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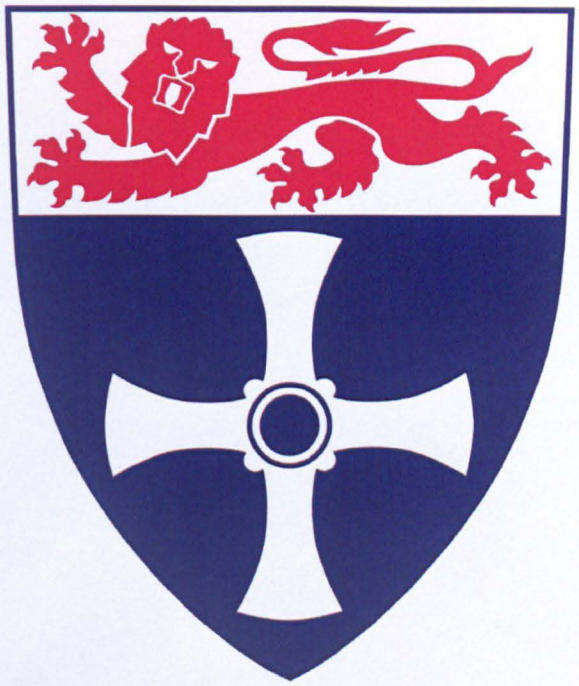

School of Agriculture, Food and Rural Development

PhD Thesis

Development of a vaccine against the poultry red mite (*Dermanyssus gallinae*)

Samuel Arkle

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ABSTRACT

The poultry red mite (PRM) (*Dermanyssus gallinae*) has been deemed the most deleterious ectoparasite in commercial housing systems for laying hens, affecting the cost of production, health and welfare. Present control of the PRM is hindered by the limited number of acaricides available, resistance by PRM to acaricides and the ability of the PRM to evade control. Therefore the aim of this thesis was to investigate the manipulation of the host immune system as a means of developing a vaccine to control the PRM. Two experiments were carried out to gain a better understanding of the relationships between natural PRM infestation, acaricide application, the immune response of laying hens and the consequences for egg production and welfare. A further two experiments were implemented to determine the effect of immunisation with PRM antigen extracts on the immune response of hens and subsequent survival and fecundity of PRM.

Experiments 1 and 2 evaluated the effect of estimated PRM populations on production parameters and the immune response of poultry across commercial laying sites. Both experiments showed significant correlations between PRM population and production parameters, specifically building temperature and hen mortality in Experiment 1 and 2 respectively. A significant reduction in PRM population following acaricide application was also observed. However, no significant relationship was found between PRM populations and either IgY or cytokine levels, although a significant negative relationship was observed between IgY and the cytokine IL-4. In both experiments, variability of data was high which may have contributed to the failure to show a relationship between PRM population and the immune response of poultry. Nevertheless, these experiments highlight the suitability of commercial egg laying systems for proliferation of PRM and the consequences for poultry.

Experiment 3 assessed the effect of immunisation with PRM antigen extracts on IgY and cytokine responses of pullets, as well as PRM survival and fecundity. There were 2 treatments: an antigen treatment which received PRM antigen extracts in Complete Freund's adjuvant (CFA), followed by two immunisations of antigen with Incomplete Freund's adjuvant (IFA) and a control treatment in which the PRM antigen was substituted for saline. Significantly higher IgY levels were observed in the antigen treatment, although an increase in IgY levels in the control treatment was also seen, resulting from non-specific antibody binding which was confirmed by western blotting. PCR performed on PRM DNA revealed that non-specific binding was a likely effect of homology between *Mycobacterium* present in both PRM and in CFA. Significantly higher IL-10 levels were seen in the antigen treatment, which was in turn thought to be responsible for the significant inhibition observed in IL-4 and IL-5. Survival and fecundity of PRM was not significantly affected by treatment.

Experiment 4 investigated the effect of immunisation of pullets with two different PRM antigen extracts, without the confounding effect of *Mycobacterium* in CFA. There were 2 control treatments receiving either PBS or IFA only, and two antigen treatments receiving IFA with either PBS or Urea-extracted PRM antigens. Levels of

IgY and IgM were not significantly different between antigen treatments, but these in turn were significantly higher than the controls. Western blotting showed several bands in the antigen treatments, which were not seen in controls. An *in vitro* feeding system revealed no significant difference between treatments for survival or fecundity of PRM, confirming that immunisation with PRM antigens did not elicit a protective host immune response.

In conclusion, this research programme has demonstrated that exposure to PRM antigens provoked an increase in immunoglobulin levels and changes to the relative expression of different cytokines in poultry. However, immunisation with PRM antigen extracts was not sufficient to cause a significant reduction in the survival or fecundity of PRM. Further research therefore is needed to identify a suitable antigen which elicits protection to laying hens from predation by the PRM.

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DECLARATION

To the best of my knowledge I declare that this thesis is entirely my own work and has not been accepted in any preceding application for a degree. All of the work was carried out by myself unless otherwise stated. All sources of information have been specifically acknowledged by means of referencing.

(Samuel Arkle)

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Chapter 1

Introduction

Modern poultry production uses fully integrated techniques that allow for production of a large number of eggs in a limited amount of space and over a relatively short period of time (Axtell and Arends, 1990). Current production systems include intensive cage and extensive floor-based systems, such as barn and free-range. All of these systems are controlled to a degree, with the former being subject to severe environmental restrictions and the latter being more representative of the birds' natural surroundings. As a result, the ecology of poultry parasites is tied to the synthetic environment in which they and the birds exist and changes in this environment which negatively effect parasites are likely to be detrimental to the birds themselves (Axtell and Arends, 1990). Since the facilities and techniques for modern poultry production are fundamentally the same throughout the world, excluding climatic and geographical variability, conditions for parasites are ideal, worldwide (Axtell and Arends, 1990).

At least 2,500 different species of mites from 40 different families are closely associated with birds, occupying all conceivable habitats on the bodies and nests of their hosts, with no avian species being free from parasitism by mites (Proctor and Owens, 2000). A number of mite species have evolved to inhabit poultry houses, of which the poultry red mite (*Dermanyssus gallinae*, De Geer, 1778), is considered to be the most profound.

It has been suggested that the poultry red mite is currently the most economically deleterious ectoparasite of laying hens in several countries (Chauve, 1998) and has been identified globally (Levot, 1991; Axtell, 1999). The poultry red mite is an obligatory haematophagous (blood-sucking) ectoparasite of both domestic and wild birds, although it has been known to engorge on a range of other species, including man (Bruneau *et al.*, 2001). The poultry red mite is referred to as a temporary parasite, since it is only found on the host when obtaining a blood-meal, with the majority of its lifecycle spent concealed in cracks and crevices of the house substructure. Subsequently, domestic poultry systems provide the poultry red mite with a wealth of potential hiding places, particularly in barn and free-range systems (Kilpinen, 2001). Therefore, the ban on cage systems within the EU proposed for

2012 is likely to indirectly reduce the welfare of hens due to the higher prevalence of the poultry red mite in extensive systems (Höglund *et al.*, 1995; Guy and Edwards, 2006).

The poultry red mite is largely seen as a problem affecting laying hens and to a lesser degree, broilers due to rapid turnover of meat birds (Kirkwood, 1967). The poultry red mite also exists as a threat in the spread of disease, since it can act as a vector for a number of pathogenic poultry infections such as *Salmonella* spp., spirochaetosis, chicken pox, Newcastle disease, fowl typhoid, fowl cholera, amongst others (Durden *et al.*, 1993; Chirico *et al.*, 2003; Moro *et al.*, 2005). However, the most profound effect of the poultry red mite is as an obligatory blood sucking parasite (Chauve, 1998). The feeding mite can cause irritation, restlessness and mild or severe anaemia, occasionally resulting in death. Subsequently this can reduce both egg production and quality, as well as compromising welfare (Urquhart *et al.*, 1996).

Under optimal conditions, the lifecycle of the poultry red mite can be completed within one week, thus rapidly establishing large populations (Soulsby, 1982). These, in conjunction with its ability to occupy small spaces makes control of the poultry red mite, typically performed using chemical sprays, extremely difficult (Bruneau *et al.*, 2001). In addition, resistance by the poultry red mite to the few registered chemicals available has been observed, which exacerbates the problem further (Beugnet *et al.*, 1997). It has recently been estimated that the cost of control for poultry red mite in the UK poultry industry alone is approximately £3.7 m per annum (Anon, 2003a). For these reasons, a range of alternative control methods have been proposed and have displayed varying degrees of success, including sorptive dusts, feeding deterrents, insect growth regulators and predatory insects (Chauve, 1998). However, one method which has shown promise for control of a number of haematophagous ectoparasite species is the use of vaccines, with particular success seen in the development of the tropical cattle tick (*Boophilus microplus*) Bm86 vaccine (Willadsen *et al.*, 1996; Pruett, 1999; Dalton and Mulcahy, 2001; Nisbet and Huntley, 2006).

The aim of this thesis therefore is to investigate the manipulation of the host immune system as a means of developing a vaccine to control the poultry red mite. In order for this to be achieved, an extensive review of literature concerning poultry red mite biology, current control methods and host-parasite immune relationships was undertaken. This was followed by a number of experiments evaluating the

relationship between poultry red mite populations, poultry production parameters and acaricide application, as well as the concurrent development of a series of immunological, proteomic and genetic laboratory techniques to determine the effects of both natural and artificial exposure to the poultry red mite. Finally, two experiments were undertaken to evaluate the protective capacity of immunisation with poultry red mite antigens in pullets.

Chapter 2

Literature Review

2.1 Classification of *Dermanyssus gallinae*

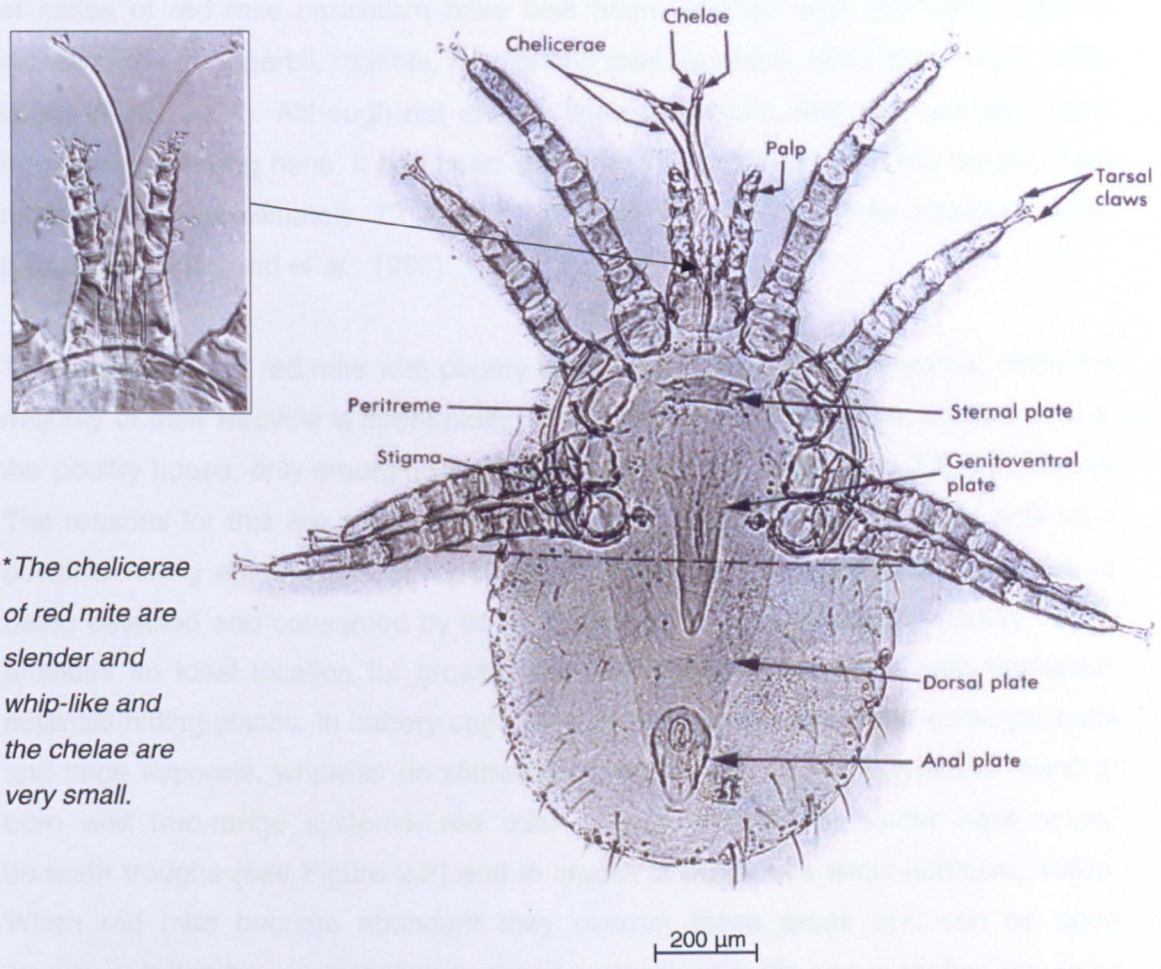
Dermanyssus gallinae is an arthropod belonging to the class Arachnida, subclass Acari, order Acarina, suborder Mesostigmata and family Dermanyssidae (Roberts and Janovy, 2000). Typical features of the members of the class Arachnida are that they have four pairs of legs, their bodies consist of a cephalo-thorax and abdomen, but the head is absent. Members of the order Acarina can be defined as arachnids with mouthparts located on the capitulum. Acarina also have two genital openings and claws on the end of the legs (Roberts and Janovy, 2000). Mesostigmata mites typically have two claws on the end of each leg, as well as no genital suckers, one pair of stigma and a body divided into anterior (gnathosoma) and posterior (idiosoma) sections (Moro *et al.*, 2005). Characterisation of *Dermanyssus* mites is based around the large dorsal shield and structure of the mouthparts (Moss, 1968), features which are described in detail below.

2.2 Morphology

The identification of *Dermanyssus gallinae* requires caution since it is closely related to other mite species, sharing many morphological characteristics, in particular with the northern fowl mite *(Ornithonyssus sylviarum)*, which also parasitizes poultry (Bruneau *et al.*, 2002). Both these mite species have stigmata (respiratory pores) in the centre of their bodies. A single stigma lies between the third and fourth coxae (leg joints) on each side of the body and is connected to a peritreme (supple membrane-like structure). The coxae (leg joints) are evenly spaced, but crowded into the anterior half of the body, the tarsi (the terminal segments of the leg) are equipped with minute claws (empodia) and the ventrum is armoured with plates (Bowman, 1995). The empodia are not synonymous with claws they are usually an additional projection between the claws. However, these two mite species can be distinguished through careful examination. *O. sylviarum* has a claw-like chelicerae (piercing mouthpart), whereas the chelicerae of *D. gallinae* are long and whip-like (see Figure 2.1) (Bruneau *et al.*, 2002). The chelae (scissor-like structures on the end of the chelicerae) of *D. gallinae* are small in comparison to those of the Northern fowl mite. Also, *D. gallinae* has a single dorsal plate and sternal plate, whereas the posterior

regions of the dorsal and sternal plates of *O. sylviarum* are thinner and the genitoventral plate is drop-like (Bruneau *et al.*, 2002).

Figure 2.1. *Ornithonyssus sylviarum (ventral view), a close relation of Dermanyssus gallinae (Left) (Bowman, 1995)*



**The chelicerae of red mite are slender and whip-like and the chelae are very small.*

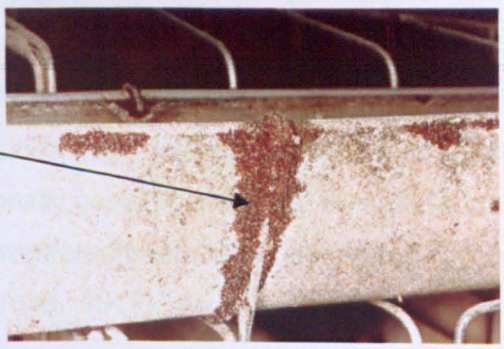
The adult female poultry red mite measures approximately 1 mm in length and 0.4 mm in width and varies in colour from grey to red depending on engorgement (Kaufman, 1996). The dorsal shield of the red mite, does not quite reach the posterior end of the body and its most posterior margin is truncated. The setae (scapular hairs) on the dorsal shield are smaller than those on the skin around the dorsal plate. Finally, the anus is on the posterior half of the anal plate, whereas that of *O. sylviarum* is on the anterior half of this plate (Soulsby, 1982).

2.3 Life cycle of the poultry red mite

The poultry red mite has been known to infest a range of avian species including the fowl, pigeon, canary, duck, turkey, pheasant, sparrow and owl (De Lope and Moller, 1993; Gicik *et al.,* 1999; Pampiglione *et al.,* 2001; Romaniuk and Owczarzak-Podziemska, 2002). However, their relationship is not exclusive to birds, as a number of cases of red mite parasitism have also been reported with alternative species including the dog, gerbil, rodents, rabbits and man (Soulsby, 1982; Bakr *et al.,* 1995; Lucky *et al.,* 2001). Although not entirely species-specific, red mite are seen most frequently on laying hens. It has been suggested that this is due to the lengthy flock turnover of approximately 72 weeks, allowing red mite time to establish large populations (Höglund *et al.,* 1995).

The relationship of red mite with poultry is referred to as a temporary one, since the majority of their lifecycle is spent hiding in cracks and crevices in the substructure of the poultry house, only emerging at night to feed on the resting bird (DEFRA, 2001). The reasons for this are suggested to be that exposure to direct sunlight acts as a powerful killing agent, causing rapid dehydration, and it also eliminates the risk of being detected and consumed by birds (Wood, 1917). Therefore, the poultry house provides an ideal location for growth and proliferation of red mite, with numerous potential hiding places. In battery cage systems, the mites hide under conveyer belts and cage supports, whereas on slatted floor units, such as those typically found in barn and free-range systems, red mite conceal themselves under nest boxes, beneath troughs (see Figure 2.2) and in cracks in the house walls (Chauve, 1998). When red mite become abundant they overrun these areas and can be seen anywhere in the house, including exposed surfaces on walls and in roofing materials (Wood, 1917).

Figure 2.2 *Poultry red mite infestation on water trough (http://www.icb.usp.br/~marcelcp/Dermanyssus.htm)*



2.3.1 Feeding habits

Several investigations into red mite feeding habits have been conducted and have reached the general consensus that red mite will consume a blood-meal lasting 0.5 to 2 hours, at intervals of 1-4 days, only feeding at night, with environmental conditions and host availability being the limiting factors (Chauve, 1998; DEFRA, 2001; Kilpinen, 2001). In heavy infestations, the red mite appears to be less selective about the conditions in which they will feed and have been observed to feed in daylight (Nakamae *et al.,* 1997). During engorgement, red mite are predominantly located on the underside of the birds wing, presumably due to a more suitable temperature, humidity and darkness (Anon., 2003b), although they can also be seen to feed on the breast and lower leg of the bird (Axtell and Arends, 1990).

2.3.2 Mating behaviour

After feeding the red mite must return to a hiding place for mating and egg deposition, which generally occurs 12 hours post blood consumption. It appears that there is little evidence of migration of red mite between hiding compartments when potential blood-meals are abundant, with mites also preferring the nesting areas of hens, therefore unequal mite distribution exists within the poultry house (Nordenfors *et al.,* 2001).

Mating behaviour of the red mite involves sperm transfer which is referred to as podospermal, where the males chelicerae are modified in the tube like spermadactyls, allowing the transfer of sperm from the males genital opening into sperm induction pores located on either side of the female's body (podosoma) near the posterior of the third coxae. The appearance of spermatozoa isolated during mating are irregularly rounded non-polar cells, with granular cell membranes, agranular cytoplasm and large, densely stained nuclei. Mating behaviour requires 14 min to 1 hour, in which one or both sperm induction pores may be used (Amano and Chant, 1978). It is thought that males are probably incapable of successfully inseminating more than three or four females in the same number of days (Hutcheson and Oliver, 1988).

2.3.3 Poultry red mite- fertilisation/egg laying

Fertilisation and oviposition (egg laying) usually takes place away from the host, although it may occasionally occur on the host. Female red mite have the capacity to feed and deposit eggs repeatedly with only one mating, but a blood-meal is required for each deposition (Wood, 1917). An average lay will result in the production of

between 2-7 eggs, laid over a period of several days (Soulsby, 1982; Nordenfors *et al.,* 1999; Bruneau *et al.,* 2001). Red mite will feed and deposit eggs in this manner several times, with each female having the capacity to lay approximately 30 eggs in its lifetime. The eggs themselves are small (400 x 270 µm), oval, smooth and pearly white (Chauve, 1998). Hatching generally occurs within 48-72 hours of being laid in summer conditions with optimum temperatures between 28-30°C and also appropriate relative humidity of around 60-90 % (Höglund *et al.,* 1995; Bucher, 1998; Chauve, 1998). Egg maturation, hatching of eggs and protonymphal maturation appears to be staggered, not synchronised in waves (Nordenfors *et al.,* 1999).

2.3.4 Poultry red mite-larva

On hatching the larvae emerges and has no requirement to feed. The young larvae are white with six legs and move around slowly, unlike other stages which are very active. The length and width of the larval body is approximately the same as the eggs. These larvae moult within 24-48 hours after hatching at a mean ambient temperature of 23°C (Wood, 1917).

2.3.5 Poultry red mite-protonymph

The young nymph backs out of its larval skin to emerge as an eight-legged protonymph. After a few hours of resting, in order to give time for the body integument to harden, the nymph becomes very active and will feed on nesting birds, mainly at night. The protonymph is slightly darker than the larva, being slightly brown in colour. The protonymph measures approximately 568 µm in length (abdomen only), a width and thickness of 338.4 and 266 µm, respectively (Wood, 1917).

