied Acarology **7**(1): 43-58.

Kenyon, F. and D. Knox (2002). "The proteinases of *Psoroptes ovis*, the sheep scab mite—their diversity and substrate specificity." Veterinary Parasitology **105**(4): 317-325.

Kew, O., V. Morris-Glasgow, et al. (2002). "Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus." Science **296**(5566): 356-359.

Keyburn, A. L., R. W. Portela, et al. (2013). "Vaccination with recombinant NetB toxin partially protects broiler chickens from necrotic enteritis." Veterinary research **44**(1): 1.

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**(3): 232-240.

Kilpinen, O., A. Roepstorff, et al. (2005). "Influence of *Dermanyssus gallinae* and *Ascaridia galli* infections on behaviour and health of laying hens (*Gallus gallus* domesticus)." British poultry science **46**(1): 26-34.

Kilpinen, O. and T. Steenberg (2009). "Inert dusts and their effects on the poultry red mite (*Dermanyssus gallinae*)." Experimental & applied acarology **48**(1-2): 51-62.

Kim, S.-I., Y.-E. Na, et al. (2007). "Contact and fumigant toxicity of oriental medicinal plant extracts against *Dermanyssus gallinae* (Acari: Dermanyssidae)." Veterinary Parasitology **145**(3): 377-382.

Kirkwood, A. C. (1967). "Anaemia in poultry infested with the red mite *Dermanyssus gallinae*." Veterinary research **80**(17): 514-516.

Kissling, R. and D. Reese (1963). "Anti-rabies vaccine of tissue culture origin." The Journal of Immunology **91**(3): 362-368.

Koenraadt, C. J. and M. Dicke (2010). "The role of volatiles in aggregation and host-seeking of the haematophagous poultry red mite *Dermanyssus gallinae* (Acari: Dermanyssidae)." Experimental & applied acarology **50**(3): 191-199.

Konecka, E. and Z. Olszanowski (2015). "A screen of maternally inherited microbial endosymbionts in oribatid mites (Acari: Oribatida)." Microbiology **161**(8): 1561-1571.

Kongsuwan, K., P. Josh, et al. (2010). "Exploring the midgut proteome of partially fed female cattle tick (*Rhipicephalus* (Boophilus) *microplus*)." Journal of insect physiology **56**(2): 212-226.

Kopáček, P., O. Hajdušek, et al. (2010). Tick innate immunity. Invertebrate Immunity, Springer**:** 137-162.

Kosma, P. and S. Müller-Loennies (2011). Anticarbohydrate antibodies: from molecular basis to clinical application, Springer Science & Business Media.

Krantz, G. W. and D. E. Walter (2009). A manual of acarology, 3rd edition, Lubbock, TX: Texas Tech University Press.

Krause, W. J. (2001). The art of examining and interpreting histologic preparations: A student handbook, CRC Press.

Kristofík, J., P. Masan, et al. (1996). "Ectoparasites of bee-eater (*Merops apiaster*) and arthropods in its nests." Biologia **51**(5): 557-570.

Kulkarni, R., V. Parreira, et al. (2008). "Oral immunization of broiler chickens against necrotic enteritis with an attenuated Salmonella vaccine vector expressing Clostridium perfringens antigens." Vaccine **26**(33): 4194-4203.

Kupsch, J.-M., N. H. Tidman, et al. (1999). "Isolation of human tumor-specific antibodies by selection of an antibody phage library on melanoma cells." Clinical cancer research **5**(4): 925-931.

Lavine, M. and M. Strand (2002). "Insect hemocytes and their role in immunity." Insect biochemistry and molecular biology **32**(10): 1295-1309.

Layton, S., M. Morgan, et al. (2011). "Evaluation of Salmonella-vectored Campylobacter peptide epitopes for reduction of Campylobacter jejuni in broiler chickens." Clinical and Vaccine Immunology **18**(3): 449-454.

Lebacq-Verheyden, A.-M., J.-P. Vaerman, et al. (1974). "Quantification and distribution of chicken immunoglobulins IgA, IgM and IgG in serum and secretions." Immunology **27**(4): 683.

LeJambre, L., R. Windon, et al. (2008). "Vaccination against *Haemonchus contortus*: performance of native parasite gut membrane glycoproteins in Merino lambs grazing contaminated pasture." Veterinary parasitology **153**(3): 302-312.

Leslie, G. A. and L. Clem (1969). "Phylogeny of immunoglobulin structure and function III. Immunoglobulins of the chicken." The Journal of experimental medicine **130**(6): 1337-1352.

Lesna, I., M. W. Sabelis, et al. (2012). "Laboratory tests for controlling poultry red mites (*Dermanyssus gallinae*) with predatory mites in small 'laying hen'cages." Experimental and Applied Acarology **58**(4): 371-383.

Lesna, I., P. Wolfs, et al. (2009). "Candidate predators for biological control of the poultry red mite *Dermanyssus gallinae*." Experimental and Applied Acarology **48**(1-2): 63-80.

Liao, M., J. Zhou, et al. (2007). "Molecular characterization of *Rhipicephalus* (Boophilus) *microplus* Bm86 homologue from *Haemaphysalis longicornis* ticks." Veterinary Parasitology **146**(1–2): 148-157.

Lienk, S. E. (1980). "European Red Mite." Tree Fruit IPM Insect Identification Sheet No. 10.

Lightowlers, M. W. (1994). "Vaccination against animal parasites." Veterinary parasitology **54**(1-3): 177-204.

Lillehoj, H. S. and J. M. Trout (1996). "Avian gut-associated lymphoid tissues and intestinal immune responses to Eimeria parasites." Clinical microbiology reviews **9**(3): 349-360.

Liu, Y. J., J. Zhang, et al. (1991). "Sites of specific B cell activation in primary and secondary responses to T cell-dependent and T cell-independent antigens." European journal of immunology **21**(12): 2951-2962.

Liu, Z., M. Li, et al. (2005). "Antigenic localization of Der p1 in the body of the house dust mite, *Dermatophagoides pteronyssinus* by immunohistochemistry." Acta Entomologica Sinica **48**(6): 833.

Lodish, H., A. Berk, et al. (2000). "Membrane Proteins." Molecular Cell Biology. 4th edition.

Lucky, A. W., C. Sayers, et al. (2001). "Avian mite bites acquired from a new source—pet gerbils: Report of 2 cases and review of the literature." Archives of Dermatology **137**(2): 167-170.

Lundqvist, M. L., D. L. Middleton, et al. (2006). "Immunoglobulins of the non-galliform birds: antibody expression and repertoire in the duck." Developmental & Comparative Immunology **30**(1): 93-100.

Luo, D. and W. M. Saltzman (2000). "Synthetic DNA delivery systems." Nature Biotechnology **18**(1): 33-37.

Lutsky, I., H. Teichtahl, et al. (1984). "Occupational asthma due to poultry mites." Journal of allergy and clinical immunology **73**(1): 56-60.

Macreadie, I. G., P. R. Vaughan, et al. (1990). "Passive protection against infectious bursal disease virus by viral VP2 expressed in yeast." Vaccine **8**(6): 549-552.

Magor, K. E. (2011). "Immunoglobulin genetics and antibody responses to influenza in ducks." Developmental & Comparative Immunology **35**(9): 1008-1017.

Malorny, B., A. Bhunia, et al. (2011). "Salmonella in pork, beef, poultry, and egg." Rapid detection, characterization, and enumeration of foodborne pathogens: 179-194.

Manzano-Román, R., S. García-Varas, et al. (2007). "Purification and characterization of a 45-kDa concealed antigen from the midgut membranes of *Ornithodoros erraticus* that induces lethal anti-tick immune responses in pigs." Veterinary Parasitology **145**(3): 314-325.

Marangi, M., V. Morelli, et al. (2012). "Acaricide residues in laying hens naturally infested by red mite *Dermanyssus gallinae*." PLoS One **7**(2): e31795.

Marangon, S. and L. Busani (2007). "The use of vaccination in poultry production." Revue Scientifique et Technique-Office International des Epizooties **26**(1): 265.

Mashaly, M., G. Hendricks, et al. (2004). "Effect of heat stress on production parameters and immune responses of commercial laying hens." Poultry Science **83**(6): 889-894.

Masteller, E. L., G. T. Pharr, et al. (1997). "Avian B cell development." International reviews of immunology **15**(3-4): 185-206.

Mathieson, B. R. F. and M. J. Lehane (2002). "Ultrastructure of the alimentary canal of the sheep scab mite, *Psoroptes ovis* (Acari: Psoroptidae)." Veterinary Parasitology **104**(2): 151-166.

Matthias, D. M., J. Robertson, et al. (2007). "Freezing temperatures in the vaccine cold chain: a systematic literature review." Vaccine **25**(20): 3980-3986.

Maurer, V., M. Bieri, et al. (1988). "Das Suchverhalten von *Dermanyssus gallinae* in Huhnerst allen." Archiv Fur Geflugelkunde **52**: 209-215.

Maurer, V. and E. Perler (2006). "Silicas for control of the poultry red mite *Dermanyssus gallinae*."

Maurer, V., E. Perler, et al. (2009). "In vitro efficacies of oils, silicas and plant preparations against the poultry red mite *Dermanyssus gallinae*." Experimental and Applied Acarology **48**(1-2): 31-41.

Mazur, P. (1984). "Freezing of living cells: mechanisms and implications." American Journal of Physiology-Cell Physiology **247**(3): C125-C142.

Mazzoni, V., A. Lucchi, et al. (2009). "Disruption of the reproductive behaviour of *Scaphoideus titanus* by playback of vibrational signals." Entomologia experimentalis et applicata **133**(2): 174-185.

McCormack, W. T., L. W. Tjoelker, et al. (1991). "Avian B-cell development: generation of an immunoglobulin repertoire by gene conversion." Annual review of immunology **9**(1): 219-241.

McDevitt, R., A. J. Nisbet, et al. (2006). "Ability of a proteinase inhibitor mixture to kill poultry red mite, *Dermanyssus gallinae* in an in vitro feeding system." Veterinary Parasitology **141**(3-4): 380-385.