2.3.6 Poultry red mite-deutonymph

After a further 24-48 hours and an additional blood-meal, the protonymph moults into a second nymphal stage called the deutonymph. As with the preceding nymphal stage, a period of hardening of the integument is required before feeding. Soon after the deutonymph becomes active it obtains a further blood-meal. The dimensions of the deutonymph before feeding are 538.2 µm in length, by a width of 297.9 µm. After feeding, the mite increases to a size of 751.9 µm in length by 461.4 µm in width (Wood, 1917). After an additional 24-48 hours, the deutonymph in turn moults into an adult red mite.

2.3.7 Poultry red mite-adult

As soon as the adult red mite crawls out of the nymphal skin they are ready to reproduce. Fertilisation can take place before or after feeding. Immediately after moulting the females will feed, but males prefer to mate before feeding, something which they are capable of doing several times in their lifespan (Wood, 1917). The complete life cycle of the red mite (egg to egg) can be completed within seven days if conditions are optimum, therefore rapidly establishing large populations in poultry houses (Soulsby, 1982).

It has been well documented that the adult red mite can survive for long periods without feeding. This was initially demonstrated by Wood (1917), where red mite survived for up to 9 months without a blood-meal. Further research supports these findings (Tucci and Guimaraes, 1998). Therefore, it is possible for a reservoir red mite population to persist in an uninhabited building to eventually re-infest a new poultry generation (Urquhart *et al.*, 1996).

2.3.8 Host evasion strategies

In order to obtain a successful blood-meal, it is essential that the red mite has some capacity to resist challenges from a variety of host mechanisms, including pain and itch responses, haemostasis and, most importantly, an immune defence (Schoeler and Wikel, 2001). The mechanisms which red mite employ to overcome these defences have not been specifically investigated. However, a series of common responses exist between arthropods and many of these are related to the pharmacological properties of their saliva (Schoeler and Wikel, 2001).

Host haemostatic defences include coagulation pathways, platelet aggregation and vasoconstriction. In response to these defences, blood-feeding insects have developed countermeasures in the form of salivary molecules that inhibit components of the coagulation pathways, block platelet aggregation and act as vasodilators (Schoeler and Wikel, 2001). One signal which alerts an animal to parasite presence is histamine, a compound which causes the sensation of itch. In order to oppose this alert blood-feeding arthropods, e.g. the kissing bug (*Rhodnius prolixus*) produce histamine-binding proteins in the salivary glands, thus rendering histamine ineffective (Schoeler and Wikel, 2001).

In addition, many arthropods are capable of modulating the host's immune defences by limiting both humoral and cellular systems. An example of this is the salivary-

gland extract of the blackfly (*Simulium villatum*), which when injected intraperitoneally inhibits the *in vitro* proliferation of both T and B-lymphocytes (Cross *et al.*, 1994).

However, since saliva is such an integral part of the success of haematophagous arthropods, it also poses a potential target for immunological control. In order to develop anti-arthropod vaccines, the immunological interactions occurring at the arthropod-host interface must be characterised in terms of the arthropod salivary constituents which are responsible for immunomodulation of hosts (Schoeler and Wikel, 2001). Manipulation of the immune system and vaccine development is discussed in more detail in Sections 2.11 and 2.13.

2.3.9 Seasonal variation

Poultry red mite have been observed to reproduce with greatest success at optimum temperatures ranging between 25-37°C, with lethal temperatures at extremes of below -20°C and above 45°C (Nordenfors *et al.*, 1999). It is not surprising then that poultry farmers suggest that greater populations of red mite can be seen in summer rather than winter months (Nordenfors *et al.*, 1999), these temperature ranges are typical of the British climate, warm in summer and occasionally reaching extremely low temperatures in winter months. Reports have shown that temperatures in modern egg production units are relatively stable from October to May, whereas daily fluctuations of approximately 10°C are apparent between May to August. This creates characteristic seasonal fluctuations in mite populations (Nordenfors and Höglund, 2000).

2.4 Control of the poultry red mite

Control of the poultry red mite is particularly problematic for a number of reasons associated with its feeding habits and innate mite biology. Also the number of pesticides registered for application in poultry houses is relatively low for reasons including, development of acaricide resistance, chemical and antibiotic residues in food and undesirable environmental effects (Dalton and Mulcahy, 2001). In addition, the predilection of the red mite to small cracks and crevices, their ability to survive for extended periods without taking a blood-meal and their prolific reproduction capacity and short life cycle make eradication very challenging (Kilpinen, 2001).

2.4.1 Chemical control

At present, the primary method of control of the poultry red mite is by the use of chemical sprays. In recent years there have been over 35 potential compounds identified or proposed for the control of red mite, including organochlorines, organophosphates, pyrethroids, carbamates, amitraz and endectocides (Chauve, 1998). However, the vast majority of these chemicals are unsuitable due to worries over food safety and their accumulation in the human food chain (Sam-Sun *et al.,* 2002). Environmental concerns have also been raised, such as the potential to poison wildlife and risk of chemicals leaching into watercourses. Alternatively, a number of chemicals which appeared to be efficient at controlling red mite in theory are actually inadequate in practice as they fail to be toxic to the mite (Chauve, 1998). Presently pyrethroids are the principal family of acaricides used to control the red mite in commercial poultry farming, preferred due to their low toxicity and minimal risk of egg contamination. Organophosphates are used to a much lesser extent as they have greater toxicity and contamination risks (Beugnet *et al.,* 1997). Table 2.1 provides a summary of the current chemicals recommended for treatment of the poultry red mite.

Since the red mite spends the vast majority of its time away from the hen, the ideal insecticide would be one with the capacity to penetrate deep into the crevices which conceal the mite. The insecticide should remain active for as long as possible so the mite is exposed to it on emergence, although it must have limited toxicity to the bird itself. Hamscher *et al.* (2003) suggested that insecticide application must be repeated twice within two weeks in order to be effective at breaking the 7-10 day life cycle.

Within Europe, regulations on the application of chemical acaricides vary between countries. In Germany for example, the chemical carbamate propoxur (2-isopropoxyphenyl-*N*-methylcarbamate) is licensed to be used as an insecticide within poultry houses and is sprayed in the presence of the live bird. The European Union and German government specify a maximum legal residue level within hen's egg of 50 $\mu g\ kg^{-1}$, as levels exceeding this are considered dangerous to human health. A German study by Hamscher *et al.* (2003), illustrated the potential risk of using such sprays on commercial poultry units. On several occasions levels of propoxur residues on eggs surpassed the legal requirement, reaching levels of up to 101 $\mu g\ kg^{-1}$. This highlights the difficulty in avoiding contamination of hen's eggs during spraying. Nonetheless, the same authors suggested that legal targets for spraying are more easily attained in free-range systems than in to battery systems, as birds have more

space to hide during application. Unfortunately, acaricide spraying at present is accepted as the most efficient way to control red mite, but it does undesirably compromise both animal and human health (Hamscher *et al.*, 2003). It should also be noted that propoxur is not currently licensed for the control of red mite in the UK.

Table 2.1. *Products currently recommended for red mite control in the UK. (ADAS, 2006)*

Product name	Active Ingredient	Comments
INSECTICIDAL		
Barricade (Sorex)	Synthetic pyrethroid (cypermethrin)	Specific approval fro red mite. Currently the only product licensed for application to the birds. Manufacturers report 95 % control achieved for 73 days.
Littac (Sorex)	Pyrethroid (alphacypermethrin)	Specific approval for red mite. Applied as a course spray to buildings containing poultry. Not to be sprayed on birds or eggs. 2-3 months residual activity.
Teremid (Scotmas Agriculture) (Plus other brand names).	Permetherin and Piperonyl Butoxide	Specific approval for red mite. Applied as a residual spray to buildings.
Actellic	Organophosphate (pirimiphos-methyl)	Licensed for 'mite' control in animal husbandry. Apply as a dilute spray to the fabric of the building. Not to be applied directly to birds and eggs must be collected before application.
Ficam W (Aventis)	Carbamate (Bendiocarb)	Apply as a dilute spray to the fabric of the building. Not to be applied directly to birds and eggs must be collected before application.
NON-INSECTICIDAL		
Decimite (Sorex) **Optimite**	Silica-based	Specific to red mite. Pesticide-free, working by physically inhibiting mite movement. Manufacturers Sorex, recommended its use as part of a rotational control programme, together with Littac.
Insecto-sec (BFREPA) **Fossil shield (Integra)** **Mitex (Agil)**	Silica-based Diatomaceous earth Diatomaceous earth	Pesticide Safety Directorate (PSD) approved. Immobilising and desiccating effects on poultry red mite. Imported desiccant – requires electrostatic applicator. Imported desiccant – requires electrostatic applicator.
Novel product (Uff Hygiene) **Breck-a-sole (Ecospray)**	Citrus extracts Garlic prill	Repellent effects Repellent and killing effect

It is for these reasons that in many countries the use of a range of chemicals to control red mite are banned, which makes the commercial situation more complex. Sweden is one particular example where presently there are no registered acaricide

sprays available in the control of ectoparasites (Chirico and Tauson, 2002). Therefore poultry farmers rely on the development of alternative control methods.

One possible substitute for the use of acaricide sprays which has been investigated in Sweden is the use of plastic strips impregnated with insecticide, such as the synthetic pyrethroid or permethrin. These strips have a low toxicity to poultry and man, require only low level concentrations and have a residual activity which is within governmental guidelines (Nordenfors *et al.*, 2001). In one particular study across several farms, plastic strips were mounted either within the housing systems, allowing birds to rub against them and the acaricide to rub off on their plumage (Treatment A), or alternatively they were attached out of the birds reach, either on perches or egg belts (Treatment B). Results showed that permethrin impregnated traps significantly reduced ($P<0.001$) red mite populations by 53 and 39 % (Treatments A and B, respectively), with a most profound reduction of 92 % with traps attached to egg-belt lids after Treatment B. However, these control levels were not adequate to achieve complete eradication, allowing red mite populations to rapidly re-establish and cause irritation to hens (Nordenfors *et al.*, 2001).

Research has also been conducted on the administration of chemicals via intramuscular or intraperitoneal injection. However, both of these methods have limited use as they are only effective for short periods and therefore require either repeated doses or administration at high levels close to toxicity in order to be effective. The provision of effective doses are expensive both in terms of labour and materials, so widespread use is unlikely (Ash and Oliver, 1989).

2.4.2 Resistance

In situations where control using acaricides does not result in a significant reduction in red mite populations, there is the risk of the development of acaricide resistance (Chirico and Tauson, 2002). Not only does this occur if insufficient red mite numbers are killed, but also if there is persistent, long term use of the same chemical. Chauve (1998) reported the suspicion of resistance by red mite to DDT and organophosphates, whilst other researchers have suggested resistance to pyrethroids in the Czeck Republic, Italy, Switzerland and more recently in France. However, many of these cases of resistance were not statistically validated, since it is often unclear whether treatment failures were due to resistance or simply insufficient acaricide application (Chauve, 1998).

A report by Beugnet *et al.* (1997) investigated the possible resistance of red mite in France to an organophosphate acaricide (dichlorvos) and pyrethroid (permethrin) using *in vitro* tests. In this trial, red mite were collected from five heavily infested farms and one farm which had no problem with red mite and therefore was used as a negative control. Red mite were then incubated on Whatman filter paper previously impregnated with one of the two chemicals. Mortality rates of red mite were determined after this and regression curves were drawn to enable the calculation of an estimated 50 % lethal concentration (LC50) i.e. the concentration at which half of the red mite population would be killed. The LC50 of the red mite from tested farms was then compared against the LC50 of red mite from the control farm and a resistance factor (RF) determined. The World Health Organization (Bramer, 1992) advised that arthropods were considered susceptible to treatment if the RF was below 3, if RF was between 3-5 then resistant genes are said to be present and if RF was above 5 resistance was apparent. Beugnet *et al.* (1997) observed that red mite were not resistant to organophosphates, with RF's were in the range 1.7-2.4, but were highly resistant to pyrethroid in all cases, with RF's between 8 and 40.

Whilst it is important to avoid situations of acaricide resistance, the current lack of new acaricides coming onto the market makes this difficult (Beugnet *et al.*, 1997). One suggestion is to employ a strategy of chemical rotation as soon as resistance is suspected. Three groups of chemicals are suggested for use in this rotation, organophosphates/carbamates, pyrethroids and endectocides. In practicing rotation the development of resistant genes, those allowing mites to remain unaffected by chemical treatment, within mite populations are limited and susceptibility genes, those permitting acaricide penetration, are promoted (Beugnet *et al.*, 1997).

(i) Mode of resistance

The mode by which a number of acaricides, such as pyrethroids and DDT eradicate their targets is by selectively modifying the activity of nerve membrane sodium channels. This is achieved by acting on an insect's nervous system by slowing the action potential decay. This initiates repetitive discharge in sensory and motor axons leading to convulsive activity, eventually resulting in paralysis and death (Morin *et al.*, 2002).

Pyrethroids in the past have been able to combine high insecticidal activity with low mammalian toxicity and so have been popular, effective acaricides. However, their persistent use has produced resistance in many insect species (Höglund *et al.*,

1995). In response to this increasing resistance, pyrethroids were mixed with organophosphates (OP's) which proved to be very effective at controlling red mite, despite high resistance to pyrethroids and OP's when applied alone (Höglund *et al.,* 1995). This reflected OP inhibition by parasite esterases, a group of hydrolytic enzymes, which among other functions cause the enhanced detoxification of pyrethroids. Detoxification of pyrethroids by esterases is one of the primary modes by which resistance occurs. However, even these combinations of pyrethroids and OP's encountered resistance after intensive application. It was discovered that further resistance involved decreased sensitivity at the sodium channel target site and is termed 'Knockdown resistance' or kdr (Soderlund and Knipple, 2003). Kdr resistance was originally reported in the 1950's and associated with the house fly (*Musca domestica* L., Busvine, 1951). However, more recently kdr has been reported in a number of other agricultural pests and disease vectors including mosquitoes (*Anopheles stephensi*), cockroaches (*Blattella germanica*) and moths (*Plutella xylostella*) (Soderlund and Knipple, 2003). Kdr resistance is brought about by mutations in the structure of two domains associated with sodium channels, through changes to specific amino acids in parasites. This brings about between a 10-30 fold increase in resistance (Morin *et al.,* 2002). Later, an even greater resistance was observed when, using the same mutations to sodium channels, but at different locations, resistance of up to 500-fold was observed. This type of resistance was designated super-kdr (Morin *et al.,* 2002; Soderlund and Knipple, 2003).

There are a number of alternative mechanisms which have been seen to mediate the occurrence of resistance, which generally involve gene mutations. One type of resistance observed is due to mutations in the sodium channel gene which confer target site insensitivity to the neurotoxic effect of pyrethroids. Similarly mutations in acetylcholinesterase, a cleavage enzyme for the neurotransmitter acetylcholine, have also been seen to confer target site insensitivity to OP's. In the fruit fly (*Drosophila melanogaster*), four point mutations in the acetylcholinesterase gene encode amino acid substitutions that render the enzyme insensitive to OP's (Morin *et al.,* 2002).

A number of metabolic mechanisms exist which also bring about resistance to acaricides. The most common is include detoxification of pyrethroids by overexpressed cytochrome P450 enzymes, a group of enzymes produced for the metabolism of toxic hydrocarbons (Lui and Scott, 1998). This was observed in research carried out on the house fly in which resistant strains were seen to transcribe 10 times the amount of P450 in comparison to non-resistant strains (Lui

and Scott, 1998). Alternatively, carboxylesterases, manufactured for the hydrolysis of endogenous and foreign compounds in parasites, are often involved in both pyrethroid and OP resistance through gene amplification and consequently overexpression or through mutations specifically increase direct hydrolytic activity to specific insecticides (Jamroz *et al.*, 2000).

2.4.3 Sorptive dust

Sorptive dusts are a group of compounds which are selective in the control of insects. These chemicals are very fine (3-9 µm), free flowing powders with a capacity to be highly absorptive. They are inert chemicals, which are harmless to animals and that do not decompose with age. Therefore arthropods are unlikely to develop physiological resistance against them. The method by which sorptive dust kills arthropods is by the absorption of the waterproof layer of lipid from the epicuticle, resulting in death by dehydration (Kirkwood, 1974). In one experiment a 40 % reduction in red mite populations within 1 hour was observed with complete eradication within 2 hours (Kirkwood, 1974). Field trials did not display complete eradication, although considerable reductions in mite populations were observed (Kirkwood, 1974). However, there are problems with application as it will only lie on horizontal surfaces unless applied using an electrostatic charge.

2.4.4 Feeding deterrence

In a number of cases the feeding patterns of red mite have been completely disrupted by the use of natural plant derived substances. This is particularly true of bay oils and citronella, which were tested in an *in vitro* feeding system to act as a repellent against the northern fowl mite (*Ornithonyssus sylviarum*), a closely related species to the red mite. However, the life habits of the two mites differ in that the northern fowl mite will spend a larger proportion of its time on the host, giving greater exposure time and making treatment more straightforward (Chauve, 1998; Nordenfors *et al.*, 2001).

Recent investigations reviewing the activity of a wide range of plant essential oils showed that there were several which could be potentially useful as control agents against red mite (Kim *et al.*, 2004). In a filter paper contact bioassay, 100 % mortality at 0.07 mg cm^2 was observed for bay, cade, cinnamon, clove bud, coriander, horseradish, lime dis 5F, mustard, pennyroyal, pimento berry, spearmint, thyme red and thyme white oils. In fumigation tests with adult red mite at 0.28 mg cm^2, cade, clove bud, coriander, horseradish and mustard oils were more effective in closed

containers than in open ones, suggesting that the effect of these essential oils is largely due to action in their vapour phase and attack via the respiratory system of the mite. Irrespective of the fact that this concealed testing is not wholly representative of field conditions, some plant essential oils might be useful as fumigants for red mite and may warrant further investigation as potential control agents (Kim *et al.*, 2004).

2.4.5 Insect growth regulators (IGR)

Studies conducted over several decades have established that peptide hormones and transmitters are key molecules in the regulation of development, complex behaviours, reproduction and other physiological processes in insects (Isaac *et al.*, 2007). IGR's are compounds that disrupt the normal development of insects by mimicking the action of these peptide hormones by interfering with hormone-regulated processes (Collins, 2006). They have been used in a variety of practical applications, largely to control storage-insects, but have been seen to be effective against a range of insects including flies, fleas and cockroaches (Chauve, 1998). Examples of IGR's include juvenile hormone mimics, chitin-synthesis inhibitors (triflumuron) and angiotensin-converting enzyme (ACE) inhibitors. Chauve (1998) referred to unpublished data where IGR's were tested on colonies of red mite and were seen to reduce mite populations. Previous research into the potential of IGR's in the control of the house dust mite (*Dermatophagoides farinae*) showed promise, particularly with the use of juvenile hormone mimics (methoprene) which work on immature stages preventing maturation (Dowling *et al.*, 1990).

2.4.6 Predatory insects

Occasional instances of other species of insect, including ants and spiders, predating upon mites have been reported (Wood, 1917). One such incidence in Sao Paulo, Brazil reported that earwigs (*Dermaptera labiidae*) were observed to consume red mite on chicken rearing units. The earwig was observed to be spread in chicken litter and farmers attributed low levels of infestation of hens by red mite to the presence of this predator. In response to these observations, a large number of earwigs were reared under laboratory conditions at a low cost with the use of cat food and dry bird feed as a source of nutrition and used as a control method (Costa *et al.*, 1994). The lesser mealworm beetle (*Alphitobius diaperinus*) has also been observed to occasionally have a role in disposal of the red mite in much the same fashion (Chauve, 1998).

2.4.7 Building design

It has been suggested that in order to prevent the establishment of red mite populations there is a need to "design out" poultry houses that promote red mite proliferation by providing refuges which are found in traditional barn and free-range systems (Drakley and Walker, 2002). This can be achieved by modifying the design of feeders and nestboxes in new buildings, or simply plugging folds and joints using silicon sealant in existing buildings (Drakley and Walker, 2002). Conversely, it is considered that it is easier to meet legal targets for residual chemical levels in eggs in free-range systems in comparison to battery cages, since in free-range conditions birds have a wider variety of places to hide during acaricide application (Hamscher *et al.*, 2003). The materials used in the design of new buildings or when upgrading existing facilities should also be considered. Bucher (1998) found that red mite showed increased longevity and greater proliferation of red mite when housed on smooth surfaces such as rubber, plastic and glass in comparison with wood, metal, stone and clay.

2.4.8 Recommended actions for red mite control (DEFRA, 2001)

A review of current research was published by DEFRA (2001) as a means of formulation a recommended best practice strategy to reduce and hopefully eradicate red mite populations in commercial laying hen systems (Table 2.2).

Table 2.2 *Current 'best practice' strategy for red mite control (DEFRA, 2001)*

Step	Action
1	Once the house is depleted and all manure has been removed, thorough cleaning using a power washer or steam-cleaner must be carried out. As many of the internal fittings as possible should be removed to facilitate good cleaning.
2	Consideration should be given to cleaning the outside of the houses as it is known that red mite are naturally found in birds' nests in the UK and will migrate in search of a house when the poultry house is empty.
3	Ensure that any obvious refuges are removed or sealed (especially in the areas surrounding the nest boxes and feeders).
4	When the house is dry, it should be sprayed with an approved acaricide, using a flat-fan spray for walls and floors, and a crack-and-crevice tool for application to small harbourages. Product use should be on a cyclical basis to reduce the risk of the development of resistance.
5	Birds should be bought from breeder flocks that can be shown to be free from red mite and care should be taken to ensure transport and staff are not carrying any parasites in small numbers from recent exposure at other sites.
6	Records of routine weekly monitoring of all houses should be kept in order to trigger spot treatments where necessary in areas where mites are found or rational treatment with other products. Treatment details must also be recorded at all times to monitor use and prevent extensive, prolonged use of any one product.