McMurtry, J. and G. Scriven (1966). "Studies on predator-prey interactions between *Amblyseius hibisci* and *Oligonychus punicae* (Acarina: Phytoseiidae, Tetranychidae) under greenhouse conditions." Annals of the Entomological Society of America **59**(4): 793-800.

Meadows, K. E. (1968). "A simple method of mosquito ovary dissection." Florida Entomologist **1**: 31-35.

Mendiola, J., M. Alonso, et al. (1996). "*Boophilus microplus*: multiple proteolytic activities in the midgut." Experimental parasitology **82**(1): 27-33.

Meng, X. (2000). "Heterogeneity of porcine reproductive and respiratory syndrome virus: implications for current vaccine efficacy and future vaccine development." Veterinary microbiology **74**(4): 309-329.

Merten, O.-W., D. Mattanovich, et al. (2013). Recombinant Protein Production with Prokaryotic and Eukaryotic Cells. A Comparative View on Host Physiology: Selected articles from the Meeting of the EFB Section on Microbial Physiology, Semmering, Austria, 5th–8th October 2000, Springer Science & Business Media.

Meyer-Kühling, B., K. Pfister, et al. (2007). "Field efficacy of phoxim 50%(ByeMite®) against the poultry red mite *Dermanyssus gallinae* in battery cages stocked with laying hens." Veterinary Parasitology **147**(3): 289-296.

Mignon, B. and B. Losson (2008). "Dermatitis in a horse associated with the poultry mite (Dermanyssus gallinae)." Veterinary dermatology **19**(1): 38-43.

Millan, C. L. B., R. Weeratna, et al. (1998). "CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice." Proceedings of the National Academy of Sciences **95**(26): 15553-15558.

Miller, P. J., E. L. Decanini, et al. (2010). "Newcastle disease: evolution of genotypes and the related diagnostic challenges." Infection, Genetics and Evolution **10**(1): 26-35.

Minnifield, N. M., J. Carroll, et al. (1993). "Antibody development against northern fowl mites (Acari: Macronyssidae) in chickens." Journal of medical entomology **30**: 360-360.

Morimura, T., R. Goitsuka, et al. (2000). "Cell cycle arrest and apoptosis induced by Notch1 in B cells." Journal of Biological Chemistry **275**(47): 36523-36531.

Moro, C. V., C. Chauve, et al. (2005). "Vectorial role of some dermanyssoid mites (Acari, Mesostigmata, Dermanyssoidea)." Parasite **12**(2): 99-109.

Moro, C. V., J. Thioulouse, et al. (2009). "Bacterial taxa associated with the hematophagous mite *Dermanyssus gallinae* detected by 16S rRNA PCR amplification and TTGE fingerprinting." Research in microbiology **160**(1): 63-70.

Mul, M., T. van Niekerk, et al. (2009). "Control methods for *Dermanyssus gallinae* in systems for laying hens: results of an international seminar." World's Poultry Science Journal **65**(04): 589-600.

Mul, M. F. and C. J. Koenraadt (2009). "Preventing introduction and spread of *Dermanyssus gallinae* in poultry facilities using the HACCP method." Experimental & applied acarology **48**(1-2): 167-181.

Mullen, G. and B. O'Connor (2009). "Chapter 25: Mites (Acari)." Medical and Veterinary Entomology, 2nd edn. Academic Press (Elsevier), Burlington, Massachusetts: 423-482.

Mullens, B., D. Soto, et al. (2012). "Northern fowl mite (*Ornithonyssus sylviarum*) control evaluations using liquid formulations of diatomaceous earth, kaolin, sulfur, azadirachtin, and *Beauveria bassiana* on caged laying hens." The Journal of Applied Poultry Research **21**(1): 111-116.

Mungube, E., S. Bauni, et al. (2008). "Prevalence of parasites of the local scavenging chickens in a selected semi-arid zone of Eastern Kenya." Tropical Animal Health and Production **40**(2): 101-109.

Munn, E. A., T. S. Smith, et al. (1997). "Vaccination against *Haemonchus contortus* with denatured forms of the protective antigen H11." Parasite Immunol **19**(6): 243-248.

Nadji, M. (1986). "Immunoperoxidase Techniques: I. Facts and Artifacts." The American Journal of Dermatopathology **8**(1): 32-36.

Nakamae H, Fujisaki K, et al. (1997). "The New Parasitic Ecology of Chicken Mites *Dermanyssus gallinae,* Parasitizing and Propagating on Chickens Even in the Daytime." Journal of the Japanese Poultry Science Association **34**(2): 110-116.

Nakao, S., H. Osada, et al. (1988). "Separation of proteins by charged ultrafiltration membranes." Desalination **70**(1): 191-205.

Niiro, H. and E. A. Clark (2002). "Regulation of B-cell fate by antigen-receptor signals." Nature Reviews Immunology **2**(12): 945-956.

Nisbet, A. J. and P. F. Billingsley (2000). "A comparative survey of the hydrolytic enzymes of ectoparasitic and free-living mites." International Journal for Parasitology **30**(1): 19-27.

Nordenfors, H. and J. Chirico (2001). "Evaluation of a sampling trap for *Dermanyssus gallinae* (Acari: Dermanyssidae)." Journal of Economic Entomology **94**(6): 1617-1621.

Nordenfors, H., J. Hoglund, et al. (1999). "Effects of temperature and humidity on oviposition, molting, and longevity of *Dermanyssus gallinae* (Acari: Dermanyssidae)." Journal of Medical Entomology **36**(1): 68-72.

Nugent, T. and D. T. Jones (2009). "Transmembrane protein topology prediction using support vector machines." BMC Bioinformatics **10**(1): 1.

Nuttall, P. and M. Labuda (2004). "Tick–host interactions: saliva-activated transmission." Parasitology **129**(S1): S177-S189.

Nuttall, P. A., A. R. Trimnell, et al. (2006). "Exposed and concealed antigens as vaccine targets for controlling ticks and tick-borne diseases." Parasite immunology **28**(4): 155-163.

Ohta, M., J. Hamako, et al. (1991). "Structures of asparagine-linked oligosaccharides from hen egg-yolk antibody (IgY). Occurrence of unusual glucosylated oligo-mannose type oligosaccharides in a mature glycoprotein." Glycoconjugate journal **8**(5): 400-413.

Oines, O. and S. Brannstrom (2011). "Molecular investigations of cytochrome c oxidase subunit I (COI) and the internal transcribed spacer (ITS) in the poultry red mite, *Dermanyssus gallinae*, in northern Europe and implications for its transmission between laying poultry farms." Med Vet Entomol **25**(4): 402-412.

Okazaki, W., H. Purchase, et al. (1970). "Protection against Marek's disease by vaccination with a herpesvirus of turkeys." Avian Diseases: 413-429.

Opdebeeck, J., J. Wong, et al. (1989). "Hereford cattle protected against Boophilus microplus with antigens purified by immunoaffinity chromatography from larval and adult ticks." Immunology **67**(3): 388.

Opitz, H. and M. Cyr (1986). "Triton X-100-solubilized Mycoplasma gallisepticum and M. synoviae ELISA antigens." Avian diseases: 213-215.

Ozaki, L. S., R. W. Gwadz, et al. (1984). "Simple centrifugation method for rapid separation of sporozoites from mosquitoes." The Journal of parasitology **70**(5): 831-833.

Pacejka, A. J., E. Santana, et al. (1996). "House wrens *Troglodytes aedon* and nest-dwelling ectoparasites: mite population growth and feeding patterns." Journal of Avian Biology: 273-278.

Pasqualini, R. and W. Arap (2004). "Hybridoma-free generation of monoclonal antibodies." Proceedings of the National Academy of Sciences **101**(1): 257-259.

Patton, T. G., G. Dietrich, et al. (2012). "Saliva, salivary gland, and hemolymph collection from *Ixodes scapularis* ticks." J Vis Exp **60**: 3894.

Perrot-Minnot, M.-J., L. R. Guo, et al. (1996). "Single and double infections with Wolbachia in the parasitic wasp Nasonia vitripennis effects on compatibility." Genetics **143**(2): 961-972.

Petrov, D. (1974). "Study of *Dermanyssus gallinae* as a carrier of *Pasteurella multocida*." Veterinarno-meditsinski nauki **12**(5): 32-36.

Pi, C., R. Z. Rou, et al. (2014). "Fair or Fowl? Industrialization of Poultry Production in China. A publication for the Global Meat Complex: The China Series, edited by Shefali Sharma and Ben Lilliston." Institute for Agriculture and Trade Policy. http://www. iatp. org/documents/fair-or-fowl-industrialization-of-poultry-production-inchina.

Pizza, M., V. Scarlato, et al. (2000). "Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing." Science **287**(5459): 1816-1820.

Pritchard, J., T. Kuster, et al. (2015). "Understanding the biology & control of the poultry red mite *Dermanyssus gallinae*: a review." Avian Pathology **44**(3): 1-42.

Ragan, C. (2012). Techniques for the analysis of membrane proteins, Springer Science & Business Media.

Rakonjac, J. (2012). "Filamentous bacteriophages: biology and applications." eLS.

Ramachandra, R. N. and S. K. Wikel (1992). "Modulation of host-immune responses by ticks (Acari: Ixodidae): effect of salivary gland extracts on host macrophages and lymphocyte cytokine production." Journal of medical entomology **29**: 818-818.

Rand, K. N., T. Moore, et al. (1989). "Cloning and expression of a protective antigen from the cattle tick Boophilus microplus." Proceedings of the National Academy of Sciences **86**(24): 9657-9661.

Ratcliffe, M. J. (2006). "Antibodies, immunoglobulin genes and the bursa of Fabricius in chicken B cell development." Developmental & Comparative Immunology **30**(1): 101-118.

Reardon, T., D. Tschirley, et al. (2014). "The Rapid Transformation of the Food Economy of Africa." Michigan State University.

Redding, L. and D. B. Weiner (2009). "DNA vaccines in veterinary use." Expert review of vaccines **8**(9): 1251-1276.

Reynaud, C.-A., V. Anquez, et al. (1985). "A single rearrangement event generates most of the chicken immunoglobulin light chain diversity." Cell **40**(2): 283-291.