2.5 Transmission of red mite

It is suggested that the primary way in which red mite are transmitted is via transportation with inanimate objects such as egg cases and trays, as well as passive transmission by personnel handling the poultry (Axtell and Arends, 1990). Therefore, in order to minimize the transmission risks between premises of laying birds it is important to implement a strict control strategy for replacement pullets. When purchasing new stock, it should a requirement that birds are free from all ectoparasite infestations (Höglund *et al.*, 1995). However it has also been observed that red mite will happily infest a range of other vertebrates that may be found in the hen house, such as rodents and wild birds. Consequently, irrespective of the comprehensiveness of control, there is always an apparent risk of infestation (Bakr *et al.*, 1995).

2.6 Pathogenicity

It is widely accepted that red mite can be a potential vector of several avian diseases, both bacterial and viral. The first report of the red mite acting as a vector was for the St Louis encephalitis virus (Smith *et al.,* 1946). Since then, a number of additional poultry pathogens have been linked with the mite, including *Salmonella* spp., spirochaetosis, chicken pox, Newcastle disease, fowl typhoid and fowl cholera, as well as a number of other livestock diseases (e.g. eastern equine encephalomyelitis virus) (Moro *et al.,* 2005). However, red mite is considered most importantly as a direct pest through its capacity as an obligatory blood-sucking parasite (Höglund *et al.,* 1995; Nordenfors *et al.,* 1999; Chauve, 1998; Bruneau *et al.,* 2001).

In order to determine the potential for red mite pathogenicity, a study by Durden *et al.* (1993) artificially induced eastern equine encephalomyelitis virus (EEE) in poultry using red mite as a vector. Red mite tested positive for EEE after engorging on blood from poultry which had previously been inoculated with EEE. The virus remained detectable by plaque assay in red mite samples for 30 days after the initial infectious blood-meal. However, virus was not observed in progeny of virus-exposed female red mite. Virus-exposed mites were then allowed to feed on naive chickens at 3, 7, 11, 15 and 30 days. EEE transmission to chickens was seen at both 3 (one transmission in four trials) and 7 days (one transmission in four trials), but not after this. These findings provide some degree of reassurance that red mite are not potent transmitters of EEE, especially between different red mite stages. Nonetheless, some red mite were capable of spreading the virus up to a week after exposure, so caution is still required. Little other research has been conducted on the capacity of red mite as a vector of disease. However, care must be taken when attributing vectoral capacity of red mite as it is possible that pathogens isolated from mites are present only in the blood-meal as a direct result of feeding and, in fact, transmission does not occur (Chirico *et al.,* 2003).

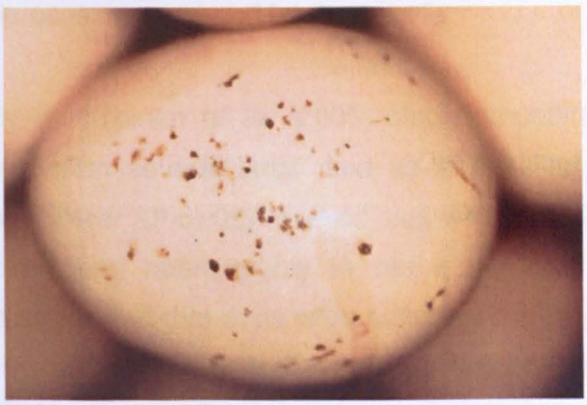
2.7 Effect on poultry

The poultry red mite has been suggested as the most important ectoparasite affecting egg laying hens in several countries (Chauve, 1998). To a lesser degree, it also affects breeding and rearing units, in both laying and broiler production (Chauve, 1998). When obtaining a blood-meal from their host, feeding nymphs and adults cause irritation, restlessness and debilitation through loss of blood and stress.

Consequently this leads to a reduced rate of bird growth, decreased egg production, poor shell integrity, blood staining (Figure 2.3) and reduced egg size (Chauve, 1998).

One such example of these debilitating capabilities was seen on a fully automated cage unit in Poland (Wojcik *et al.,* 2000). Here birds showed all the typical symptoms of red mite infestation including a decrease in body weight, anaemia and exhaustion. Mortality of birds rose from 1 to 4 % and egg production was at a level of 92 % in un-infested farms compared to 81 % on parasitized units (Wojcik *et al.,* 2000). A similar case was seen in France on a 60,000 hen cage unit, where a significant decrease ($P<0.001$) in egg production was recorded (85 %) and an increase in hen mortality of 52 % (Cosoroaba, 2001).

Figure 2.3 *Blood spotting occurs as a result of eggs rolling over and squashing* D. gallinae *on the shell surface*



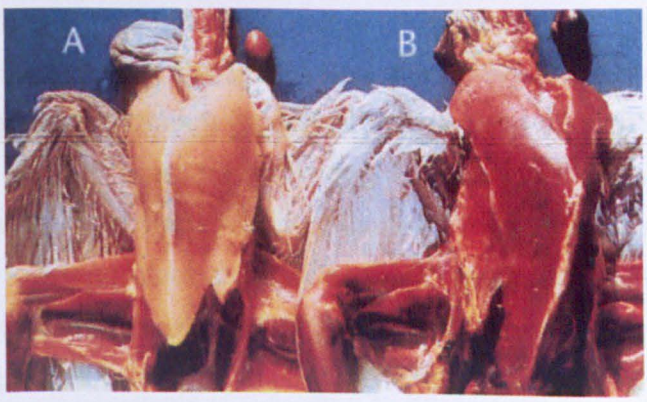
Red mite have also been observed to affect the behaviour of birds, with red mite infestation leading to increases in stereotypical behaviours including feather pecking self-grooming, head scratching behaviours and an overall reduction in welfare as well as a significant decrease ($P<0.01$) in bodyweight of parasitized birds (Kilpinen, 1999; Kilpinen *et al.* (2005).

In heavy mite infestations severe and occasionally fatal anaemia can ensue (Figure 2.4) (Soulsby, 1982; Urquhart *et al.,* 1996; Kaufman, 1996; Bruneau *et al.,* 2002). However, only a limited number of studies have examined the changes in blood composition of infested birds which may result in anaemia. A paper by Kirkwood (1967) describes observations on the erythrocyte values of poultry exposed to infestations of red mite to evaluate the parasitic impact. In the investigation, both 14-week-old and 2-year old birds were maintained in small cages. A minimum of 10,000 red mite per bird were sustained, although red mite number reached as high as

165,000 per young chick and 956,000 per adult. Birds were periodically bled from the brachial vein and the number of erythrocytes determined. Several birds died within 16 hours from the onset of the experiment due to extensive anaemia, with all birds showing depletion in erythrocyte numbers. However, once marginally anaemic birds had been returned to mite-free cages they were seen to recover rapidly. There were several limitations to this research. Firstly, exposure of birds to red mite was vast and sudden, whereas under field conditions exposure is likely to be much more gradual and allows physiological compensation for erythrocyte numbers. These findings are substantiated by Kilpinen (1999) who also observed that the rate of increase of a red mite population is the most important factor in the onset of anaemia. The second limitation was that Kirkwood (1967) used small experimental cages which were not representative of those used commercially, therefore allowing a much elevated mite-host interaction causing exaggerated loss of blood. Despite these limitations, this paper is useful as a model to establish the potential threat of the poultry red mite as a debilitating parasite in causing rapid and fatal anaemia.

A more recent study by Kilpinen *et al.* (2005) also demonstrated the debilitating capacity of red mite, when several birds died as a result of the rapid onset of anaemia. Significantly lower ($P<0.05$) packed cell volumes (PCV) and reduced concentrations of circulating haemoglobin in red blood cells were observed in infested birds. It was suggested that birds were suffering from regenerative anaemia, where blood is lost faster than the rate of haematopoiesis. It was estimated that at peak infestation, birds were parasitized with between 150,000 to 200,000 red mite, which was suggested to be equivalent to that seen in a poultry house under severe attack. Again this highlights the potential risks and costs associated with red mite infestation.

Figure 2.4 *(A) Anaemia caused by heavy* Dermanyssus gallinae *infection. (B) Negative control (Kaufman, 1996)*



2.8 Human-mite interactions

A number of incidents of red mite parasitizing humans have been recorded. Humans come into contact with the red mite whilst working in poultry houses or alternatively the mite may enter rooms when migrating from wild birds' nests in the eves of residential houses (Urquhart *et al.*, 1996). When bitten, symptoms in humans range from minor irritation, to skin lesions and dermatitis and in one case multiple erythematous papules (inflamed lesions) accompanied by severe pruritus (itching) were observed. Red mite readily infect other animals of both domestic and wild species and have been seen to cause erythema (reddening of the skin) and intense pruritus in cats (Urquhart *et al.*, 1996, Bruneau *et al.*, 2001; Bruneau *et al.*, 2002; Rosen *et al.*, 2002).

2.9 Host detection

In many parasite species an array of senses are used in order to locate potential hosts which act as a source of food. Usually ectoparasites rely on several host-related stimuli, each showing variable importance depending on the context in which they are detected (Kilpinen, 2005). These host detection strategies used in other species are thought to remain consistent with those used by red mite (Zeman, 1988). It is important to note that the ease with which red mite reach the host has an effect on the speed of population increase. Hungry mites, even though they may be positioned near to the host, have great difficulty in locating the host unless the route of access is direct. This accounts for the predilection of red mite for nest boxes and perches, in order to be in close proximity of their food source (Zeman, 1988).

2.9.1 Environmental stimuli

Heat is a well documented stimulus involved in host detection and location processes of many ectoparasites (Kilpinen, 2001). There has been some interest in the link between temperature and predation by red mite. Kilpinen (2001) carried out a study to determine the sensitivity of the poultry red mite to temperature change and also response to temperature gradient (speed of temperature change). Results showed that temperature was a powerful activating stimulus, with rises in temperature resulting in increases in red mite activity. However, mites were seen to lack a well-defined response to temperature change itself, but respond much more distinctly to a temperature gradient. Temperature changes of 0.2°C failed to activate mites when the temperature gradient was below 0.004°C/s, whereas temperature changes as low

as 0.04°C were successful at higher gradients starting at around 0.005°C/s. This sensitivity of the red mite to heat may be used to direct them towards their potential host, although temperature can only act as a close range measure since a bird will generally have a surface temperature close to that of the surrounding building. However, in close proximity to a nesting bird the body temperature is distinctly higher than ambient temperature. Therefore potentially guiding the mite towards the birds by the heat transmitted through the nesting substrates. Kilpinen (2001) also suggests that heat from exhaled air from the bird could also be used for host detection.

Temperature is closely related to humidity regarding the survival and fecundity of red mite, with an optimum red mite proliferation seen at a relative humidity of 70 %, similar to that in a poultry house (Nordenfors *et al.*, 1999). Since both are so closely linked it is likely that humidity also plays a role in host detection (Kilpinen, 2005), although little research has been conducted in this area.

CO_2 is also considered to be a host attractant for a wide range of ectoparasites, although the behavioural response can vary depending on other external factors (Kilpinen, 2005). For example, responsiveness of the tick *Amblyomma hebraeum* is immediately enhanced by CO_2 contact, whilst the tick *Ornithodoros concanensis* initially react with a negative response, followed by habituation with extended exposure. A further tick species, *Argas cooleyi* does not react to CO_2 unless it is already active (Kilpinen, 2005). The response of red mite to CO_2 stimulation in daylight is to freeze and remain motionless, even with the activating stimulus of heat. If vibrations were applied during CO_2 immobility, then mites start moving but only for the duration of the vibrations. This is a mechanism which apparently allows red mite to avoid been eaten by the host. When light intensities were reduced, where birds would not be able to see mites, the freezing response is abandoned and only the simultaneous joint effect of heat and vibration can cause changes to activity levels (Kilpinen, 2005).

It is clear that host detection is a complicated procedure involving interaction between several different host-related stimuli (temperature, humidity, vibration and CO_2). All of these stimuli can effect behaviour of the ectoparasite depending on the context in which they are applied (Kilpinen, 2005). An understanding of the impact of these environmental stimuli on mite development and survival may have potential implications in future red mite control, although it must be remembered that any

changes to environmental conditions employed as control strategies, may hinder bird performance (Nordenfors *et al.*, 1999).

2.9.2 Surface skin lipids

In addition to changes in environmental conditions, the poultry red mite appears to have other means of host detection. One such way was studied by Zeman (1988) in which red mite affinity to surface skin lipids of birds was investigated. Extracts from various tissues, plumage and the uropygial glands (located close to the skin surface) were removed from hens, dissolved in solvents and subsequently spread onto skin (dissected from birds) or synthetic Parafilm® M membranes, in order to search for the presence of a kairomone (a naturally produced pheromone) for attracting red mite. Membranes impregnated with feather and uropygial gland extracts, as well as non-impregnated natural skin were highly attractive to red mite, but muscle and fatty tissues on the other hand produced no response. Thus, it was apparent that mites were attracted to a component of the uropygial gland secretions. Subsequent chromatography and *in vitro* assessment of gland secretions revealed that, in fact both diesters of fatty acids and skin surface lipids, comprising of ester wax, appeared to attract red mite. Therefore, these components were observed to possess essential properties enabling them to play a key role in the host-parasite relationship. The structures of these skin surface lipids have also been observed to show specificity to different avian species and so can assist in taxonomic classification.

In addition to specific molecules involved in host detection, other membrane characteristics such as texture, elasticity and affinity to lipids, amongst others, also seem to be required in host recognition by mites. This was concluded from *in vitro* studies which showed that red mite displayed very little interest in synthetic membranes of the type generally accepted by many other blood-sucking arthropods (Butler *et al.*, 1984). Red mite have also shown this extreme selectivity even when using skin from other avian species (Bruneau *et al.*, 2001).

2.10 Housing systems

It is commonly acknowledged that red mite infestation levels vary between production systems (Kilpinen, 1999). In battery-cage farms the red mite hide under conveyer belts and cage supports, whereas in slatted floor systems, such as the ones found typically in barn and free-range units, red mite conceal themselves under nest boxes, beneath troughs and in cracks in the house walls (Chauve, 1998). However, in recent

years research on the prevalence of red mite and its association to certain types of housing system has been limited. This is largely due to the fact that in the past commercial egg production on a worldwide scale was mainly carried out in battery cage systems. Within the EU, less than 1 % of eggs produced in 1995 came from systems other than laying cages (Höglund *et al.,* 1995). However, there has been a recent shift towards egg production in extensive systems. For example, the UK currently possesses a high proportion of non-cage systems with approximately 25 % free-range and 50 % barn (CEAS, 2004).

Although not wholly conclusive, some research has been conducted to establish the preferred housing systems for red mite. A study across a range of farms in Sweden revealed that only 4 % of cage systems were parasitized, whereas red mite infections were more common and widespread in deep-litter flocks (33 % infested). It has been suggested that this can be attributed to the presence of more potential hiding places for the mites in these systems (Höglund *et al.,* 1995). A similar study by Kilpinen (1999) revealed that the occurrence of red mite in free-range systems was as high as 68 %, with only around one third of cage systems showing signs of red mite infestation. Studies in the UK substantiate these findings showing the distribution of red mite to be approximately 60 % in free-range, 32.5 % in barn and 7.5 % in cage systems (Anon, 2003c).

It is also generally accepted that since the introduction of cages in the 1950's, problems with haematophagous mites of laying hens have become less frequent (Axtell and Arends, 1990; Höglund *et al.,* 1995), although this assumption lacks statistical justification. However, the recent ban on the use of battery cages throughout the EU, under the European Council Directive (1999/74/EC), has been observed to indirectly reduce bird welfare. This occurs by promoting the use of alternative systems which provide optimal conditions for red mite proliferation, thus amplifying the current problem (Chirico and Tauson, 2002). Observations of this nature have also been seen in Denmark through the increasing popularity of organic systems (Permin and Nansen, 1996). This legislation will be enforced in the remaining EU countries, and will take effect in the UK in 2012 (Guy and Edwards, 2006).

2.11 Economic/production impact

Red mite have been described as one of the most economically important ectoparasites of domestic fowl (Nordenfors, 1999). It has been estimated that red mite infestations cost the UK egg industry £3.7 million per year (Anon., 2003a), with cost of ectoparasitic resistance to acaricides in the US estimated at as much as $1.4 billion (Lui and Scott, 1998). A well documented effect of red mite infestation is a reduction in the economic efficiency of egg producing units due to a decline in egg laying capacity (Chauve, 1998; DEFRA, 2001). Red mite infestation can typically lead to a drop of 5 to 10% in overall egg production (Anon., 2003b). It has also been suggested by Hutchinson *et al.* (2004) that costs accumulate due to increased expenditure on excessive washing to remove red blemishes left by mites on eggs, a condition known as 'egg spotting'. However, washing of eggs is permitted in only a small number of countries due to infectious risks (Hutchinson *et al.,* 2004). Coupled with the implications of a direct downgrading of egg quality, these circumstances result in considerable losses for egg production (Anon., 2003b).

Due to the substantial economic burden and compromise to welfare of hens it is important that strategies for controlling red mite are investigated further (Beugnet *et al.,* 1997). One possible approach which has shown promise with other ectoparasite species is the development of an arthropod vaccine (Willadsen, 2001). However, in order to understand and exploit previously implemented vaccine technologies an introduction to the immune function of domestic fowl is necessary.

2.12 Immunology

2.12.1 Immune system function

The immune system can initially be divided into two distinct categories, innate and adaptive. The innate system is composed of the skin, cilia in mucous membranes, tears, nasal secretions and saliva and a number of phagocytic cells. Innate immunity is directed against any kind of pathogen entering a host. If the pathogen is able to evade the innate defences, the body could launch an adaptive or specific attack against that specific antigen. The adaptive system is divided into two further sections, humeral and cellular. The humoral immune system is associated with the production of immunoglobulins to eliminate pathogens and their specific products, whereas the cellular system targets pathogens infecting cells directly. The cells which form the basis of both types of immune response are lymphocytes which originate in the bone

marrow and migrate to different lymphoid organs. All lymphocytes are derived from the bone marrow, but their future function is related to their destination for subsequent maturation. Those that pass through the thymus become T-lymphocytes (T-cells) and are responsible for cell-mediated immunity. In contrast those which pass through the bursa of fabricius in birds and the bursa equivalent in humans, thought to be red bone marrow, become B lymphocytes (B-cells) (Abbas *et al.*, 2003).

Both humoral and cellular systems work closely with T-helper cells ($CD4^+$T cells), a sub-group of lymphocytes involved in the activation and direction of other immune cells. However, $CD4^+$T cells are unable to kill infected cells, having no cytotoxic or phagocytic capacity. Mature $CD4^+$T cells can differentiate into two major subtypes of cells known as Th1 and Th2 cells. These subtypes are defined on the basis of the specific cytokines they produce. Cytokines are proteins made and secreted by cells, which act as mediators of growth, differentiation and activation, playing a pivotal role in the immune system. Th1 cells produce the cytokines interferon-gamma (IFN-γ), tumor necrosis factor-beta (TNF-β) and interleukin-12 (IL-12), whilst Th2 cells produce the cytokines interleukin-4 (IL-4), interleukin-5 (IL-5) and interleukin-13 (IL-13) among numerous other cytokines.

It has been frequently acknowledged that both Th1 and Th2 -type cytokine profiles appear to be biased in the type of immune stimulation that they promote. Th1-type cytokines tend to produce the pro-inflammatory responses attributed to killing intracellular parasites by increasing proliferation of macrophage and Cytotoxic T cells ($CD8^+$ T cells) which are both involved in the cellular immune response (Berger, 2000). Th2-type cytokines on the other hand are involved in the stimulation of B-cell proliferation and also increased antibody class-switching and production. These are all functions associated with the humoral immune system (Abbas *et al.*, 2003; Berger, 2000). Since numerous mechanisms and pathways exist between cytokines and other immune molecules, a complex network of both the stimulation and inhibition of Th1 and Th2 -type immunity arises. Therefore, the following sections will describe in more detail the immune response of hens to pathogens, which is well described in the text by Abbas and Litchman (2003).

2.12.2 Humoral immune response

Humoral immunity is based around the action of B-lymphocytes (B-cells). Each B-cell contains multiple copies of one kind of antibody in the form of surface receptors that

are specific to an epitope on a particular antigen. As one population, B-cells have a vast scope in binding to millions of specific antigens. The B-cells themselves however are not involved in the reaction (Abbas *et al.*, 2003).

After binding to the correct antigen, B-cells are stimulated to undergo proliferation and differentiation, a process known as colonal selection. The resulting cells then develop into one of two types, either short-lived plasma cells, which produce vast numbers of identical antibodies and release them into circulation or memory cells, which possess the ability to manufacture small amounts of antibody long after the initial infection. This process of clonal selection usually requires interaction with T-helper cells since immunoglobulin receptors on B-cells are capable of recognising epitopes on the antigen surface, but unable to proliferate and differentiate unless prompted by the action of these $CD4^{+}T$ cells. Memory cells remain in greater numbers than the initial B cells, allowing the body to quickly respond to a second exposure of that antigen. However, in the absence of further stimuli, memory cells show depletion in numbers over a period of time (Abbas *et al.*, 2003).

(i) Immunoglobulins

As stated above, the basic units of humoral immunity are immunoglobulins or antibodies, which are glycoproteins found in all the jawed vertebrates. They are highly conservative in their overall structure, consisting of 4 peptide chains, 2 heavy and 2 light, but differ in their detailed structure and subsequent function (Tizard, 2002). Antibodies are capable of binding a phenomenal amount of structurally different antigen molecules due to the variability of the antigen-binding regions between individual antibodies. In mammals every individual is thought to have between 10^7 and 10^9 different antibody molecules, each having a unique amino acid sequence within their antigen-combining sites (Abbas *et al.*, 2003).