Reynaud, C.-A., B. Bertocci, et al. (1994). "Formation of the chicken B-cell repertoire: ontogenesis, regulation of Ig gene rearrangement, and diversification by gene conversion." Advances in Immunology **57**: 353-378.

Reynaud, C. A., B. Imhof, et al. (1992). "Emergence of committed B lymphoid progenitors in the developing chicken embryo." The EMBO journal **11**(12): 4349.

Ribeiro, J. (1987). "Role of saliva in blood-feeding by arthropods." Annual review of entomology **32**(1): 463-478.

Richards, S. A., C. Stutzer, et al. (2015). "Transmembrane proteins – Mining the cattle tick transcriptome." Ticks and tick-borne diseases **6**(6): 695-710.

Roberts, D. J. and M. Waelbroeck (2004). "G protein activation by G protein coupled receptors: ternary complex formation or catalyzed reaction?" Biochemical Pharmacology **68**(5): 799-806.

Rohde, B., T. M. Paris, et al. (2013). "Responses of *Diaphorina citri* (Hemiptera: Psyllidae) to conspecific vibrational signals and synthetic mimics." Annals of the Entomological Society of America **106**(3): 392-399.

Rosen, S., I. Yeruham, et al. (2002). "Dermatitis in humans associated with the mites *Pyemotes tritici, Dermanyssus gallinae, Ornithonyssus bacoti* and *Androlaelaps casalis* in Israel." Medical and Veterinary Entomology **16**(4): 442-444.

Ross, M. H. and W. Pawlina (2006). Histology, Lippincott Williams & Wilkins.

Roy, L. and T. Buronfosse (2011). "Using mitochondrial and nuclear sequence data for disentangling population structure in complex pest species: a case study with *Dermanyssus gallinae*." PLoS One **6**(7): e22305.

RUMA (2015). "Responsible use of vaccines and vaccination in poultry production."

Sachs, J. N. and D. M. Engelman (2006). "Introduction to the membrane protein reviews: the interplay of structure, dynamics, and environment in membrane protein function." Annu. Rev. Biochem. **75**: 707-712.

Sahibi, H., O. Sparagano, et al. (2008). Dermanyssus gallinae: Acari parasite highly aggressive but still ignored in Morocco. BSP spring, trypanosomiasis/leishmaniasis and malaria meetings. March 30th, April 2nd, Newcastle Upon Tyne.

Santillan-Galicia, M. T., R. Carzaniga, et al. (2008). "Immunolocalization of deformed wing virus particles within the mite *Varroa destructor*." The Journal of general virology **89**(Pt 7): 1685-1689.

Santos, C. D., A. F. Ribeiro, et al. (1986). "Differential centrifugation, calcium precipitation, and ultrasonic disruption of midgut cells of *Erinnyis ello* caterpillars. Purification of cell microvilli and inferences concerning secretory mechanisms." Canadian journal of zoology **64**(2): 490-500.

Sayegh, C. E., S. L. Demaries, et al. (2000). "The chicken B-cell receptor complex and its role in avian B-cell development." Immunological reviews **175**(1): 187-200.

Schicht, S., W. Qi, et al. (2013). "The predicted secretome and transmembranome of the poultry red mite *Dermanyssus gallinae*." Parasites & vectors **6**(1): 259.

Schicht, S., W. Qi, et al. (2013). "Whole transcriptome analysis of the poultry red mite *Dermanyssus gallinae* (De Geer, 1778)." Parasitology **141**(3): 1-11.

Schoeler, G. and S. Wikel (2001). "Modulation of host immunity by haematophagous arthropods." Annals of Tropical Medicine and Parasitology **95**(8): 755-771.

Scopes, R. K. (2013). Protein purification: principles and practice, Springer Science & Business Media.

Scott, J. K., D. Loganathan, et al. (1992). "A family of concanavalin A-binding peptides from a hexapeptide epitope library." Proceedings of the National Academy of Sciences **89**(12): 5398-5402.

Scott, L., M. J. Schell, et al. (1993). "Isolation of plasma membrane sheets and plasma membrane domains from rat liver." Biomembrane Protocols: I. Isolation and Analysis: 59-69.

Seddon, A. M., P. Curnow, et al. (2004). "Membrane proteins, lipids and detergents: not just a soap opera." Biochimica et Biophysica Acta (BBA)-Biomembranes **1666**(1): 105-117.

Sharma, J. and B. Burmester (1982). "Resistance of Marek's disease at hatching in chickens vaccinated as embryos with the turkey herpesvirus." Avian Diseases: 134-149.

Shaw, K. E., G. Davidson, et al. (2002). "Laboratory bioassays to assess the pathogenicity of mitosporic fungi to *Varroa destructor* (Acari: Mesostigmata), an ectoparasitic mite of the honeybee, *Apis mellifera*." Biological Control **24**(3): 266-276.

Sheeler, P. (1981). Centrifugation in biology and medical science, Wiley.

Shi, W.-B. and M.-G. Feng (2004). "Lethal effect of *Beauveria bassiana*, *Metarhizium anisopliae*, and *Paecilomyces fumosoroseus* on the eggs of *Tetranychus cinnabarinus* (Acari: Tetranychidae) with a description of a mite egg bioassay system." Biological Control **30**(2): 165-173.

Shirinov, F. B., A. I. Ibragimova, et al. (1972). "The dissemination of the fowl-pox by the mite *Dermanyssus gallinae*." Veterinarya **4**: 48-49.

Siettou, C. and S. B. C. Biosciences (2016). Avian Influenza: outbreaks and the impact on UK consumer demand for poultry. 90th Annual Conference, April 4-6, 2016, Warwick University, Coventry, UK, Agricultural Economics Society.

Sigma-Aldrich (2016). "Tween 20 detergent."

Singer, S. and G. L. Nicolson (1972). "The fluid mosaic model of the structure of cell membranes." Membranes and Viruses in Immunopathology; Day, SB, Good, RA, Eds: 7-47.

Skerra, A. and A. Pluckthun (1988). "Assembly of a functional immunoglobulin Fv fragment in Escherichia coli." Science **240**(4855): 1038.

Smith, S. M. (2011). "Strategies for the purification of membrane proteins." Protein Chromatography: Methods and Protocols: 485-496.

Smith, W. and D. Pettit (2004). "Immunization against sheep scab: preliminary identification of fractions of *Psoroptes ovis* which confer protective effects." Parasite immunology **26**(6): 307-314.

Smucker, R. A. and R. M. Pfister (1975). "Liquid nitrogen cryo-impacting: a new concept for cell disruption." Applied microbiology **30**(3): 445-449.

Sobotnik, J., G. Alberti, et al. (2008). "Ultrastructure of the digestive tract in *Acarus siro* (Acari: Acaridida)." Journal of Morphology **269**(1): 54-71.

Sojka, D., Z. Franta, et al. (2013). "New insights into the machinery of blood digestion by ticks." Trends in Parasitology **29**(6): 276-285.

Sokół, R., A. Szkamelski, et al. (2008). "Influence of light and darkness on the behaviour of *Dermanyssus gallinae* on layer farms." Polish Journal of Veterinary Sciences **11**(1): 71-73.

Soon-Il, K., Y. Jee-Hwan, et al. (2004). "Acaricidal activity of plant essential oils against *Dermanyssus gallinae* (Acari: Dermanyssidae)." Veterinary Parasitology **120**(4): 297-304.

Sparagano, O., A. Pavlicevic, et al. (2009). "Prevalence and key figures for the poultry red mite *Dermanyssus gallinae* infections in poultry farm systems." Experimental & applied acarology **48**(1-2): 3-10.

Sparagano, O. A., D. R. George, et al. (2014). "Significance and Control of the Poultry Red Mite, *Dermanyssus gallinae*." The Annual Review of Entomology **59**: 447-466.

Steenberg, T. and O. Kilpinen (2003). "Fungus infection of the chicken mite *Dermanyssus gallinae*." IOBC-WPRS Bulletin **26**(1): 23-26.

Steinitz, M. (2000). "Quantitation of the blocking effect of tween 20 and bovine serum albumin in ELISA microwells." Analytical biochemistry **282**(2): 232-238.

Strambini, G. B. and E. Gabellieri (1996). "Proteins in frozen solutions: evidence of ice-induced partial unfolding." Biophysical journal **70**(2): 971.

Strome, C., P. DeSantis, et al. (1980). "A convenient technique for the dissection of mosquito salivary glands." Journal of tissue culture methods **6**(1): 9-11.

Sugumaran, M. (2002). "Comparative biochemistry of eumelanogenesis and the protective roles of phenoloxidase and melanin in insects." Pigment Cell Research **15**(1): 2-9.

Swanepoel, R. and J. Coetzer (2004). "Rift valley fever." Infectious diseases of livestock **2**: 1037-1070.

Takács, B. J. (2000). Protein purification: Theoretical and methodological considerations, Wiley Online Library.

Tarigan, S. and J. F. Huntley (2005). "Failure to protect goats following vaccination with soluble proteins of *Sarcoptes scabiei*: evidence for a role for IgE antibody in protection." Veterinary Parasitology **133**(1): 101-109.

Tarnowski, B. I. and L. B. Coons (1989). "Ultrastructure of the midgut and blood meal digestion in the adult tick *Dermacentor variabilis*." Experimental & applied acarology **6**(4): 263-289.

Tavassoli, M., M. Allymehr, et al. (2011). "Field bioassay of *Metarhizium anisopliae* strains to control the poultry red mite *Dermanyssus gallinae*." Veterinary Parasitology **178**(3–4): 374-378.

Tellam, R., D. Kemp, et al. (2002). "Reduced oviposition of Boophilus microplus feeding on sheep vaccinated with vitellin." Veterinary Parasitology **103**(1): 141-156.

Tellam, R. L., D. Smith, et al. (1992). "Vaccination against ticks." Animal Parasite Control Utilizing Biotechnology: 303-331.

Tennant, S. M. and M. M. Levine (2015). "Live attenuated vaccines for invasive Salmonella infections." Vaccine **33**: C36-C41.

Thompson, C. B. and P. E. Neiman (1987). "Somatic diversification of the chicken immunoglobulin light chain gene is limited to the rearranged variable gene segment." Cell **48**(3): 369-378.