The effector functions of antibodies are exploited for the identification and neutralization of foreign objects such as bacteria and viruses. Each antibody recognizes a specific antigen unique to its target. By binding to their specific antigens antibodies can elicit agglutination/precipitation of antibody-antigen products, promote the coating of microorganisms in opsonins (opsonization) for subsequent phagocytosis by macrophages and other cells, block viral receptors, and stimulate the complement pathway (Abbas *et al.*, 2003).

Antibodies are classified on the basis of differences in their heavy chain regions. The diversity in structure also allows for an array of alternative functions to be mediated by different antibodies through binding of heavy chain regions to a range of cell receptors, including phagocytes, natural killer cells and mast cells (Abbas *et al.*, 2003). In mammals immunoglobulins take the form of one of several isotypes, namely IgA, IgD, IgG, IgE or IgM (Abbas *et al.*, 2003). However, the avian immune system possesses only three classes of immunoglobulin, namely IgA, IgM and IgY (Hamal *et al.*, 2006). Both IgA and IgM are thought to mediate similar functions in mammalian and avian immune systems. IgA is a secretory, dimeric immunoglobulin (i.e. consists of 2 subunits) that appears predominantly that on body surfaces, especially mucus membranes in intestinal and respiratory tracts, and is responsible for the protection of these surfaces against invasion (Abbas *et al.*, 2003). Antigens inducing an IgA response must be deposited on the body surface and ideally invade it. For example, live organisms such as parasitic worms, are needed (Tizard, 2002; Muleke *et al.*, 2007).

IgM is a polymeric immunoglobulin consisting of 5 subunits, in theory allows the potential binding of 10 antigen epitopes at any one time. IgM is predominantly located in blood serum and is formed during the primary immune response immediately after the first exposure to an antigen (Tizard, 2002). However, IgM responses rapidly decline within 4-8 days and are replaced by the production of monomeric IgY.

The overall structure of IgY has been determined, through molecular cloning techniques, to be the evolutionary ancestor of IgG and also IgE, with several marked differences (Warr *et al.*, 1995). For example IgY has a slightly greater molecular mass than IgG, 180 kDa vs. 150 kDa (Karlsson *et al.*, 2004). The effector functions of IgY include coating antigens for subsequent phagocytosis by neutrophiles, as well as complement activation and opsonization (Warr *et al.*, 1995; Karlsson *et al.*, 2004). The complement system is a series of serum and membrane proteins which are activated in a cascade reaction to bring about degradation of antigens.

Extensive research has been conducted on IgY and it has recently been recognised as having several advantages over mammalian-raised polyclonal antibodies (Karlsson *et al.*, 2004). One of the principal advantages of using poultry antibodies in immunological research is derived from the simple phylogenic differences between mammalian and avian species (Davalos-Pantoja *et al.*, 2000). This evolutionary gap

means that there is no immunological cross-reactivity between chicken IgY and mammalian IgG thus enhancing immunogeneity (Karlsson *et al.*, 2004). Some of the other advantageous characteristics, such as increased yields and better stability, that IgY offers over the use of mammalian IgG are listed in Table 2.3 (Zrein *et al.*, 1988; Zhang, 2003; Karlsson *et al.*, 2004).

Other subtle differences exist between avian and mammalian immune systems arising from evolutionary divergence. These include the lack of lymph nodes and functional eosinophils in chickens and also interleukin- 5 (IL-5) appears not to have a functional role and is actually a pseudogene role (Kaiser *et al.*, 2005).

Table 2.3. *Comparison of IgG and IgY antibodies (modified from Zhang, 2003).*

Features of comparison	**IgG**	**IgY**
Animal	Mammals	Birds, reptiles, amphibians
Sources	Blood serum	Egg yolk/blood serum
Molecular weight determined by SDS-PAGE	Whole: 150 kDa Light chains: 22 kDa x 2 Heavy chains: 50 kDa x 2	Whole: 180 kDa Light chains: 21 kDa x 2 Heavy chains: 70 kDa x 2
Basic structure differences	Flexible hinge region, shorter Fc stem with 2 pairs of carbohydrate groups	Shorter and less flexible hinge, longer Fc region with 3 pairs of carbohydrate groups
Immune response to mammalian antigens	Adversely affected by phylogenic homology	Enhanced by phylogenic differences
Quantity (yield per month/ per animal)	Milligrams with 1-10 % specific antibodies if mice or rabbits used	Grams with 2-10 % specific antibodies
Cross reactivity	Reaction to mammalian immunoglobulins and complement factors	No binding to mammalian immunoglobulins and complement factors
Stability	Good, stable at pH 3-10, up to 70°C	Good, stable at pH 4-9, up to 65°C
Productivity	Limited in quantity if mice and rabbits are hosts	High with greater quality and long duration
Monoclonal antibodies	Have been well developed	2 cases reported, more development needed

2.12.3 Cellular immune response

Cell-mediated immunity is particularly effective against fungi, parasites, intracellular viral infections, cancer cells and foreign tissue and it is T-lymphocytes (T-cells) which are responsible for this system of defence. Cell-mediated immunity works independently of antibodies and involves activation of macrophages and natural killer cells (NK), production of antigen-specific CD8+ T cells and release of various cytokines in response to antigens, which are typically displayed on the cell surface membranes of infected cells (Abbas *et al.,* 2003).

Macrophages are cells derived from monocytes residing within tissues. Their role is to phagocytize (engulf and then digest) a wide variety of particulate molecules and pathogens. Not only do they act in specific cell-mediated immunity, but also in non-specific innate defences. They also act as secretory cells releasing large numbers of cytokines, enzymes, inflammatory mediators and microbacterial agents (Herbert *et al.,* 1995).

NK cells are a subset of cytotoxic lymphocytes that function in innate immune responses to kill microbe infected cells by direct lytic mechanisms. Activation of NK cells is not restricted but due to their strong cytolytic capacity is closely regulated and requires stimulation by cytokines, particularly IFNγ and cell surface stimulatory and inhibitory receptors, which work in close proximity to the Major Histocompatibility Complex (MHC), which is a large genomic region found in most vertebrates (Abbas *et al.,* 2003).

CD8+ T cells are also a sub-group of T lymphocytes which can initiate cell death via apoptosis (cell death), usually in cells infected with viruses. As with NK cells, CD8+ T cells require activation by MHC molecules bound to antigen presenting cells in order to carry out their effector function (Herbert *et al.,* 1995).

Many of the cells involved in cellular immunity require stimulation/activation by forming complexes with the MHC. MHC molecules are embedded in the cell membrane, where they display short polypeptides which are complementary to T-cells, via receptors on the T-cell surface. These polypeptides may be self (i.e. native to the host), which should be ignored by T-cells, or non-self (i.e. foreign to the host), originating from foreign/pathogenic molecules, which should stimulate the appropriate effector response in T-cells. Two structurally distinct types of MHC exist,

Class I and Class II, which are present on nucleated cells and antigen presenting cells, respectively (Herbert *et al.*, 1995; Abbas *et al.*, 2003).

2.13 Vaccine development

Both humoral and cellular-mediated immunity have been observed to play an essential role in the successful development of protective responses against ectoparasites and subsequent vaccine production (Nisbet and Huntley, 2006). However, despite the extreme importance of ectoparasites in disease transmission and economic losses, only vaccines against the African cattle tick (*Boophilus microplus*) are commercially available (Willadsen *et al.*, 1999; Nisbet and Huntley, 2006). This is a reflection of the difficulty which is encountered in isolating suitable antigenic targets (Trimnell *et al.*, 2002). However, there is a drive and urgency for this to change in the future since there are many problems associated with alternative chemical anti-parasitic control methods. Problems largely relate to intrinsic characteristics, such as development of parasitic drug-resistance, chemical and antibiotic residues in food and concerns over undesirable environmental affects. Vaccines on the other hand do not pose such problems and are regarded as a much safer means of control, provided there is isolation of an appropriate antigen (Dalton and Mulcahy, 2001).

Two approaches of manipulating the immune system in order to elicit host protection to parasitic species have been employed in arthropod vaccine development. Firstly, exploration of naturally acquired antigen immunity and secondly vaccination against 'concealed' antigens (Trimnell *et al.*, 2002).

2.13.1 Naturally acquired immunity

Naturally acquired immunity is a commonly exploited area and involves exposure of a host to repeated antigen stimulus following parasitic engorgement. In some cases there is no response at all whilst in others this can result in an elevated immune response to subsequent stimulation with the same antigen (Dalton and Mulcahy, 2001). Typically, in the case of endoparasites these antigens are located on their surface, e.g. worms, flukes, protozoa, burrowing mites etc. Alternatively they are associated with feeding and often found in saliva, e.g. mites, fleas, ticks, flies etc. (Gillespie *et al.*, 2000). On entering the host these antigens can elicit Th1 and/or Th2-type immune responses, although this is not guaranteed to provide protection (Nisbet and Huntley, 2006). There have been a number of attempts to utilize the strategy of

naturally acquired immunity to find suitable antigen candidates for both mite and other ectoparasitic species, with varying degrees of success.

The sheep scab mite (*Psoroptes ovis*) has been the focus of much research in recent years, due to the ability of the host to display substantial protective immunity following primary infestations (Van den Broek *et al.*, 2000). However, the mechanisms behind this protective immunity are poorly understood, although it is speculated that they are related to increases in hypersensitive IgE responses. In an effort to improve the understanding of the underlying immune mechanisms behind protection, Van den Broek *et al.* (2003) examined isotype-specific antibody responses in naturally infested sheep. Antigen-specific ELISA revealed significant increases in both IgG and IgM, but not IgA. However, IgA antibody response to ectoparasitic antigens has not yet been documented (Van den Broek *et al.*, 2000). Western blots on fractionated whole mite proteins indicated that IgG and IgE antibodies reacted with a range of immunodominant antigens, with the number increasing as the infestations progressed (Van den Broek *et al.*, 2000). A more recent study by the same group demonstrated that inhibition of Th2-type inflammatory responses with the immunosuppressant drug, cyclosporin A resulted in significant ($P<0.05$) reductions in mite numbers, thus suggesting that inflammation is important in parasite survival (Huntley *et al.*, 2005). *P. ovis* research has also demonstrated cross-reactivity of antibodies with several other mite species including, swine mange mite (*Sarcoptes scabiei*), the bovine mange mite (*Chorioptes bovis*) and the cat mange mite (*Notoedres cati*) which is a desired vaccine characteristic (Matthes *et al.*, 1995).

Similarly, infestation with the mange mite (*Sarcoptes scabiei*) has been observed to provoke mite-specific circulating antibodies leading to subsequent immunological memory, reduction of parasite numbers and failure of re-infestation. *S. scabiei* antigens induce both a Th2-type immune response, characterised by initial increases in both IgG and IgE, with pronounced cellular responses during re-infestation, marked by rapid increases in mononuclear cells, neutrophils and mast cells, paralleled by clearance of mites (Nisbet and Huntley, 2006).

Immunoglobulin responses of poultry to natural infestations of the Northern fowl mite (NFM) (*Ornithonyssus sylviarum*), a closely related species to the poultry red mite, have also been characterised. Western blot analysis demonstrated the appearance of a mite-specific antibody in chicken blood sera approximately proportional to the

time of appearance and intensity of estimated NFM populations (Devaney and Augustine, 1987). Mite populations declined significantly ($P<0.05$) around 3-6 weeks, although IgY was detected until the close of the study, suggesting the occurrence of antibody-induced protective immunity to natural antigens. Other studies have similarly shown the production of antibodies against Northern Fowl mite populations to have linear relations with mite numbers so that as the infestation burden increased so did the strength of the immunological response of the chicken host (Murano *et al.*, 1989).

In addition to responses observed in mite species, there has also been substantial research into the immunological effect of natural exposure to a number of other haematophagous ectoparasites. For example, the development of resistance following natural exposure to ticks has been demonstrated on a number of occasions by several host species, including guinea pigs, rabbits, mice, raccoons and cattle (Craig *et al.*, 1996). Often antigens responsible for eliciting protection have been isolated from the tick saliva and it appears that mast cells, T-cells and immunoglobulins IgG and IgE all play key roles in conferring protection (Craig *et al.*, 1996). It has also been observed that protection, in terms of reduced survival and engorgement weights, in ticks is generally induced under Th1-type cellular immunity. Whereas, immune response to tick antigens directed towards Th2-type humoral immunity do not develop measurable resistance (Ogden *et al.*, 2002).

Immunological response of hosts to parasitism by mosquitoes and subsequent vaccine development is also a frequently researched topic, a consequence of the current human malaria epidemic which infects 300-500 million people per year (Greenwood and Mutabingwa, 2002). Studies have investigated the development of an anti-mosquito vaccine as a means of control since the mosquito is the vector of malaria. It has previously been illustrated that significantly higher ($P<0.001$) levels of both IgG and IgM anti-*Anopheles* mosquito antibodies can be detected in hosts after natural exposure to the parasite (Waitayakul *et al.*, 2006). These increases have been observed to induce deleterious effects on feeding, fecundity and survival (Gillespie *et al.*, 2000). Subsequent investigation using western blotting and immunohistochemical staining showed that antibodies once again reacted specifically with components of *Anopheles* salivary glands (Waitayakul *et al.*, 2006). Saliva/salivary glands are regarded as an important component of bloodfeeding ectoparasites' artillery in obtaining successful engorgement and consequently have been investigated as potential antigenic targets (Schoeler and Wikel, 2001).

Despite multiple reports of natural exposure to parasitic species leading to protective immunity, other reports have not confirmed this protection. Examples of failed resistance include cats and dogs exposed to fleas (*Ctenocephalides felis*), sheep parasitized by blowfly (*Lucilia cuprina*) and infestation of cattle by the warble fly (*Hypoderma lineatum*). In all of these examples strong Th2-type (typically immunoglobulin-G) responses are observed. Therefore it is more likely that Th1-type cellular immunity would be responsible for protection (Nisbet and Huntley, 2006).

Therefore, using this kind of naturally acquired immunity as the basis for vaccine development does have limitations, in that the hosts are naive to the majority of potentially protective ectoparasite antigens with exception to those which are secreted during engorgement or are external. The immune responses necessary for protection may therefore differ to those generated during natural infection (Dalton and Mulcahy, 2001). Recent studies have demonstrated that many ectoparasites possess the ability manipulate the hosts immune responses in their favour by stimulating/inhibiting expression of cytokines associated with either Th1/Th2-type immunity (Schoeler and Wikel, 2001). Consequently natural antigens rarely induce complete protective immunity and so much of the recent research into ectoparasitic vaccines has been focused on isolating 'concealed' antigens, those to which the host is rarely, if ever, exposed (Nisbet and Billingsley, 1999).

2.13.2 Concealed immunity

The second vaccine approach is via the use of internal components, used for critical physiological purposes within the parasite, referred to as 'concealed' antigens. Concealed antigens are generally proteins located in the internal/gut tissue of the parasite, which under normal circumstances are not exposed to the host. However, isolation, purification and immunisation of these antigens commonly elicits a strong immune response, which often leads to protection (Lee *et al.*, 1999).

This concept of using 'concealed' antigens was most notably used to form the basis of the successful, commercially produced tropical cattle tick (*Boophilus microplus)* vaccine (TickGARD™, Intervet, Australia) with the discovery of the Bm86 antigen. The Bm86 antigen is a membrane-bound glycoprotein present in very low abundance in extracts of partially-engorged adult female tick guts (Willadsen *et al.*, 1999). The uptake of this antigen during feeding by the cattle tick leads to severe damage to the parasite gut. Microgram quantities of this antigen have been effective in protecting

cattle against the parasite, characterised by a reduction in engorgement weights and egg laying capacity of the surviving female ticks. Immunoglobulins to the antigenic epitopes bind to the surface of midgut cells of the feeding tick. As a result of the reaction with these antibodies, the endocytotic activity of these cells a critical step in blood-meal digestion in this tick, is strongly and rapidly inhibited (Willadsen *et al.*, 1999). Subsequent research into the protective capacity of Bm86 showed promising results by reducing engorgement weights and subsequent oviposition of on a range of other tick species, including *Boophilus decoloratus, Hyalomma anatolicum anatolicum* and *Hyalomma dromedarii* (De Vos *et al.*, 2001). An additional study into the resistant ability of synthetic oligopeptides derived from different regions of the Bm86 glycoprotein was undertaken by Patarroyo *et al.* (2002). Results showed a significant increase ($P<0.01$) in mean IgG levels following immunisation which generated a high vaccine efficacy (81 %), measured as the percentage reduction in adult females. Current research continues to exploit the initial success of the Bm86 vaccine and as such has formed the basis of additional *Boophilus microplus* vaccines namely TickGARD Plus® in Australia and Gavac® in Cuba (Nijhof *et al.*, 2007).

As shown with the Bm86 antigen located in the midgut section, fractions of the digestive systems of invertebrate parasites and pests make excellent potential targets for control agents, through the inhibition of digestive enzymes or food absorption (Nisbet and Billingsley, 2000). A detailed knowledge of the pest's digestive system would therefore be useful in order to exploit such control approaches. Nisbet and Billingsley (2000) took extracts of ectoparasitic mites found on birds to assess the presence of hydrolytic enzymes used in digestion, including red mite. Many of the enzymes identified in this study were found in the principal areas of digestion (the ventriculus, diverticulae and caecae) and are known to be involved in the hydrolysis of food within the digestive tract (Evans, 1992). Red mite showed considerable uniformity in their range of phosphatase, esterase and aminopeptidase enzymes, in agreement with those seen in several other mite species, which suggests that the red mite is highly adapted to an animal parasitic lifestyle (Nisbet and Billingsley 2000). The same enzymes had been previously isolated in *Boophilus microplus,* suggesting similar modes of digestion. Therefore, immunisation of hosts with these enzymes may potentially induce protective immunity by the mechanisms observed previously with successful *Boophilus microplus* vaccines (Nisbet and Billingsley, 2000).

More recently, a number of attempts at recreating the success of the Bm86 midgut proteins have been made in a range of other invertebrate parasites. Some success has been seen with the tick family, for example the bush tick (*Haemaphysalis longicornis*), where a series of monoclonal IgG antibodies (mAbs) showed cross-reactivity with a 76 kDa midgut protein in certain mice. Adult ticks feeding on these animals immunised with this midgut protein subsequently failed to oviposit (Nakajima *et al.*, 2003). Similarly, mAbs raised against mosquito midguts of *Plasmodium falciparum* and *P. vivax*, both malarial vectors, reduced survival and fecundity as well having a parasite transmission-blocking capacity (Lal *et al.*, 2001).

Progress has also been made in immunological control of the sheep scab mite (*Psoroptes ovis*), with the initial development of a cDNA expression library, allowing identification and characterisation of potential target antigens (Lee *et al.*, 1999). This was followed by the immuno-localisation of host IgG in engorged mite midgut sections examined by cryosectioning, highlighting the potential susceptibility of *P. ovis* to the 'concealed' antigen route of vaccination (Pettit *et al.*, 2000). Subsequently, several attempts have been made to immunise sheep against *P. ovis* using concealed antigens. In one study, whole mite proteins were extracted using saline, Tween, urea and CHAPS and immunised into sheep, which produced varying degrees of success (Smith *et al.*, 2002). All immunised animals showed higher levels of increased circulating IgG responses when compared control sheep injected with adjuvant only. However, only the saline and Tween extracts stimulated significant two-fold reductions in mean lesion areas ($P<0.02$ and $P<0.01$, respectively) and significant decreases in mite numbers ($P<0.01$ and $P<0.02$, respectively). Further investigation of immunisation with whole mite extracts purified by both Fast Protein Liquid Chromatography (FPLC) and ultrafiltration to increase antigen specificity also showed considerable variation between individual sheep (Smith and Pettit, 2004). However, animals immunised with more highly purified forms of *P. ovis* extract, particularly after FPLC showed significantly higher (P-values not given) levels of protection than those immunised with whole mite extract, suggesting that there had been a concentration of protective components within the extract. SDS-PAGE profiles of these fractions displayed that they still showed multiple protein bands at different weights, suggesting that further purification is necessary in order to determine the protective components (Smith and Pettit, 2004). *P. ovis* antigens have since been observed to share homology and elicit strong IgG and IgE reactions with major allergens previously characterised in the house dust mite (*Dermatophagoides farinae*) (Lee *et al.*, 2002; Huntley *et al.*, 2004). These allergens include tropomyosin,

paramyosin, apolipophorin and cystine proteases, which have all been seen to show promise as vaccine candidates (Nisbet and Huntley, 2006).

Host resistance to a range of other ectoparasites has also been documented. For example, Arlain *et al.* (1995) observed resistance to the burrowing mite *Sarcoptes scabiei* in rabbits immunised with extracts from whole house dust mites, which had previously shown cross-reactivity between the two species. Results indicated that the balance between Th1 and Th2-type immunity played a pivotal role in the pathogenesis and expression of protection to scabies. It appeared that antigens induced a stronger Th1-type response in resistant hosts, illustrated by the increased expression of neutrophils. Conversely, the Th2-type response was up-regulated in non-resistant hosts, characterised by the immunoglobulin levels. Progress has also been made with immunising host species with a range of other concealed antigens from *Sacrcoptes scabiei*, such as, glutathione S-transferase, paramyosin and cysteine proteases (Ljunggren, 2005). It has also been observed that eliciting the correct type of immune response is essential in providing protection. Tarigan and Huntley (2005) reported failure to protect sheep against *Sacrcoptes scabiei* after immunising with extracts purified with ion exchange chromatography. They observed a strong increase in IgG, but no significant change in IgE levels, which had been previously correlated to a reduction in mite numbers.

Increased immunoglobulin levels but failure to reduce parasite numbers has also been observed in the Northern fowl mite, (*Ornithonyssus sylviarum*). In a study by Minnifield *et al.* (1993), mite proteins were obtained by affinity chromatography and used for immunisation. Western blot analysis identified several proteins that were reactive with sera from antigen immunised birds with a particularly immunodominant band at 100 kDa, which indicated that serum antibodies to the mite had been produced. However, this immunity did not decrease the level of mite infestation on birds or reduce survival in an *in vitro* feeding device.