Tiller, G. E., T. J. Mueller, et al. (1984). "Hydrogenation of Triton X-100 eliminates its fluorescence and ultraviolet light absorption while preserving its detergent properties." Analytical biochemistry **141**(1): 262-266.

Tizard, I. (2002). The avian antibody response. Seminars in Avian and Exotic Pet Medicine, Elsevier.

Trimnell, A. R., G. M. Davies, et al. (2005). "A cross-reactive tick cement antigen is a candidate broad-spectrum tick vaccine." Vaccine **23**(34): 4329-4341.

Trimnell, A. R., R. S. Hails, et al. (2002). "Dual action ectoparasite vaccine targeting 'exposed'and 'concealed'antigens." Vaccine **20**(29): 3560-3568.

Tucci, E. (1997). "A laboratory method for the rearing of *Dermanyssus gallinae* (DeGeer, 1778)(Acari, Dermanyssidae)." Arquivos do Instituto Biologico: 1-4.

Tucci, E., A. Prado, et al. (2008). "Development of *Dermanyssus gallinae* (Acari: Dermanyssidae) at different temperatures." Veterinary Parasitology **155**(1): 127-132.

Ullmann, A., C. Lima, et al. (2005). "Genome size and organization in the blacklegged tick, Ixodes scapularis and the Southern cattle tick, Boophilus microplus." Insect molecular biology **14**(2): 217-222.

Valiente Moro, C., C. Chauve, et al. (2007). "Experimental infection of *Salmonella Enteritidis* by the poultry red mite, *Dermanyssus gallinae*." Veterinary Parasitology **146**(3): 329-336.

Valiente Moro, C., C. J. De Luna, et al. (2009). "The poultry red mite (*Dermanyssus gallinae*): a potential vector of pathogenic agents." Experimental & applied acarology **48**(1-2): 93-104.

Valiente Moro, C., J. Thioulouse, et al. (2009). "Bacterial taxa associated with the hematophagous mite *Dermanyssus gallinae* detected by 16S rRNA PCR amplification and TTGE fingerprinting." Research in microbiology **160**(1): 63-70.

Valle, M. R., L. Mèndez, et al. (2004). "Integrated control of Boophilus microplus ticks in Cuba based on vaccination with the anti-tick vaccine Gavac TM." Experimental & applied acarology **34**(3-4): 375-382.

Van Bronswijk, J. and R. Sinha (1971). "*Pyroglyphid* mites (Acari) and house dust allergy: a review." Journal of Allergy and Clinical Immunology **47**(1): 31-52.

Van Emous, R. (2005). "Wage war against the red mite!" Poultry International **44**: 26–33.

Van Overbeke, I., K. Chiers, et al. (2001). "Effect of Endobronchial Challenge with Actinobacillus pleuropneumoniae Serotype 9 of Pigs Vaccinated with a Vaccine Containing Apx Toxins and Transferrin-binding Proteins." Journal of Veterinary Medicine, Series B **48**(1): 15-20.

Vaswani, P. S. (2015). "Indian Poultry Industry: Challenges and Opportunities." Global Journal of Fundamental and Applied Life Sciences **1**(1).

Vaughan, A. M. and S. H. Kappe (2012). "Malaria vaccine development: persistent challenges." Current opinion in immunology **24**(3): 324-331.

Vercruysse, J., D. P. Knox, et al. (2004). "Veterinary parasitic vaccines: pitfalls and future directions." TRENDS in Parasitology **20**(10): 488-492.

Volossiouk, T., E. J. Robb, et al. (1995). "Direct DNA extraction for PCR-mediated assays of soil organisms." Applied and Environmental Microbiology **61**(11): 3972-3976.

von Jagow, G. (1994). A practical guide to membrane protein purification, Gulf Professional Publishing.

Wales, A., J. Carrique Mas, et al. (2010). "Review of the carriage of zoonotic bacteria by arthropods, with special reference to *Salmonella* in mites, flies and litter beetles." Zoonoses and public health **57**(5): 299-314.

Walker, J. M. (2009). "The bicinchoninic acid (BCA) assay for protein quantitation." The Protein Protocols Handbook: 11-15.

Wall, R. and D. Shearer (1997). Mites (Acari). Veterinary Entomology, Springer**:** 43-95.

Wallin, E. and G. V. Heijne (1998). "Genome-wide analysis of integral membrane proteins from eubacterial, archaean, and eukaryotic organisms." Protein Science **7**(4): 1029-1038.

Walter, D. E. and H. C. Proctor (1999). Mites: ecology, evolution, and behaviour, Springer.

Wang, F., M. Wang, et al. (2010). "Survey of prevalence and control of ectoparasites in caged poultry in China." Vet Rec **167**(24): 934-937.

Wang, S., C. Zhang, et al. (2008). "The relative immunogenicity of DNA vaccines delivered by the intramuscular needle injection, electroporation and gene gun methods." Vaccine **26**(17): 2100-2110.

Wang, X. W., N. S. Tan, et al. (2006). "Evidence for the ancient origin of the NF-κB/IκB cascade: its archaic role in pathogen infection and immunity." Proceedings of the National Academy of Sciences of the United States of America **103**(11): 4204-4209.