Similar observations were made with poultry immunised with red mite extracts (Sam-Sun *et al.*, 2002). Here, antigen characterisation performed by western blotting revealed several prominent proteins recognised by IgY. Subsequently, the immune effect of somatic antigens on birds immunised with the whole mite extracts and then challenged with red mite was assessed. ELISA optical density readings revealed both significantly higher ($P<0.05$) IgY levels and significantly lower ($P<0.05$) levels of

mites in birds immunised with red mite antigens when compared to control birds (Sam-Sun *et al.*, 2002).

Many more examples of immune response to immunisation with ectoparasitic components exist showing a variety of responses, ranging from those which have no impact on parasite survival through to those which result in almost complete protection. However, it appears that host protection is particularly sensitive to the precise characteristics of the immunised antigens and vaccine constituents. For example, discreet changes to the tertiary structure of proteins have been attributed to the difference between the reduction and non-reduction of a parasitic burden (Tellman *et al.*, 2001), as have changes in the type of adjuvant used (Dalton and Mulcahy, 2001). Parasites have also become very adept at evading and manipulating host immune responses, often through saliva components, in order to obtain a blood-meal and in turn improve their survival (Gillespie *et al.*, 2000).

2.13.3 Dual action antigens

The use of concealed antigens is particularly effective in reducing ectoparasite populations in the short term. However, protective immunity can be short-lived, as natural infestations do not invoke a response to concealed antigens due to the absence of a sustained stimulus. In addition, the greatest problem associated with natural antigens is their inability to provoke a substantial protective immunological response. Therefore, the ideal ectoparasite vaccine would integrate both the concept of the concealed antigen whilst inducing an immune response reflective of natural infestation, eliciting a natural booster effect and thus eliminating the need for repeated vaccination (Trimnell *et al.*, 2002). Trials for such a vaccine have been implemented in a study using a putative tick cement protein (64P) from the African brown ear tick (*Rhipicephalus appendiculatus*). The 64P protein is thought to be involved in anchoring the tick mouthparts into the skin of the host during feeding and purified forms of the 64P protein have displayed an immunogenic nature (Trimnell *et al.*, 2002).

In order to expose immunogenic regions and also assess the efficacy of these 64P antigens, a series of both truncated and full length clones were used to immunise guinea pigs parasitized by *R. appendiculatus*. In addition, antigenic cross reactivity between gut sections and 64P were assessed separately through western blotting. It was observed that ticks which had consumed blood from guinea pigs immunised with versions of the 64P protein showed significantly higher ($P<0.001$) mortality rates

ranging between 56-70 % compared to controls. Moreover, antibodies raised against *R. appendiculatus,* cross reacted with antigenic epitopes from salivary glands and midgut sections of unfed adult female ticks. Subsequent research by the same group also revealed cross-protection against other tick species, *Rhipicephalus sanguineus, Amblyomma variegatum* and *Ixodes ricinus* (Trimnell *et al.,* 2005). The same authors also suggested that protection was invoked due to immunised animals stimulating local inflammatory immune responses, involving basophils, eosinophils, lymphocytes, mast cells, macrophages and dendritic-like cells. This is a novel approach whereby truncation of a recombinant form of secreted/external protein used as an anti-parasitic vaccine, exposes similar epitopes found on 'concealed' antigens as those seen on the internal portion of the ectoparasite. On combining this with the effect of the parasite naturally feeding, an innate immune boosting is provided. This enhances the effectiveness of a single vaccination by employing a dual action strategy of targeting the parasite both externally and internally, through vaccination and natural boosting (Trimnell *et al.,* 2002).

2.14 Summary and conclusions

The poultry red mite is one of the most important welfare and economic concerns of current poultry production, with the situation likely to become more serious after the prohibition of cages and continued withdrawal of acaricides. As such, there is the need for a new and sustained approach to control this parasite. Previous research in other haematophagous ectoparasite species has demonstrated the possibility of utilising the host immune system to elicit host protection by way of immunisation. Several approaches for the extraction and purification of arthropod antigens for use in such immunisation studies have been attempted. Therefore, this thesis aims to exploit these techniques in the potential development of a novel poultry red mite vaccine. This requires the attainment of a number of objectives including:

- The evaluation of the relationship between red mite populations, poultry production parameters and acaricide application.

- The development of techniques for the optimum extraction of red mite antigens and IgY from egg yolks.

- The optimisation and validation of immunological assays to evaluate the effect of natural red mite exposure to hens, including ELISA, SDS-PAGE and western blotting.

- The establishment of both *in vivo* and *in vitro* red mite feeding systems to determine survival and reproductive parameters of red mite.

- Finally, compile these techniques to assess the effect of immunisation with red mite antigens on avian immune response and the subsequent impact on survival and fecundity of red mite populations.

Chapter 3

Techniques

3.1 Poultry red mite antigen extraction

3.1.1 Introduction

A number of the assays used to evaluate the immunological aspects of vaccine development require red mite protein extracts including ELISA, SDS-PAGE and western blotting. The same red mite proteins can be used as vaccine antigens which, if the vaccine is to be successful, have the primary function of eliciting a sufficient protective host immune response.

Some of the most important technical challenges of proteomics involve overcoming the difficulties associated with protein solubilisation and purification. Moreover, immunologically relevant proteins frequently represent a minute fraction of a pathogen's proteome (Zintl *et al.,* 2006). An additional problem in the isolation of antigens from the poultry red mite relate to its size (0.75 x 0.46 mm) which eliminate the possible use of dissection of body parts, a strategy used for the successful development of other ectoparasite vaccines (Willadsen *et al.,* 1999). Therefore, this study set out to compare less direct whole mite protein extraction methods, which have previously been used to induce protection of host species against parasitic infections (Jayawardena *et al.,* 2000; Smith *et al.,* 2002).

Many different protein extraction methods have been developed and documented previously in the literature. These methods are generally based around both physical and chemical degradation of ectoparasites and are aimed at the isolation of soluble or membrane-bound proteins.

The initial step in preparation of antigens from ectoparasites commonly involves physically collecting samples from the field. Once in the laboratory, these ectoparasite samples are generally counted and collected via aspiration (Fletcher and Axtell, 1991). A washing step follows, usually in a non-ionic detergent, such as 1-10 % Sodium Dodecyl Sulfate (SDS) or Tween-20. This allows disruption and

removal of loosely bound debris which may contain contaminating polypeptides (Huntley *et al.,* 2004; Smith and Pettit, 2004).

The next step is often a disruptive one which may involve freezing in liquid nitrogen (Matthes *et al.,* 1995), sonication (Nakajima *et al.,* 2003) or homogenisation, either with a micro-pestle or milling using glass beads (Devaney and Augustine, 1987). This disruptive step is typically conducted in the presence of protease inhibitors, which are in turn placed in an extraction buffer to prevent excessive denaturation of proteins by proteases. Extraction buffers may contain any number of compounds suitable for disrupting protein structure such as Triton X, SDS, urea, Tween-20, EDTA, CHAPS, although many are simply extracted in saline (Laemmli, 1970; Devaney and Augustine, 1987; Ogden *et al.,* 2002; Van den Broek *et al.,* 2003; Huntley *et al.,* 2004). Samples are then filtered or centrifuged to pellet any remaining debris and the protein-containing supernatant can then be used in either immunological assays or as a vaccine antigen.

Occasionally, in order to purify protein fragments further, immunoaffinity columns are used. Here specific antigen fragments are bound to columns pre-coated with antibody and then eluted for subsequent use (Minnifield *et al.,* 1993; Shelver *et al.,* 1998; Tarigan and Huntley, 2005).

Since such an array of extraction protocols exist, the aim of this section is therefore to evaluate a number of these to determine the optimal extraction method for red mite antigens, both yielding high antigen concentrations and generating an array of proteins at different molecular weights.

3.1.2 Materials and methods

3.1.2.1 Source of poultry red mite

Red mite were collected using the trap sampling method outlined in Section 4.2.3 from commercial laying units around Northumberland which had a history of red mite infestation. Before being used in any of the extraction procedures, fed red mite were left in the dark in sealed polythene bags for approximately 10 days, to allow them to digest their previous blood-meals and remove blood proteins which may be a potential source of contamination.

3.1.2.2 Chemical extraction

All methods of both chemical and physical extraction of red mite antigens, outlined in this section, were repeated 5 times to ensure reproducibility.

(i) Urea extraction

The first chemical protein extraction method used a urea based membrane organelle solubilizing kit (Sigma, St. Louis, US) which targets both soluble and membrane bound proteins. Some 1,000 unfed poultry red mite were suspended in 1 ml of urea solubilizing reagent containing protease inhibitors (4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), E-64, bestatin, leupeptin, aprotinin, and sodium EDTA; Sigma, St. Louis, US), which was immediately reduced using tributylphosphine (final concentration 5 mM) and homogenised using a micro-pestle for 10-15 min on ice, until no whole mites were detectable microscopically. Proteins were subsequently alkylated by adding iodoacetamide (final concentration 15 mM) and the resulting suspension was then centrifuged at 14,000 rpm for 10 min, at room temperature. The supernatant was then removed, ready for protein concentration measurement using a Bradford assay.

(ii) CelLyltic™ extraction

The second chemical extraction method used was a CelLyltic™ extraction reagent (Sigma, St. Louis, US), which also targets both soluble and membrane bound proteins and contains 40 mM Tris-HCl (pH 8.0) and a proprietary formulation of zwitterionic detergents (which help to maintain a neutral solution). Again 1,000 unfed adult red mite were aspirated into an eppendorf tube and 1 ml extraction reagent along with DTT (5 mM final concentration) was added. Red mite were homogenised using a micro-pestle and held at room temperature on an orbital shaker for 30 min. The lysed red mite cells were centrifuged for 10 min at 14,000 x *g*, at room temperature to pellet the cellular debris. The protein-containing supernatant was transferred to a clean tube and stored at -20°C for subsequent use.

(iii) PBS, SDS and Tween extraction

All subsequent extraction protocols employed the same general methodology, however the buffer in which the protein was extracted varied. As before 1,000 unfed red mite were submerged in 1 ml of buffer, containing protease inhibitors (Sigma, St. Louis, US) and either PBS, 10 % SDS in PBS or 0.1 % Tween-20 in PBS. The suspension was then homogenised on ice, using a micro-pestle for 10-15 min, until no whole mites were detectable microscopically. Samples were then centrifuged at

14,000 x *g* for 10 min, at room temperature in order to pellet debris. The supernatant was then removed and retained for protein concentration determination using a Bradford assay.

3.1.2.3 Physical extraction

The two protocols for the chemical extraction which yielded the highest protein concentration were evaluated using different physical extraction methods (see Figure 3.1), namely sonication and freezing in liquid nitrogen. Sonication was performed following homogenisation in extraction buffer for 10 min in ice cold water and 60 Sonics/min (Branson ultrasonic cleaner, model 2210, Branson, Connecticut, USA). Freezing with liquid nitrogen, on the other hand was conducted on live red mite after being aspirated into eppendorf viles and before homogenisation. Subsequent methodology was conducted as previously described in Section 3.1.2.2.

3.1.2.4 Protein yield

The concentration of proteins extracted by all chemical and physical methods was compared using a Bradford assay according to the manufacturer's instructions. The absorbance values of each extract were read using an eppendorf Biophotometer (Eppendorf, Hamburg, Germany). The protein concentration of each sample was determined from a standard curve generated by a range of bovine serum albumin BSA) (Sigma, St. Louis, US) concentrations from 0.2 to 1.0 mg/ml.

3.1.2.5 Protein precipitation

Once optimal conditions for protein extraction had been established, in order to avoid contamination, improve purity and generate higher yields, proteins were precipitated using both trichloroacetic acid (TCA) and sodium deoxycholate (DOC). Subsequently both protein yield and purity of precipitated proteins were compared with those extracted by the standard methods outlined above.

A sample of 100 µl protein extract was brought to 1 ml by adding 900 µl 0.2 % (w/v) DOC. This solution was vortexed and incubated at room temperature for 10 min. Then 100 µl of 100 % (w/v) TCA was added before further vortexing, after which the solution was left to stand for 20 min at 4°C. The solution was then centrifuged at 14,000 x *g* for 10 min and the supernatant was decanted. The pellet was dispersed by vortexing and washed in 1 ml ice cold acetone solution. This solution was then centrifuged at 14,000 x *g* for 5 min, the supernatant was decanted and the wash procedure repeated once again. The remaining pellet was then allowed to dry by

exposing it to room temperature for 30 min and the pellet re-dissolved in either PBS for protein concentration or in sample buffer for subsequent SDS-PAGE analysis.

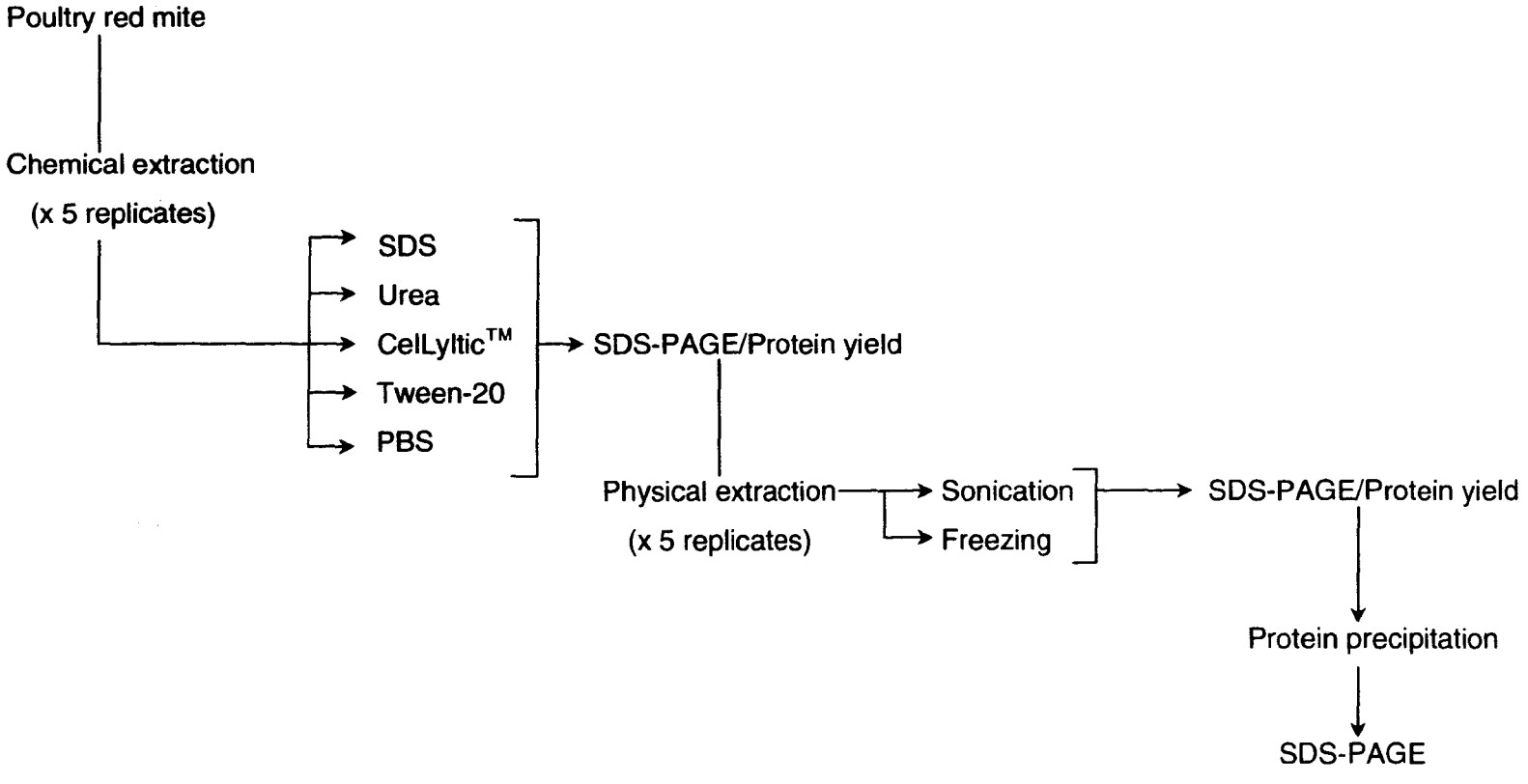
3.1.2.6 Comparison of extraction methods

Following the determination of yield both antigen extraction and also protein precipitation methods were analysed by SDS-PAGE in order to compare protein profiles (see Section 3.3). Briefly, 20 µg of each red mite protein extract was added to sample buffer (NuPAGE® sample buffer, NuPAGE® reducing agent, deionized water; Invitrogen, Paisley, UK). Samples were then heated to 70°C for 10 min and loaded into Novex 4-12 % Bis-Tris mini-gel lanes. Proteins were fractionated by electrophoresis for approximately 1 hour at 200 V and protein bands stained using Coomassie Blue stain (Safestain®, Invitrogen, Paisley, UK). A summary of all protein extraction protocols is given in Figure 3.1.

3.1.2.7 Statistical analysis

Antigen extraction protocols were assessed by analysis of variance (ANOVA) using the statistical package, MINITAB (v14), imputing protein yields of both chemical and physical extraction as response variables.

Figure 3.1 *Summary of poultry red mite protein extraction protocol*



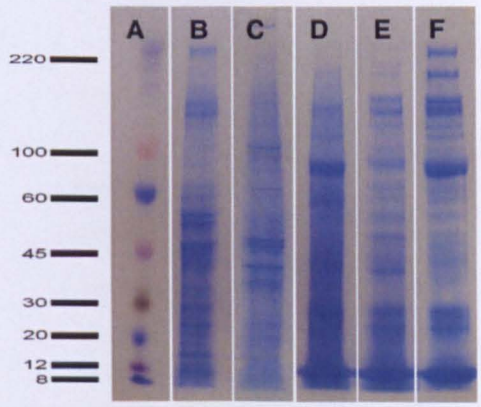
3.1.3 Results

3.1.3.1 Chemical extraction

Comparison of protein extraction methodologies using SDS-PAGE revealed that most, if not all bands, were common to all extracts, although the various proteins were represented at different concentrations (Figure 3.2). A number of common bands could be seen when comparing lanes, in particular a heavy band was seen at around 100 kDa. Resolution of bands was generally good, excluding extraction using CelLyltic™, which demonstrated a high degree of smearing, possibly as a result of protein degradation.

The protein banding patterns of the 10 % SDS, 0.1 % Tween-20 and PBS extracts were very similar, spread out evenly over the 8 to 220 kDa size range. PBS showed the most prominent bands at a series of molecular weights, in particular the heavier end of the scale ranging between 100 and 220 kDa. Extraction using urea solution, on the other hand, produced several strong bands at lighter molecular weights, around 40 to 50 kDa.

Figure 3.2 *Comparison of poultry red mite protein extraction method by SDS-PAGE; Lane A: Molecular weight marker (kDa); Lane B: 10 % SDS; Lane C: Urea; Lane D: CelLyltic™; Lane E: 0.1 % Tween-20; Lane F: PBS*



Significant differences were not observed between chemical extraction methods (Table 3.1). However, the PBS extraction method yielded the highest concentration of protein giving between 2.1 to 0.4 mg/ml extra total protein compared to the other extraction methods.

Table 3.1 *Comparison of mean poultry red mite protein yields using different extraction buffers*

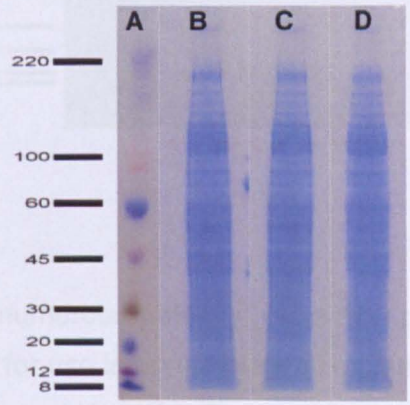
Extraction Method	Protein Yield (mg/ml)	S.E. Mean
PBS	3.2	0.17
Urea	2.8	0.16
10% SDS	2.2	0.08
0.1% Tween-20	1.4	0.21
CelLyticTM	1.1	0.15
Significance	NS	-

NS: no significant difference

3.1.3.2 Physical extraction

The physical process of extracting proteins was compared by SDS-PAGE and visualised using Coomassie Blue stain. The resulting protein bands generated showed no difference in conformation (Figure 3.3). Extraction by homogenisation, freezing in liquid nitrogen and sonication all produced bands ranging from 8 to 220 kDa, with particularly concentrated bands at around 100 kDa.

Figure 3.3 *Comparison of physical poultry red mite protein extraction method by SDS-PAGE; Lane A: Molecular weight marker (kDa); Lane B: PBS homogenisation; Lane C: Liquid nitrogen + PBS homogenisation; Lane D: Sonication + PBS homogenisation*



Surprisingly, neither freezing in liquid nitrogen or sonication yielded significantly higher protein levels over those extracted in PBS alone. In fact, sonication resulted in the lowest protein concentration numerically (Table 3.2).

Table 3.2 *Mean poultry red mite protein yields achieved with different physical extraction methods*

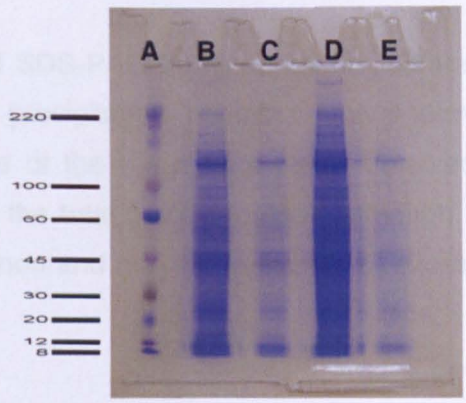
Extraction Method	Protein Yield (mg/ml)	S.E. Mean
PBS	3.2	0.17
PBS + Liquid nitrogen	3.2	0.19
PBS + Sonication	3.0	0.23
Significance	NS	-

NS: no significant difference

3.1.3.3 Protein precipitation

Precipitation of proteins did not result in additional purity of protein extracts when evaluated by SDS-PAGE. This was apparent since profiles of precipitated proteins appeared to be exact replicates of non-precipitated protein extracts (Figure 3.4).

Figure 3.4 *Comparison of precipitated poultry red mite proteins by SDS-PAGE, Lane A: Molecular weight marker (kDa), Lanes B and C: Non-precipitated proteins (10 and 1 µg, respectively), Lanes D and E: Precipitated protein (10 and 1 µg, respectively)*



3.1.4 Discussion

At present there are numerous potential extraction protocols available for preparing ectoparasitic proteins for use in immunological experiments (Uhlir, 1992; Minnifield *et al.*, 1993; Jayawardena *et al.*, 1998; Tarigan and Huntley, 2005). Therefore, the aim of this section was to evaluate these methods to determine the optimal extraction protocol for red mite antigens, both yielding high antigen concentrations and generating an array of proteins at different molecular weights.

After performing extraction of proteins, firstly by comparing buffer components, it was apparent that extraction with either PBS or SDS/Tween-20 generated the greatest number and widest range of bands over an extensive range (8 to 220 kDa). However, PBS alone produced several additional protein bands, and also gave the highest resolution, although this observation was entirely subjective. This configuration of red mite protein band patterns has previously been observed over molecular weights ranging from 9 to 170 kDa (Sam-Sun *et al.,* 2002). Extraction of red mite proteins using the urea buffer also produced a number of concentrated bands between 35 and 50 kDa which were not present using alternative methods. This perhaps contributed to both PBS and urea extraction protocols producing the highest protein yields, although not significantly so.

Isolation of proteins using the CelLyltic™ kit on the other hand produced both fewer protein bands and also appeared to cause excessive degradation. This is likely to be due to the abrasive nature of the components, Tris-HCl and zwitterionic detergents causing extreme disruption to protein structure as previously observed (Zintl *et al.,* 2006).

Further analysis of SDS-PAGE gels comparing different physical extraction methods and also protein precipitation revealed little improvement over simple chemical extraction in terms of the range of proteins detected. Sonication in fact caused a slight reduction in the total protein yielded, although this was not significant. Again, this is not uncommon and may be indicative of excessive protein disruption (Zintl *et al.,* 2006).

Therefore, it would appear that targeting soluble proteins using PBS extraction alone was the most effective method of extraction. Despite the fact that this method extracts only soluble proteins it has been observed in other arthropod species to elicit greater protection against infestation when compared to membrane-bound antigens (Nisbet and Huntley, 2006).

In conclusion, from these results it appears that using the PBS extraction method generated the greatest array and abundance of individual proteins which demonstrated suitability for use in immunological assays. Urea extraction of red mite antigens also provided a useful alternative by generating high protein yields, and an alternative protein profile to PBS.

3.2 Enzyme linked immunosorbent assay

3.2.1 Introduction

The enzyme-linked immunosorbent assay (ELISA) is a widely used procedure, most commonly applied for the recognition/quantification of antibody responses to specific pathogens (Mansheim *et al.*, 1980). It is a particularly useful technique and is used on both laboratory and commercial scales due to its sensitivity and high throughput (Wadhwa *et al.*, 2003). At present several poultry ELISA assays have been developed, typically for viral and bacterial pathogens, including *Salmonella* and *Mycoplasma* infections (Wunderwald and Hoop, 2002). However, availability of assays for serological detection of parasitic species such as the poultry red mite, are not common.

Immunoglobulin-Y (IgY) is the predominant antibody produced in poultry against prolonged exposure to specific antigens (Hamal *et al.*, 2006), such as chronic infestation by red mite. The aim of this section therefore was to describe the process of optimisation of a non-competitive, capture ELISA for the detection of anti-poultry red mite IgY. The validation experiments reported here were based on a factorial design with respect to several features of the assay.

3.2.2 Materials and methods

In order to determine optimum parameters for ELISA using poultry red mite extracts, a series of pilot studies were conducted. These allowed the establishment of optimum concentrations and dilutions of red mite protein, blocking agent, primary and secondary antibodies. These parameters were investigated for both serum- and yolk-derived IgY as shown in Figure 3.5. Parameters for the current ELISA assay were based on several previously documented parasitic ELISA assays (Silber *et al.*, 2002; Smith and Pettit, 2004; Cortes *et al.*, 2006; Pokharel *et al.*, 2006).

3.2.2.1 Materials and reagents

ELISA was performed using 96-well microtitre plates (Nunc, Denmark), which were coated using unfed whole red mite antigen, the extraction of which is described in Section 3.1. PBS-Tween-20 (0.15 M NaCl, 0.02 M Na_2HPO_4, 0.01 % Tween-20, pH 7.2) was used for both washing and dilution (Sigma, St Louis, USA). Bovine serum

albumen (BSA), secondary anti-chicken IgY peroxidase conjugated antibody and Tetramethylbenzidine (TMB) were also used (Sigma, St Louis, USA). Optical densities of wells were read on a microplate reader (Bio-tek Instruments, Winooski, USA).

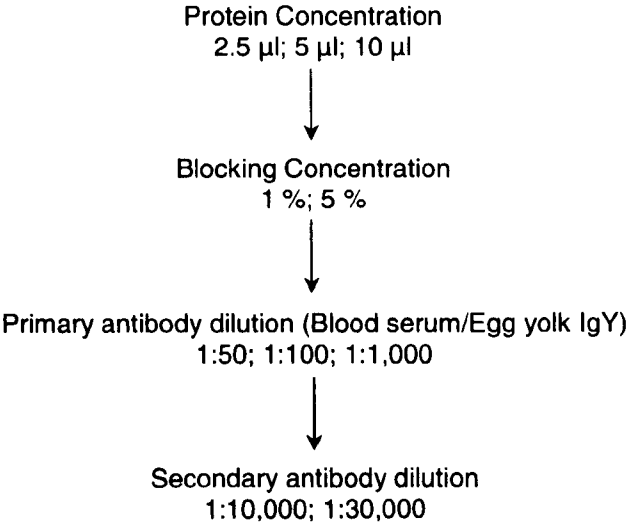
3.2.2.2 Immunoglobulin samples

Sources of both positive blood serum and yolk IgY were obtained from birds on farms which had previously been determined, via trap sampling, as having high levels of poultry red mite infestation. Negative serum on the other hand was collected from 7 week-old broiler chickens which had previously, to our knowledge, never been exposed to red mite. Initial ELISA optimisation was performed using pooled serum/yolk extract from both positive and negative birds (n= 15).

3.2.2.3 Assay design and optimisation

The 96-well plates were coated with a series of concentrations of unfed whole red mite antigen diluted in 0.1 M $NaHCo_3$ (pH 9.5) and incubated overnight in an orbital shaker at 4°C. The following day, the plates were washed 3 times in 200 µl PBS-Tween-20 and then blocked in a series of concentrations of BSA (see Figure 3.5). Following another three washes in 200 µl PBS-Tween-20, 100 µl of serum/yolk IgY was serially diluted in PBS-Tween-20 and left to incubate for 1.5 hours, then subsequently washed three times in PBS-Tween-20 as before. Each well then received 100 µl of a known concentration of secondary antibody, diluted in PBS-Tween-20 (see Figure 3.5) and was incubated for 1 hour and once again washed three times in PBS-Tween-20. Finally, 100 µl of TMB substrate was added to each well and the colour allowed to develop, after which optical density (O.D.) was read on a microplate reader. Plates were read kinetically at 630 nm, every 2 min for 40 min, in order to establish the optimal point of O.D.

Figure 3.5 *Schematic diagram of ELISA parameters for optimisation*



3.2.2.4 Optimisation parameters and replication

Assay optimisation involved investigating all combinations of various parameters in order to establish which ones produced the greatest signal obtained for positive samples, minus that of the negative value (Mire-Sluis *et al.,* 2004). IgY binding ratios (positive/negative O.D.) of samples were also employed as determinants for optimisation, with higher ratios being preferred (Dubois *et al.,* 2006). This was carried out for both blood sera and yolk IgY. Each optical density value expressed in the results section is a mean of two duplicate wells, repeated three times over successive days, unless otherwise stated.

3.2.2.5 Cut-off point and normalisation

The cut-off point of an assay is the level of response of the assay at or above which a sample is defined to be positive and below which it is defined to be negative (Mire-Sluis *et al.,* 2004). It is recommended that the cut-off point is set at an upper negative limit of around 95 % of the negative optical density value, which is achieved by adding 1.645 standard deviations (S.D.) to the mean (Mire-Sluis *et al.,* 2004; Dubois *et al.,* 2006). This value was calculated over three independent runs (three separate days), in order to compensate for inter-day variability. Also, inter-bird variability was accounted for by analysing samples from 15 individual negative samples. Cut-off points were determined for both serum and yolk IgY samples independently.

In order to standardise plates on consecutive runs, a normalisation factor was calculated. This value was determined as the ratio between the mean cut-off point of

the negative serum samples (n=15), divided by the mean optical density of a negative serum pool plus 1.645 S.D. (Mire-Sluis, *et al.*, 2004). The resulting value could subsequently be used for calibration of future assays.

3.2.3 Results

3.2.3.1 Optimisation of kinetic parameters

All combinations of ELISA parameters were subject to kinetic development for a period of 40 min with read intervals of 2 min. Table 3.3 displays optical density values over this period and shows that at 15 min the binding ratio is at its highest point, whilst the positive/negative difference is not maximised, although it is above the mean of 1.45.

Table 3.3 *Optimisation of kinetic ELISA parameters for serum IgY (numbers in bold represent optimum conditions)*

	Negative OD		Positive OD			
Time (min)	Mean	S.D.	Mean	S.D.	Pos-Neg	Binding Ratio
5	0.112	0.0551	0.506	0.0764	0.39	4.52
7	0.144	0.0341	0.822	0.0100	0.68	5.71
9	0.170	0.0578	1.077	0.0420	0.91	6.34
11	0.211	0.0651	1.321	0.1003	1.11	6.26
13	0.246	0.0544	1.531	0.0492	1.29	6.22
15	**0.272**	**0.0438**	**1.742**	**0.1025**	**1.47**	**6.40**
17	0.324	0.0432	1.879	0.0257	1.55	5.79
19	0.375	0.0476	1.993	0.0123	1.62	5.31
21	0.420	0.0487	2.109	0.0596	1.69	5.03
23	0.464	0.0287	2.200	0.0714	1.74	4.74
25	0.509	0.0541	2.300	0.0541	1.79	4.52
27	0.554	0.0341	2.385	0.0368	1.83	4.31
29	0.598	0.0131	2.461	0.0438	1.86	4.11
31	0.643	0.0506	2.534	0.0509	1.89	3.94
33	0.688	0.0487	2.590	0.0099	1.90	3.77
35	0.733	0.0497	2.640	0.0304	1.91	3.60
37	0.777	0.0483	2.720	0.0297	1.94	3.50
39	0.822	0.0488	2.779	0.0564	1.96	3.38

Figure 3.6 shows the kinetic reaction of positive against negative serum. There is an initial rapid increase in optical density of positive serum until approximately 13 min, at which point the rate of increase slows, but still continues to rise at a decreased rate until time expires at 39 min. Negative serum on the other hand appears to maintain a slow, yet constant increase for the duration of the kinetic read.

Figure 3.6 *Optimisation of kinetic ELISA parameters for serum IgY*

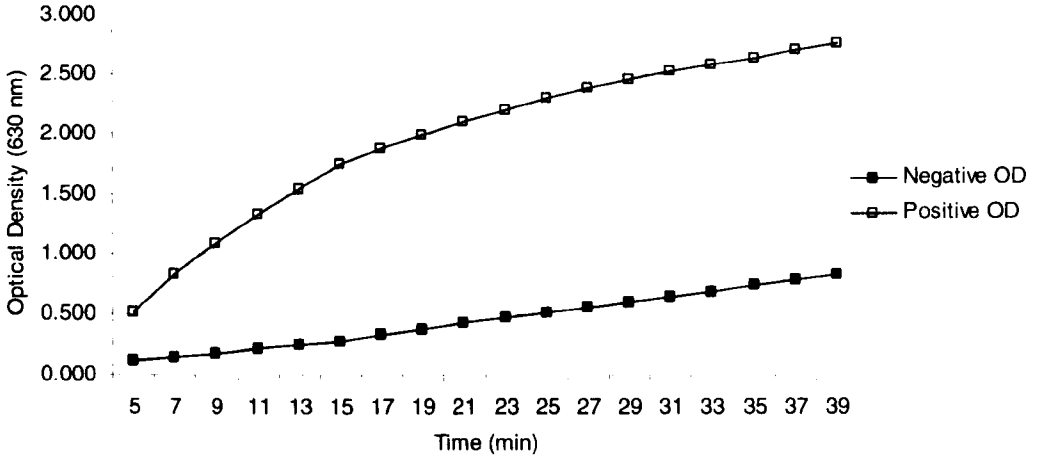


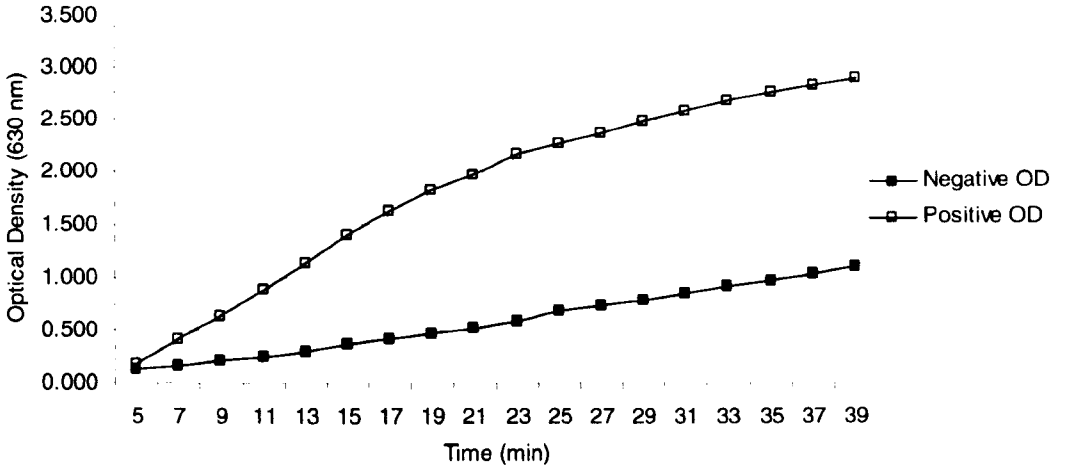
Table 3.4 also illustrates kinetic optimisation over a read period of 40 min, using IgY extracted from egg yolks. Here both optimal binding ratio and a positive-negative difference above the mean of 1.26 are observed at 19 min.

Table 3.4 *Optimisation of kinetic ELISA parameters for Yolk IgY (numbers in bold represent optimum conditions)*

	Negative OD		Positive OD			
Time (min)	Mean	S.D.	Mean	S.D.	Pos-Neg	Binding Ratio
5	0.129	0.0273	0.188	0.0771	0.06	0.46
7	0.172	0.0332	0.407	0.0354	0.23	1.36
9	0.211	0.1096	0.636	0.0693	0.43	2.02
11	0.244	0.0156	0.876	0.0700	0.63	2.59
13	0.298	0.0325	1.129	0.0707	0.83	2.78
15	0.358	0.0064	1.395	0.0304	1.04	2.89
17	0.412	0.0212	1.626	0.0601	1.21	2.94
19	**0.462**	**0.0262**	**1.826**	**0.0453**	**1.36**	**2.95**
21	0.513	0.0283	1.973	0.0757	1.46	2.85
23	0.583	0.0296	2.169	0.0396	1.59	2.72
25	0.676	0.0323	2.273	0.0679	1.60	2.37
27	0.734	0.0346	2.374	0.0608	1.64	2.23
29	0.783	0.0304	2.491	0.0014	1.71	2.18
31	0.844	0.0472	2.593	0.1527	1.75	2.07
33	0.906	0.0306	2.682	0.0474	1.78	1.96
35	0.967	0.0341	2.772	0.0806	1.81	1.87
37	1.028	0.0264	2.842	0.0813	1.81	1.76
39	1.090	0.0198	2.901	0.0141	1.81	1.66

As for serum, positive yolk IgY samples display an initial rate of optical density increase which appears to be exponential with unit time, which subsequently slows, but continues to increase at a steady rate until the end of the reaction. Negative yolk IgY also maintains a steady rate of increase for the read duration (Figure 3.7).

Figure 3.7 *Optimisation of kinetic ELISA parameters for Yolk IgY*



3.2.3.2 Serum IgY optimisation

ELISA outputs for serum IgY suggested that protein concentration had a large bearing on the subsequent optical densities (see Tables 3.5-3.8). As protein concentration rose from 2.5-10 µg so did the optical density values, but a subsequent increase to 15 µg caused no further rise. The optimum concentration appeared to be at 10 µg of protein per well (Table 3.7), giving rise to both the largest difference in positive and negative values and also greatest binding ratios. There was no further increase with addition of protein up to 15 µg per well.

There was a steady decline in optical density values as the blocking concentration, primary and secondary antibody dilutions increased. These rises in blocking and antibody dilution also had the effect of increasing the positive/negative difference and binding ratio until saturation, which appeared to be at an antigen concentration of 10 µg per well.

Therefore the series of parameters which yielded optimal results was, 10 µg red mite antigen per well, blocking with a concentration of 5 % BSA, incubation with primary antibody diluted 1:100 and secondary antibody diluted 1:30,000.

Table 3.5 *Optimisation of ELISA parameters using 2.5 µg red mite protein per well for serum IgY*

Primary Antibody	Blocking %	Secondary Antibody	Negative OD		Positive OD		Pos-Neg	Binding Ratio
			Mean	S.D.	Mean	S.D.		
		1:10,000	1.269	0.0177	1.669	0.0269	0.40	1.32
	1	1:30,000	0.964	0.0078	1.492	0.0184	0.52	1.55
		1:10,000	1.012	0.0318	1.484	0.0658	0.48	1.47
1:50	5	1:30,000	0.887	0.0163	1.477	0.0198	0.59	1.67
		1:10,000	0.865	0.0453	1.475	0.0438	0.61	1.71
	1	1:30,000	0.773	0.0665	1.433	0.0898	0.66	1.85
		1:10,000	0.442	0.0594	1.122	0.0912	0.68	2.54
1:100	5	1:30,000	0.313	0.0778	1.113	0.0601	0.80	3.56
		1:10,000	0.599	0.0170	0.969	0.0304	0.37	1.62
	1	1:30,000	0.424	0.0219	0.894	0.0099	0.47	2.11
		1:10,000	0.366	0.0467	0.886	0.0757	0.52	2.42
1:1,000	5	1:30,000	0.293	0.0049	0.843	0.0262	0.55	2.88

Table 3.6 *Optimisation of ELISA parameters using 5 µg red mite protein per well for serum IgY*

Primary Antibody	Blocking %	Secondary Antibody	Negative OD		Positive OD		Pos-Neg	Binding Ratio
			Mean	S.D.	Mean	S.D.		
		1:10,000	1.699	0.0283	2.699	0.0495	1.00	1.59
	1	1:30,000	0.989	0.1096	2.099	0.0092	1.11	2.12
		1:10,000	1.104	0.0877	2.094	0.0629	0.99	1.90
1:50	5	1:30,000	1.750	0.0502	2.840	0.0106	1.09	1.62
		1:10,000	0.685	0.0163	1.955	0.0212	1.27	2.85
	1	1:30,000	0.774	0.0085	1.954	0.1633	1.18	2.52
		1:10,000	0.581	0.0707	1.901	0.0643	1.32	3.27
1:100	5	1:30,000	0.419	0.0113	1.749	0.0516	1.33	4.17
		1:10,000	0.863	0.0205	1.643	0.0283	0.78	1.90
	1	1:30,000	0.921	0.1351	1.611	0.0990	0.69	1.75
		1:10,000	0.537	0.0757	1.307	0.0544	0.77	2.43
1:1,000	5	1:30,000	0.296	0.0361	1.136	0.0297	0.84	3.84

Table 3.7 *Optimisation of ELISA parameters using 10 µg red mite protein per well for serum IgY*

Primary Antibody	Blocking %	Secondary Antibody	Negative OD		Positive OD		Pos-Neg	Binding Ratio
			Mean	S.D.	Mean	S.D.		
		1:10,000	2.519	0.0269	3.499	0.057	0.98	1.39
	1	1:30,000	1.767	0.1937	2.987	0.021	1.22	1.69
		1:10,000	1.056	0.0544	2.346	0.0389	1.29	2.22
1:50	5	1:30,000	0.805	0.0764	2.105	0.0587	1.30	2.61
		1:10,000	0.775	0.0071	2.055	0.0361	1.28	2.65
	1	1:30,000	0.655	0.0049	1.955	0.2475	1.30	2.98
		1:10,000	0.308	0.0467	1.748	0.0417	1.44	5.68
1:100	5	1:30,000	0.272	0.0438	1.742	0.025	**1.47**	**6.40**
		1:10,000	0.553	0.0311	1.433	0.0028	0.88	2.59
	1	1:30,000	0.685	0.2206	1.405	0.0071	0.72	2.05
		1:10,000	0.534	0.0467	1.374	0.0304	0.84	2.57
1:1,000	5	1:30,000	0.379	0.0933	1.219	0.0707	0.84	3.22

Numbers in bold represent optimum conditions

Table 3.8 *Optimisation of ELISA parameters using 15 µg red mite protein per well for serum IgY*

Primary Antibody	Blocking %	Secondary Antibody	Negative OD		Positive OD		Pos-Neg	Binding Ratio
			Mean	S.D.	Mean	S.D.		
		1:10,000	1.722	0.0410	2.732	0.0021	1.01	1.59
	1	1:30,000	1.469	0.2715	2.709	0.0134	1.24	1.84
		1:10,000	1.581	0.0368	2.691	0.0226	1.11	1.70
1:50	5	1:30,000	1.414	0.1131	2.684	0.0764	1.27	1.90
		1:10,000	1.442	0.0007	2.672	0.0438	1.23	1.85
	1	1:30,000	1.314	0.0113	2.614	0.2970	1.30	1.99
		1:10,000	1.126	0.0255	2.506	0.0304	1.38	2.23
1:100	5	1:30,000	1.112	0.0771	2.502	0.1294	1.39	2.25
		1:10,000	1.451	0.0453	2.431	0.0042	0.98	1.68
	1	1:30,000	1.359	0.2892	2.359	0.0141	1.00	1.74
		1:10,000	1.224	0.0318	2.244	0.0191	1.02	1.83
1:1,000	5	1:30,000	0.866	0.1252	1.986	0.0714	1.12	2.29

3.2.3.3 Yolk IgY optimisation

As for blood sera, yolk IgY displayed a continual decline in optical density values as the blocking concentration, primary and secondary antibody dilutions increased, with increases in optical density resulting from increases of antigen concentration until saturation at 10 µg of mite protein per well (see Tables 3.9-3.12). Again, the difference between positive and negative sera was seen to rise and peak with antigen concentration up to 10 µg of protein per well at which point a plateau was reached. Binding ratios, however, were initially observed to decrease from 2.5 to 5 µg of mite protein per well, but increase thereafter, with an optimum reached at 10 µg antigen per well.