Wegner, Z. (1975). "Laboratory study on some parasitic hematophagous arthropods as possible subsidiary links of the biocenosis of tick-borne encephalitis." Bulletin of the Institute of Maritime and Tropical Medicine in Gdynia **27**(1): 75-85.

Weir, E. and K. Hatch (2004). "Preventing cold chain failure: vaccine storage and handling." Canadian Medical Association Journal **171**(9): 1050-1050.

White, N. D. G. and D. S. Jayas (1991). "Control of insects and mites with carbon dioxide in wheat stored at cool temperatures in nonairtight bins." Journal of Economic Entomology **84**(6): 1933-1942.

WHO/FAO (1983). "The use of veterinary vaccines for prevention and control of Rift Valley fever: memorandum from a WHO/FAO meeting." Bull World Health Organ **61**(2): 261-268.

Willadsen, P. (2004). "Anti-tick vaccines." Parasitology **129 Suppl**: S367-387.

Willadsen, P. (2006). "Tick control: Thoughts on a research agenda." Veterinary Parasitology **138**(1–2): 161-168.

Willadsen, P. (2006). "Vaccination against ectoparasites." Parasitology **133 Suppl**: S9-S25.

Willadsen, P., P. Bird, et al. (1995). "Commercialisation of a recombinant vaccine against *Boophilus microplus*." Parasitology **110**: S43-50.

Willadsen, P., R. V. McKenna, et al. (1988). "Isolation from the cattle tick, *Boophilus microplus*, of antigenic material capable of eliciting a protective immunological response in the bovine host." International Journal for Parasitology **18**(2): 183-189.

Willadsen, P., G. A. Riding, et al. (1989). "Immunologic control of a parasitic arthropod. Identification of a protective antigen from *Boophilus microplus*." Journal of immunology **143**(4): 1346-1351.

Williams, R., W. Carlyle, et al. (1999). "The efficacy and economic benefits of Paracox®, alive attenuated anticoccidial vaccine, in commercial trials with standard broiler chickens in the United Kingdom." International Journal for Parasitology **29**(2): 341-355.

Willis, C., K. Fischer, et al. (2006). "Scabies mite inactivated serine protease paralogues are present both internally in the mite gut and externally in feces." American Journal of Tropical Medicine and Hygiene **75**(4): 683-687.

Wilson, K. and J. Walker (2010). Principles and techniques of biochemistry and molecular biology, Cambridge university press.

Wizemann, T. M., J. H. Heinrichs, et al. (2001). "Use of a whole genome approach to identify vaccine molecules affording protection against Streptococcus pneumoniae infection." Infection and Immunity **69**(3): 1593-1598.

Wood, H. P. (1917). The chicken mite: its life history and habits, US Dept. of Agriculture.

Wright, H. W., K. Bartley, et al. (2009). "The testing of antibodies raised against poultry red mite antigens in an in vitro feeding assay; preliminary screen for vaccine candidates." Experimental & applied acarology **48**(1-2): 81-91.

Wright, H. W., A. J. Nisbet, et al. (2011). "Identification of vaccine candidates against the Poultry Red Mite, *Dermanyssus gallinae*." from http://hdl.handle.net/1842/5703.

Wright, K. and I. Newell (1964). "Some Observations on the Fine Structure of the Midgut of the Mite Anystis sp." Annals of the Entomological Society of America **57**(6): 684-693.

Wu, J. S. and L. Luo (2006). "A protocol for dissecting *Drosophila melanogaster* brains for live imaging or immunostaining." Nature protocols **1**(4): 2110-2115.

Xu, Y., J. F. Bruno, et al. (2005). "Identification of novel tick salivary gland proteins for vaccine development." Biochemical and biophysical research communications **326**(4): 901-904.

Yan, J. P., J. H. Ko, et al. (2004). "Generation and characterization of a novel single-chain antibody fragment specific against human fibrin clots from phage display antibody library." Thrombosis research **114**(3): 205-211.

Zeman, P. (1988). "Surface skin lipids of birds—a proper host kairomone and feeding inducer in the poultry red mite, *Dermanyssus gallinae*." Experimental and Applied Acarology **5**(1): 163-173.

Zeman, P., V. Stika, et al. (1981). "Potential role of *Dermanyssus gallinae* De Geer, 1778 in the circulation of the agent of pullurosis-typhus in hens." Folia Parasitologica **29**(4): 371-374.

Zeman, P. and J. Zelezny (1985). "The susceptibility of the poultry red mite, *Dermanyssus gallinae* (De Geer, 1778), to some acaricides under laboratory conditions." Experimental & applied acarology **1**(1): 17-22.

Zemskaya, A. A. and A. A. Pchelkina (1967). "Gamasoid mites and Q fever. In: Markevich." Problemy Parazitologii: 258–259.

Zhang, Q., J. R. Ongus, et al. (2007). "Detection and localisation of picorna-like virus particles in tissues of *Varroa destructor*, an ectoparasite of the honey bee, *Apis mellifera*." Journal of invertebrate pathology **96**(2): 97-105.

Zhu, Y., S. Thangamani, et al. (2005). "The ancient origin of the complement system." The EMBO journal **24**(2): 382-394.