Converse, to serum IgY, optimal optical density values for yolk IgY were observed when blocked with 1 % BSA, followed by incubation with primary antibody at 1:100 dilution and finally secondary antibody at 1:10,000 dilution (Table 3.11).

Table 3.9 *Optimisation of ELISA parameters using 2.5 µg red mite protein per well for yolk IgY*

Primary Antibody	Blocking %	Secondary Antibody	Negative O.D.		Positive O.D.		Pos-Neg	Binding Ratio
			Mean	S.D.	Mean	S.D.		
		1:10,000	0.827	0.0127	1.167	0.0460	0.34	1.41
	1	1:30,000	0.759	0.0127	1.139	0.0509	0.38	1.50
		1:10,000	0.630	0.0049	1.020	0.0198	0.39	1.62
1:50	5	1:30,000	0.447	0.0120	0.877	0.0785	0.43	1.96
		1:10,000	0.343	0.0304	0.873	0.0276	0.53	2.55
	1	1:30,000	0.391	0.0297	0.801	0.0127	0.41	2.05
		1:10,000	0.263	0.0071	0.763	0.0170	0.50	2.90
1:100	5	1:30,000	0.299	0.0495	0.692	0.0226	0.39	2.31
		1:10,000	0.300	0.0276	0.611	0.0552	0.31	2.04
	1	1:30,000	0.315	0.0170	0.605	0.0622	0.29	1.92
		1:10,000	0.201	0.0120	0.461	0.0311	0.26	2.29
1:1,000	5	1:30,000	0.179	0.0311	0.449	0.0672	0.27	2.51

Table 3.10 *Optimisation of ELISA parameters using 5 µg red mite protein per well for yolk IgY*

Primary Antibody	Blocking %	Secondary Antibody	Negative O.D.		Positive O.D.		Pos-Neg	Binding Ratio
			Mean	S.D.	Mean	S.D.		
		1:10,000	1.394	0.0184	1.944	0.0481	0.55	1.39
	1	1:30,000	1.229	0.0141	1.809	0.0629	0.58	1.47
		1:10,000	1.109	0.0163	1.709	0.0255	0.60	1.54
1:50	5	1:30,000	0.997	0.0156	1.657	0.0134	0.66	1.66
		1:10,000	0.814	0.0559	1.534	0.0325	0.72	1.88
	1	1:30,000	0.742	0.0665	1.372	0.0156	0.63	1.85
		1:10,000	0.650	0.0332	1.310	0.0212	0.66	2.02
1:100	5	1:30,000	0.659	0.0375	1.309	0.0042	0.65	1.99
		1:10,000	0.744	0.0212	1.294	0.0375	0.55	1.74
	1	1:30,000	0.770	0.0148	1.270	0.0643	0.50	1.65
		1:10,000	0.626	0.0177	1.196	0.0085	0.57	1.91
1:1,000	5	1:30,000	0.514	0.0120	0.994	0.0007	0.48	1.93

Table 3.11 *Optimisation of ELISA parameters using 10 µg red mite protein per well for yolk IgY*

Primary Antibody	Blocking %	Secondary Antibody	Negative O.D.		Positive O.D.		Pos-Neg	Binding Ratio
			Mean	S.D.	Mean	S.D.		
		1:10,000	1.155	0.0410	2.045	0.0205	0.89	1.77
	1	1:30,000	0.951	0.0156	1.941	0.0884	0.99	2.04
		1:10,000	0.857	0.0283	1.917	0.0240	1.06	2.24
1:50	5	1:30,000	0.798	0.0064	1.908	0.0049	1.11	2.39
		1:10,000	0.462	0.0262	1.826	0.0453	**1.36**	**3.96**
	1	1:30,000	0.786	0.0771	1.796	0.0177	1.01	2.28
		1:10,000	0.555	0.0134	1.685	0.0396	1.13	3.04
1:100	5	1:30,000	0.787	0.0021	1.647	0.0106	0.86	2.09
		1:10,000	0.606	0.0431	1.476	0.0191	0.87	2.44
	1	1:30,000	0.674	0.0163	1.474	0.0940	0.80	2.19
		1:10,000	0.639	0.0346	1.419	0.0262	0.78	2.22
1:1,000	5	1:30,000	0.548	0.0106	1.358	0.0106	0.81	2.48

Numbers in bold represent optimum conditions

Table 3.12 *Optimisation of ELISA parameters using 15 µg red mite protein per well for yolk IgY*

Primary Antibody	Blocking %	Secondary Antibody	Negative O.D.		Positive O.D.		Pos-Neg	Binding Ratio
			Mean	S.D.	Mean	S.D.		
		1:10,000	1.126	0.0481	1.946	0.0219	0.82	1.73
	1	1:30,000	0.951	0.0205	1.801	0.0884	0.85	1.89
		1:10,000	0.774	0.0424	1.714	0.0304	0.94	2.21
1:50	5	1:30,000	0.671	0.0071	1.701	0.0078	1.03	2.54
		1:10,000	0.484	0.0184	1.604	0.0530	1.12	3.31
	1	1:30,000	0.578	0.0948	1.558	0.0255	0.98	2.70
		1:10,000	0.535	0.0269	1.435	0.0495	0.90	2.68
1:100	5	1:30,000	0.606	0.0106	1.416	0.0085	0.81	2.34
		1:10,000	0.516	0.0516	1.356	0.0269	0.84	2.63
	1	1:30,000	0.457	0.0240	1.227	0.0813	0.77	2.68
		1:10,000	0.479	0.0460	1.219	0.0389	0.74	2.54
1:1,000	5	1:30,000	0.378	0.0078	1.008	0.0127	0.63	2.67

3.2.3.4 Serum IgY cut-off point and normalisation

The cut-off point or level of response at which a sample is defined as positive or negative was determined using serum/yolk IgY samples from birds which had previously never been exposed to poultry red mite. This was carried out using serum/yolk IgY from 15 individual birds, over three consecutive ELISA runs and both mean and standard deviation (S.D.) calculated for each day (see Tables 3.13-3.14).

For serum IgY (Table 3.13) the mean cut-off value (Mean + 1.645 S.D.), calculated as the mean of all serum samples taken across all days, had a moderate variability (mean O.D. 0.333; C.V. 10 %). This fluctuation in O.D. within the pool of negative

samples resulted in the generation of a small number of false-positive results, although it encompassed 91 % of samples.

Table 3.13 *Determination of cut-off value for serum IgY (O.D. values in bold exceed the cut-off point)*

Sample	Day1	Day 2	Day 3
1	0.184	0.179	**0.318**
2	0.205	0.321	0.227
3	0.277	0.325	0.215
4	0.167	0.207	0.172
5	**0.419**	0.230	0.223
6	0.245	0.317	0.227
7	0.212	0.166	0.278
8	0.175	0.204	0.188
9	0.198	0.191	0.219
10	0.204	0.197	0.227
11	0.214	0.266	**0.310**
12	0.263	**0.372**	0.207
13	0.137	0.134	0.204
14	0.209	0.349	0.134
15	0.207	0.213	0.227
Mean:	0.221	0.244	0.225
S.D.	0.065	0.074	0.048
Mean+ 1.645 S.D.	0.328	0.367	0.304
Negative pool + 1.645 S.D.	0.315	0.264	0.279
Normalization factor	1.21	1.39	1.09

In order to correct the inherent inter-day drift, the cut-off point was normalised by comparing it to that of a pool of negative serum. The mean normalisation ratio (mean cut-off point/mean negative pool + 1.645 S.D.) for serum IgY was calculated at 1.23± 0.15.

3.2.3.5 Yolk IgY cut-off point and normalisation

In contrast to serum IgY, the variation of mean yolk IgY cut-off values calculated as the mean of all samples and successive reads, (Table 3.14) was relatively high (mean O.D. 0.664; C.V. 24 %). There was also the presence of a small number of false positives as a result of the discrepancy between negative optical density values, but as for serum IgY still managed to account for 91 % of all samples.

Table 3.14 *Determination of cut-off value for Yolk IgY (O.D. values in bold exceed the cut-off point)*

Sample	Day1	Day 2	Day 3
1	0.278	0.456	0.316
2	0.372	0.621	0.292
3	0.364	0.388	0.306
4	0.259	0.525	0.447
5	0.392	0.760	0.335
6	0.278	0.480	0.254
7	0.281	0.458	0.485
8	0.306	**0.988**	0.532
9	**0.588**	0.378	0.370
10	0.294	0.412	0.375
11	0.360	0.512	**0.754**
12	0.205	0.423	0.533
13	0.237	0.751	0.308
14	0.359	0.354	**0.669**
15	0.488	0.362	0.457
Mean:	0.337	0.524	0.429
S.D.	0.099	0.181	0.145
Mean+ 1.645 S.D.	0.501	0.823	0.668
Negative pool + 1.645 S.D.	0.531	0.596	0.517
Normalization factor	0.94	1.38	1.29

The normalisation factor (mean cut-off point/mean negative pool + 1.645 S.D.) for yolk IgY was calculated to be slightly lower than that of serum IgY at 1.21± 0.23.

3.2.4 Discussion

This section was aimed at determining optimum conditions for establishment of blood serum and yolk IgY ELISA assays. Results demonstrated that concentrations/dilutions and time parameters varied for respective IgY assays. Optimisation was determined at the point where both binding ratios were highest and also the difference between positive and negative samples was above the mean value. For blood serum this was apparent when using 10 µg red mite antigen per well, blocking with a concentration of 5 % BSA, incubation with primary antibody diluted 1:100 and secondary antibody diluted 1:30,000 and also subsequent to 15 min development in TMB.

The yolk-derived IgY ELISA assay displayed optimal results again when coating wells with 10 µg red mite antigen per well. However, this time blocking with a concentration of 1 % BSA. As with serum, incubation with primary antibody diluted

1:100, but secondary antibody diluted 1:10,000 and finally exposure with TMB for 20 min displayed highest binding ratios and positive-negative difference.

Following plate optimisation, validation of the cut-off point for negative samples was undertaken on 15 separate serum samples over three successive read points. Mire-Sluis *et al.* (2004) recommended that the cut-off value is determined as the overall mean negative optical density, plus 1.645 S.D., which in the case of the present study incorporated 91 % of negative samples for both serum and yolk IgY ELISA. By taking this cut-off value and dividing it against a pool of negative serum, a normalisation factor can be calculated. In this study the normalisation value was optimised at 1.23± 0.15 for serum IgY and 1.21± 0.23 for yolk IgY, both of these values can be used as a function to standardise future assays and where necessary will be recalculated for subsequent negative standards.

It was also evident from ELISA plate optimisation that there is a degree of non-specific binding resulting in a relatively high background optical density. This occurred irrespective of the level of blocking or antibody dilution and may result from the unusually high affinity of avian serum to plastic and polystyrene surfaces, and/or the nature of the antigen coated in the plate wells (Bauer *et al.*, 1999).

This section therefore illustrates the development of a functional immunoassay for direct screening of immunoglobulin samples. Subsequent studies using this ELISA assay, with either blood serum or yolk-derived IgY will employ the optimal parameters outlined here.

3.3 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.3.1 Introduction

SDS-PAGE is a common procedure used for the separation of proteins according to their molecular size, which gives rise to specific protein configurations. Once fractionated, SDS gels can act as a simple measure of comparing protein profiles between samples or can be further applied to immunoblotting/protein sequencing.

First described by Laemmli (1970), the application of SDS-PAGE is used as the foundation of much of the present day proteomics. SDS-PAGE has previously been used for the fractionation of proteins of a range of mite species, including those of the northern fowl mite (*Ornithonyssus sylviarum*, Devaney and Augustine, 1987), house dust mite (*Dermatophagoides pteronyssinus*, Stewart and Fisher, 1986), scabies mite (*Sarcoptes scabiei*, Petterson *et al.*, 2005) and sheep scab mite (*Psoroptes ovis*, Smith *et al.*, 2002).

Generally, an SDS-PAGE protocol involves initially extracting the desired protein, as described previously in Section 3.1. Once the protein of interest has been successfully obtained and quantified, a known concentration, typically 5-50 µg protein (Matthes *et al.*, 1996) is then added to a loading buffer. Loading buffers usually consist of 2-mercaptoethanol, SDS, glycine, Tris-HCl and bromophenol blue (Hou *et al.*, 2006), components which both reduce and denature proteins to allow their progression through gels. The 2-mercaptoethanol reduces the intra and inter-molecular disulfide bonds, whilst the SDS detergent denatures the proteins and gives each subunit an overall negative charge so that they will separate based on size along an electric gradient. The bromophenol blue serves as a dye front that runs ahead of the proteins and also assists sample visualisation during loading. Finally, the glycerol increases the density of the sample so that it will lay in the sample well (Sigma, Technical Bulletin, 2006). Samples are then further heat denatured at between 70-100°C for 2-10 min.

At the same time acrylamide-containing gels are constructed, typically consisting of 4 % acrylamide stacking gels and 8-20 % acrylamide running gels (Craig *et al.*, 1996; Huntley *et al.*, 2004), depending on the assumed size and nature of the proteins

being denatured. Once set, after approximately 10-20 min, these gels are submerged in an electrophoresis tank containing a running buffer, typically Tris-HCl, Glycine and SDS. Samples are then loaded into gel lanes and run at between 100-200 V, until the leading dye front reaches the bottom of the gel (Lee *et al.,* 2002). Following this electrophoretic separation, protein bands are visualised via staining, which is performed using Coomassie Blue or Silver stain, each involving fixing, staining and destaining steps. Once protein bands are stained the protein profiles can be compared. The aim of this chapter was, therefore, to describe the development of a protocol for the fractionation of poultry red mite extracts using SDS-PAGE, and in particular the evaluation of a new mini-gel electrophoresis system.

3.3.2 Materials and methods

This section describes the comparison of two SDS-PAGE systems, a vertical gel unit and a NuPAGE® mini-gel electrophoresis system, as well as the evaluation of both staining and loading preparations of protein extracts. Also, SDS-PAGE fractionation of proteins was carried out on soluble unfed whole mite proteins extracted in PBS buffer, as described previously (see Section 3.1).

3.3.2.1 Vertical gel unit SDS-PAGE

For initial electrophoretic separation, a vertical gel unit (Fisher scientific, Loughborough, UK) was utilized. In this system 20 x 17 cm 30 % acrylamide-bisacrylamide (29:1) gels were used, consisting of 4 % stacking and 12 % running gels. Protein extracts were mixed 2:1 with Laemmli sample buffer (4 % SDS, 20 % glycerol, 10 % 2-mercaptoethanol, 0.004 % bromophenol blue and 0.125 M Tris HCl, pH approx. 6.8) (Sigma, St. Louis, US) and denatured by boiling for 10 min at 100°C.

Three different titrations of red mite protein extract, approximately 10, 5 and 1 µg, were loaded into gel lanes and electrophoretic separation was performed in a running buffer (containing 25 mM Tris-HCl, 200 mM Glycine, 0.1 % (w/v) SDS). Electrophoresis was performed for 1 hour at 100 V until the dye front had reached the stacking/running gel interface. After which gels were run for a further 12 hours at 250 V at room temperature until the leading dye front reached the bottom of the gel.

3.3.2.2 NuPAGE® mini-gel electrophoresis system

This method of SDS-PAGE is a more recently developed system and has been used successfully on several occasions for fractionating proteins extracted from

ectoparasites (Lee *et al.*, 2002; Huntley *et al.*, 2004; Nisbet *et al.*, 2006a). As before, titrations of approximately 10, 5 and 1 µg mite protein extract were reduced in NuPAGE® LDS sample buffer (Invitrogen, Paisley, UK) and denatured for 10 min at 70°C. Samples were then loaded into pre-cast gradient 8 x 8 cm, NuPAGE® 4-12 % Novex Bis-Tris mini-gels (composed of Bis-Tris-HCl (pH 6.4), acrylamide, Bis-acrylamide, ammonium persulfate and ultrapure water). These were then run in NuPAGE® MOPS SDS (Invitrogen, Paisley, UK) running buffer for approximately 1 hour at 200 V and room temperature until the leading dye front reached the gel bottom.

3.3.2.3 Comparison of gel staining

Staining protocols for gels were compared on Novex Bis-Tris mini-gels, using either Coomassie Blue (Simplyblue stain, Invitrogen, Paisley, UK), or alternatively using a Silver staining kit (Sigma, St. Louis, US). Staining using Coomassie Blue involved submerging gels in 100 ml ultrapure water and microwaving for 1 min, which was repeated twice. After which gels were incubated with Coomassie Blue stain (Invitrogen, USA) for 10 min, at room temperature on an orbital shaker. Destaining was performed by placing gels in ultrapure water overnight, at room temperature to give optimum resolution.

Silver staining involved, firstly fixing proteins on gels using fixing solution (10 % 1.5 M acetic acid, 40 % ultrapure water, 50 % ethanol). This was followed by a wash step using an excess of ultrapure water. Subsequently, gels were washed in a sensitizer solution and a wash buffer. Finally, gels were developed using developer solution and stopped using 0.1 M HCl. Gel staining techniques were subsequently compared for protein profiles, and intensity/resolution of protein bands.

3.3.2.4 Loading preparations of protein extracts

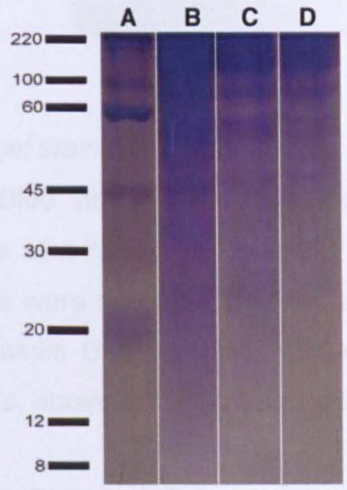
After establishing optimum gel and staining conditions, the means by which proteins were treated prior to electrophoretic separation, via either reduction or denaturation, was investigated. Using the NuPAGE® mini-gel electrophoresis system fractionation of red mite protein extracts was carried out under either reduced or non-reduced conditions. Non-reduced conditions were established by the emission of NuPAGE antioxidant (Invitrogen, Paisley, UK), but were otherwise the same as the reduced conditions. As for the comparison of denaturation, red mite protein extracts were considered non-denatured if samples were not heated for 10 min at 70°C step and samples were instead loaded directly into gel lanes.

3.3.3 Results

3.3.3.1 Vertical gel unit system

Analysis of gels following migration on 12 % acrylamide gels using the vertical gel unit and Coomassie Blue staining generated poor results (Figure 3.8). Gel lanes showed heavy smearing of proteins generating no clear bands. However, smearing was limited by reducing the concentration of protein per gel lane.

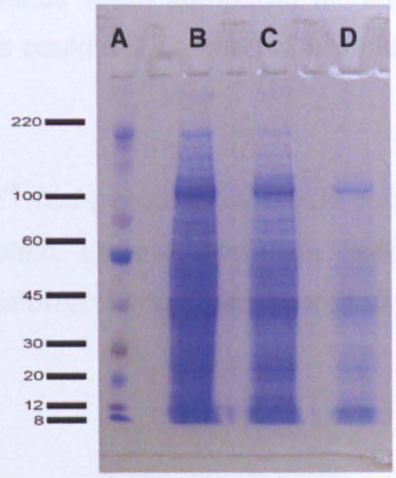
Figure 3.8 *SDS-PAGE gel of different red mite protein concentrations using vertical gel unit; Lane A, Molecular weight Marker (kDa); Lanes B, C and D unfed mite protein (10, 5 and 1 µg, respectively)*



3.3.3.2 NuPAGE® mini-gel electrophoresis system

For the NuPAGE® mini-gels, the greatest range and resolution of protein bands appeared when using 5 µg of protein per lane. Higher protein concentrations resulted in streaking, whereas lower concentrations failed to generate such an array of bands. Gel lanes containing 5 µg protein produced bands with a variety of molecular weights ranging from low (8 kDa) to high (220 kDa). At least 20 different sized bands were revealed, with several major bands identified at 20, 45, 60 and 120 kDa (Figure 3.9).

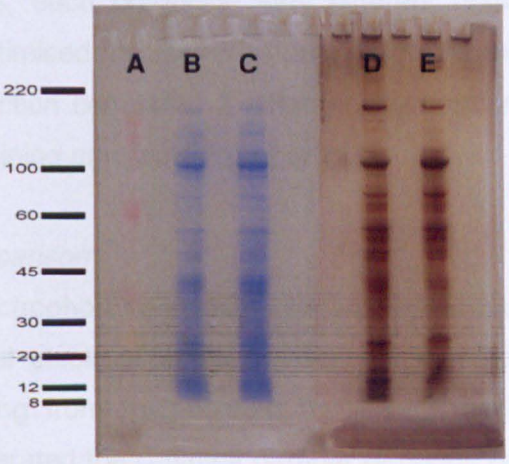
Figure 3.9 *SDS-PAGE gel of different red mite protein concentrations using NuPAGE® mini-gel electrophoresis system; Lane A, Molecular weight Marker (kDa), Lanes B, C and D unfed mite protein (10, 5 and 1 µg, respectively)*



3.3.3.3 Comparison of gel staining

Both the Coomassie Blue and Silver stain methods of staining NuPAGE gels provided multiple bands at a range of molecular weights (8 to 220 kDa). However, several additional bands were recognised when using Silver staining which were not revealed by the Coomassie Blue staining. These bands were seen particularly at heavy molecular weights, above 100 kDa (Figure 3.10).

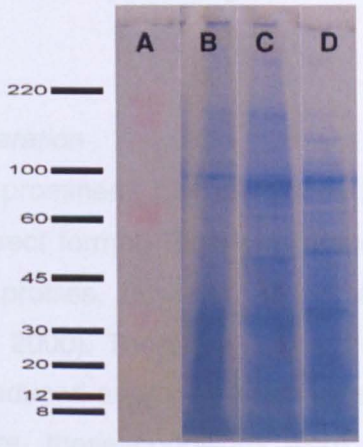
Figure 3.10 *Coomassie Blue and Silver staining using 5 µg of red mite protein on NuPAGE gel; Lane A, Molecular weight Marker (kDa), Lanes B and C, Coomassie Blue stained, Lanes D and E, Silver stained unfed mite protein*



3.3.3.4 Loading preparations of protein extracts

Investigation of protein preparation using non-reduced/non-denatured and reduced/non-denatured protein resulted in an increase in smearing and reduction in the total amount of bands when compared to reduced/denatured protein extracts. However, major bands could still be seen at around 20, 45, 60 and 120 kDa (Figure 3.11).

Figure 3.11 *NuPAGE® mini-gel electrophoresis comparing protein preparation using 5 µg of red mite protein. Lane A, Molecular weight marker (kDa); Lane B, non-reduced and non-denatured; Lane C, reduced and non-denatured; Lane D, reduced and denatured*



3.3.4 Discussion

Since there are numerous potential conditions for the fractionation of proteins using SDS-PAGE systems, each producing very different results, it was essential that parameters were optimised for use with poultry red mite extracts. In order for this to be achieved this section compared 2 different electrophoresis systems, as well as methods for both staining and protein preparation.

3.3.4.1 System comparison

Two SDS-PAGE electrophoresis systems were compared, both a vertical gel unit and a NuPAGE® mini-gel electrophoresis system. On comparison of Coomassie Blue stained gels resulting from fractionation using both systems, NuPAGE® mini-gel electrophoresis generated the greatest number of bands ranging from 8 to 220 kDa, with high resolution and intensity. The vertical gel unit on the other hand generated smeared streaks, a likely effect of extensive degradation resulting prolonged

electrophoresis running time necessary to achieve sufficient protein fractionation (Ortwerth *et al.*, 1988).

3.3.4.2 Comparison of staining

Comparison of stained gels revealed that Silver staining improved sensitivity of protein recognition as several additional bands were uncovered, particularly at higher molecular weights (100 to above 220 kDa). This difference in detection sensitivity is frequently observed with Coomassie Blue often failing to detect low-abundance proteins (Dzandu *et al.*, 1984; Lopez *et al.*, 2000). Therefore, Silver staining is often preferred for proteomic applications which require a high level of sensitivity, such as MALDI-TOF protein sequencing (Kang *et al.*, 2002). However, one draw back of Silver staining is that it requires a greater intensity of labour and higher cost of reagents.

3.3.4.3 Protein preparation

In order to achieve prominent, high resolution bands, proteins must be loaded into gel lanes in the correct format. Extensive reducing and denaturing often generates impressive protein profiles, however, is not always representative of the native protein (Pettit *et al.*, 2000). Therefore, it is not uncommon for proteins to be loaded into gels in a non-reduced and/or non-denatured form (Pettit *et al.*, 2000; Litman *et al.*, 2002). Therefore, these conditions were investigated using poultry red mite protein extracts. Contrary to previous research, stained gels revealed that optimal parameters for protein preparation appeared to be when proteins were both reduced and denatured (Pettit *et al.*, 2000; Litman *et al.*, 2002). Under these conditions the greatest array, resolution and intensity of proteins was observed.

3.3.5 Conclusion

Overall assessment of parameters for SDS-PAGE revealed that optimal conditions for fractionation of poultry red mite proteins resulted from using a NuPAGE® mini-gel electrophoresis system in combination with Silver staining, as well as reduction and denaturation of proteins.

3.4 Western blotting

3.4.1 Introduction

Western blotting is a fundamental immunological tool used for the screening of specific host antibodies against invading antigens. A great deal of research has been conducted on the immune response of hosts to either natural or artificial exposure to parasitic antigens, for which western blotting has been used widely (Lee *et al.*, 2002; Weber *et al.*, 2003). Western blotting is frequently employed in vaccine development, in particular for the recognition of potential antigen candidates and has been successfully used for the characterisation of a range of ectoparasitic antigens (Arlain *et al.*, 1995; Lal *et al.*, 2001; Sam-Sun *et al.*, 2002; Van der Broek *et al.*, 2003).

Since western blotting forms an integral part of the screening of vaccine candidates, a western blot was developed in this study for the analysis of immunogenicity of poultry red mite antigens. The methodology set out here was modified from a number of previously documented western blot protocols which utilized western blotting for a number of different arthropod species (Minnifield *et al.*, 1993; Matthes *et al.*, 1995; Lee *et al.*, 2002; Huntley *et al.*, 2004).

3.4.2 Materials and methods

3.4.2.1 Protein transfer and blotting

Following protein fractionation using SDS-PAGE, as described previously in Section 3.3, gels were subject to western blotting. Electrophorised protein bands were transferred to 7 x 10 cm nitrocellulose membranes (Sigma, St Louis, US) in a mini-gel transfer unit in ice cold NuPAGE® transfer buffer (Invitrogen, Paisley, UK) at 30 V for 50 min at room temperature. They were then submerged in western blocker solution (Sigma, St Louis, US), placed on a shaking platform and held overnight at 4°C. The following day, membranes were cut into strips and placed in blood sera diluted 1:100 in western blocker solution and held for 45 min in an orbital shaker at room temperature. Blood serum had been previously collected from birds shortly after cervical dislocation and bleed directly from the heart. Serum was deemed as either negative control serum, i.e. from day-old chicks which had not previously been exposed to poultry red mite. Positive serum was collected from 71 week-old laying hens which had been previously exposed to red mite infestation. For this experiment three replicates were used for both control and positive serum.

After incubation with serum, nitrocellulose strips were then washed 3 times with Tris Buffered Saline-Tween-20 (TBS-T) (20 mM Tris, pH approx. 7.4, and 0.9 % NaCl) (Sigma, St. Louis, US) and incubated for a further 45 min with horse radish peroxide labelled rabbit anti-chicken IgY (Sigma, St. Louis, US) at a series of dilutions (1:15,000, 1:30,000, 1:50,000 and 1:100,000) again at room temperature. Following incubation with secondary antibody, nitrocellulose strips were again washed 3 times in TBS-T and visualised by one of three staining methods.

3.4.2.2 Membrane development

Typically methods are based on either simple colorimetric or chemiluminescent detection (Constantine *et al.*, 1994; Cusak *et al.*, 2001; De Bleser *et al.*, 2003; Cepok *et al.*, 2005). These were compared here using colorimetric 3,3',5,5'-Tetramethylbenzidine (TMB; Sigma, St Louis, US) and 3,3-diaminobenzidine tetrahydrochloride (DAB; Sigma, St Louis, US) development and enhanced chemiluminescent ECL (Amerham, Buckinghamshire, UK).

TMB is supplied as a ready to use substrate, which is poured directly onto the membrane and the reaction stopped when bands are visible by adding distilled water.

DAB was prepared by diluting 25 mg of DAB in 2 ml of distilled water. This solution was then mixed with 48 ml phosphate buffered saline containing 45 µl hydrogen peroxide. Blots were incubated at room temperature with this solution for 1–3 min and the reaction terminated by adding excesses of distilled water (Artuch *et al.*, 2003).

ECL detection was carried out by adding equal volumes of pre-formulated developer solutions to nitrocellulose membranes for 1 min at room temperature. The membrane was removed and placed between 2 sheets of transparent film, which was in turn placed inside an x-ray film cassette, protein side up. The next stage was carried out under red light, whereby a sheet of autoradiography film (Kodak, Rochester, USA) was placed on top of the membrane. The cassette was then closed and the autoradiography film exposed for 15 seconds. Exposed films were then submerged in a developer solution (Kodak, Rochester, USA) for 5 min, followed by submersion in a fixing and replenishing solution (Kodak, Rochester, USA) for 5 min, and a final wash in distilled water.

3.4.2.3 Non-specific binding of secondary immunoglobulins

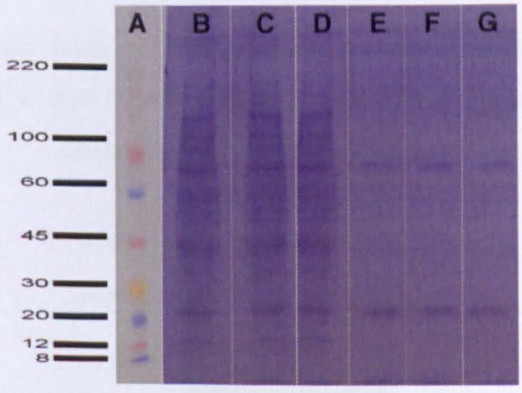
In order to test for non-specific binding of secondary horse radish peroxide labelled rabbit anti-chicken IgY, blots were performed with the expulsion of primary serum antibodies. Rather than incubating with serum, subsequent to red mite antigen fractionation and transfer using both PBS and urea mite extracted protein, nitrocellulose membranes were immediately incubated with secondary antibody and then developed using TMB.

3.4.3 Results

3.4.3.1 Protein transfer and blotting

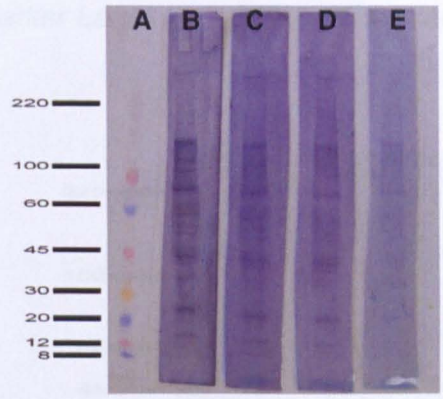
Western blots using serum from red mite exposed birds showed multiple bands where mite antigens were recognised by polyclonal serum immunoglobulins (Figure 3.12). A complex series of bands was observed at a range of 8-220 kDa, with major bands at 15, 20, 40, 70 and 120 kDa. Control serum did not display the same number of bands, although some non-specific recognition was seen at approximately 60 and 20 kDa.

Figure 3.12 *Western blot comparing 3 naturally exposed chicken sera (Lanes B-D) against control sera from 3 control birds (Lanes E-G) and molecular weight marker (kDa) (lane A)*



In order to optimise blotting profiles, decreasing concentrations of HRP labelled secondary rabbit anti-chicken IgY immunoglobulins were used (Figure 3.13). The effect that this had on band recognition was simply to reduce overall intensity, although at higher concentrations this difference was only very slight.

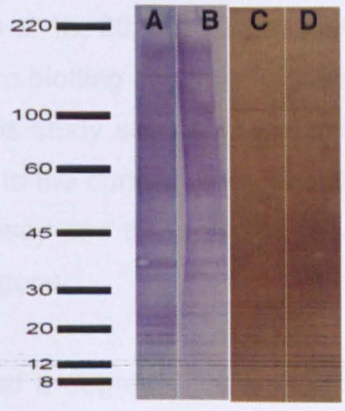
Figure 3.13 *Western blot showing decreasing concentrations of HRP labelled rabbit anti-chicken IgY secondary antibody (lane B: 1:15,000; Lane C: 1:30,000; Lane D: 1:50,000; Lane E: 1:100,000) with molecular weight marker (kDa) (Lane A)*



3.4.3.2 Membrane development

Blots were successfully visualised using TMB and DAB (Figure 3.14), however, ECL failed to recognise protein bands and remained blank. Both TMB and DAB allowed the detection of major bands at 15, 20, 40, 70 and 120 kDa. However, in order to generate sufficient banding using DAB, development was carried out over several hours, increasing background staining significantly, which compromised overall visualisation. TMB staining generated an overall greater sensitivity with the detection of numerous minor bands ranging between 8-220 kDa in comparison to DAB development.

Figure 3.14 *Western blots following development using; Lanes A and B were visualised with TMB, C and D with DAB with molecular weight marker (kDa)*

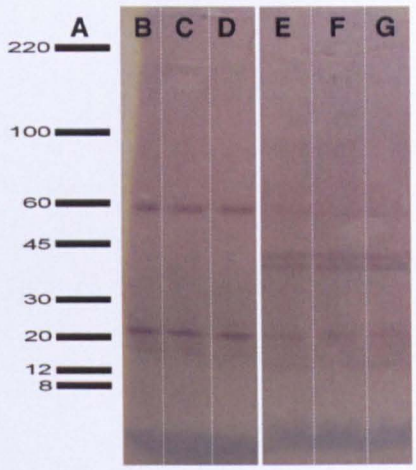


3.4.3.3 Non-specific binding of secondary immunoglobulins

Western blotting with the expulsion of serum revealed antibodies binding directly to mite antigens at 60 and 20 kDa for PBS fractionated protein. Urea fractionated

proteins on the other hand revealed a dimmer at approximately 40 kDa and faint bands at both 20 and 60 kDa (Figure 3.15).

Figure 3.15 *Western blot analysis with the elimination of polyclonal serum; Lane A, molecular weight marker Lanes B-D (kDa), PBS mite extract; Lanes C-G, urea mite extract*



3.4.4 Discussion

Western blotting using serum from chickens which had been naturally exposed to the poultry red mite was successfully conducted in the current study. Visualisation of proteins was improved when using higher concentrations of secondary anti-chicken antibodies (1:15,000-1:30,000) and TMB colorimetric detection.

Optimisation of western blotting protocol allowed detection of a series of immunodominant bands at 15, 20, 40, 70 and 120 kDa. To date only one other study has documented western blotting on whole mite antigens from poultry red mite (Sam-Sun *et al.*, 2002). In this study several major antigens were recognised (17 to 110 kDa) in a similar range to the current study (8 to 220 kDa). Thus illustrating both the reproducibility of the assay and the potential for subsequent use of whole red mite extracts as vaccine antigens.

This study also detected a degree of non-specific antigen recognition, where blots were performed without polyclonal serum. Here secondary anti-chicken antibodies directly reacted with mite fractions. This has previously been observed and was attributed non-specific recognition of chicken blood protein residues present in mite extracts from remnants of previous blood-meals (Devaney and Augustine, 1987).

Therefore, future analysis using western blotting took this into consideration. However, the general methodology outlined in this section was used as the standard protocol for subsequent western blots.

3.5 Immunoglobulin-Y (IgY) extraction

3.5.1 Introduction

IgY is the predominant avian immunoglobulin, equivalent to mammalian IgG. It is transferred from serum to egg yolk to confer passive immunity to embryos and neonates (Karlsson *et al.,* 2005). The advantages IgY offers over conventional antibody production are well documented (Akita and Nakai, 1992; Gee *et al.,* 2003; Zhang, 2003; Karlsson *et al.,* 2004; Guang-Ping *et al.,* 2005). Such advantages arise largely due to phylogenetic distance between birds and mammals, resulting in elimination of cross-reactivity between avian IgY and mammalian IgG (Jensenius *et al.,* 1981; Svensden *et al.,* 1995). IgY is a readily available, easily accessible source of antibody, with production levels being much greater than the mammalian equivalent, IgG (Schade *et al.,* 1994). Such a high level of production also reduces the requirement for large numbers of animals when sampling, and eradicates the invasive and painful collection of blood (Schade *et al.,* 1996). In addition the level of yolk-derived IgY has been shown to reflect fluctuations found in blood serum and so acts as a gauge of humoral immunity (Mohammed *et al.,* 1986; Hagan *et al.,* 2004).

Numerous methods for the extraction and purification of functionally active avian antibodies from egg yolk have been documented all of which differ in the yield, purity and material cost (Jensenius *et al.,* 1981; Polson *et al.,* 1985; Akita and Nakai, 1992). Several methods are used based on the separation of livetins, proteins which are believed to be IgY, from lipoproteins and the rest of the yolk lipids using organic solvents such as chloroform (Ntakarutimana *et al.,* 1992). Other methods are based on affinity chromatography (Verdoliva *et al.,* 2000), ion exchange chromatography or salt precipitations using salts such as ammonium sulfate, polyethylene glycol (PEG), dextran sulfate, dextran blue, sodium sulfate, caprylic acid and sodium citrate (Bizhanov *et al.,* 2004).

The requirement for the current study was to create a reproducible, cost effective and rapid method of determining IgY level for subsequent use in immunological assays. Therefore, two of the most routinely used extraction protocols were explored, ammonium sulfate precipitation (Akita and Nakai, 1992) and PBS/chloroform extraction (Mohammed *et al.,* 1986).

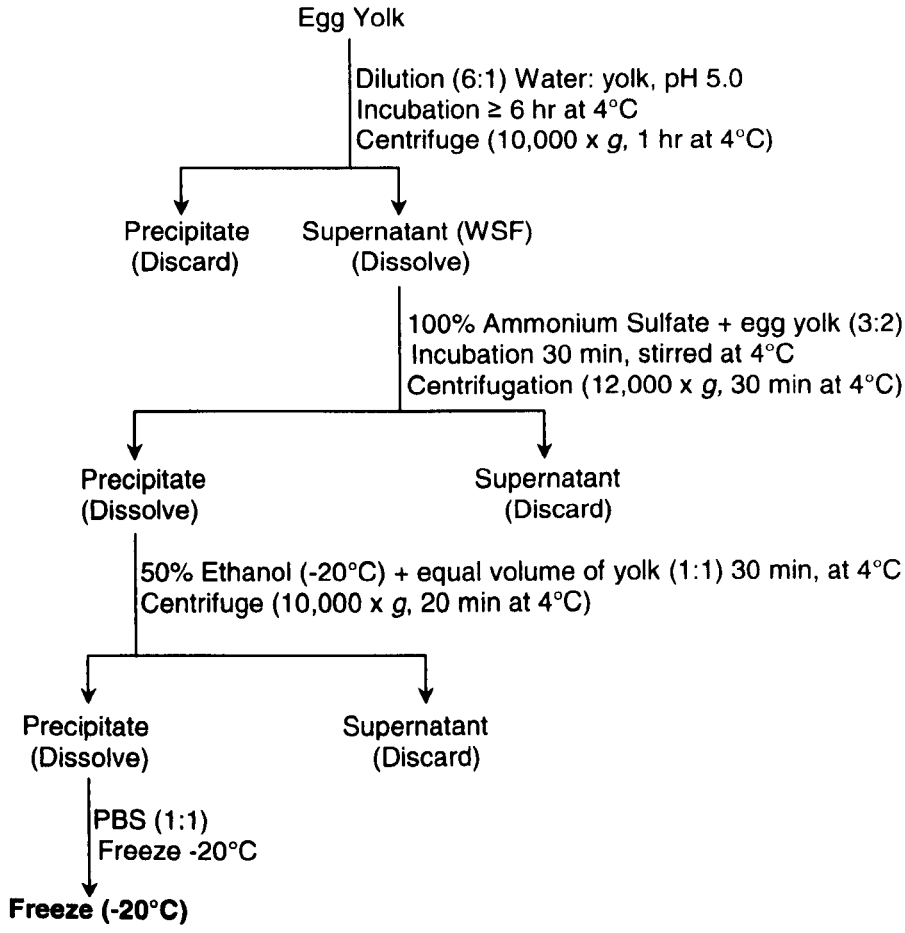
3.5.2 Materials and methods

3.5.2.1 Ammonium sulfate precipitation of egg yolk IgY

Antibodies were isolated from egg yolk following a protocol modified from Akita and Nakai (1992). All eggs were obtained from a commercial laying flock in Northumberland and brought to the laboratory, where they were cracked open to allow separation of the yolk from the white, followed by washing with distilled water and rolling on Whatman filter paper to remove any adhering albumen. The yolk membrane was then punctured using a glass Pasteur pipette and the yolk allowed to flow into a graduated centrifuge tube and the yolk membrane disposed. Egg yolk was then diluted 6-fold with distilled water (acidified with 0.1N HCl, to give a pH of 5.0) and held for 6 hours at 4°C before centrifugation (10,000 x *g* for 1 hour at 4°C).

The resulting immunoglobulin-containing supernatant was further purified using ammonium sulfate precipitation. Here ammonium sulfate was added to the extract at 60 % saturation (0.370 g/ml ammonium sulfate solution) and held for 30 min at 4°C. The antibody precipitate was spun out (12,000 x *g*, 30 min, at 4°C), the supernatant was discarded and the pellet re-dissolved in an equal volume of 50 % ethanol pre-cooled to -20°C. The suspension was then held at 4°C for 30 min before being centrifuged (10,000 x *g* for 20 min at 4°C). The final solution was then added to equal volumes of PBS and stored at -20°C until required for subsequent use (see Figure 3.16). This method of extraction was performed on 10 replicate egg yolk samples.

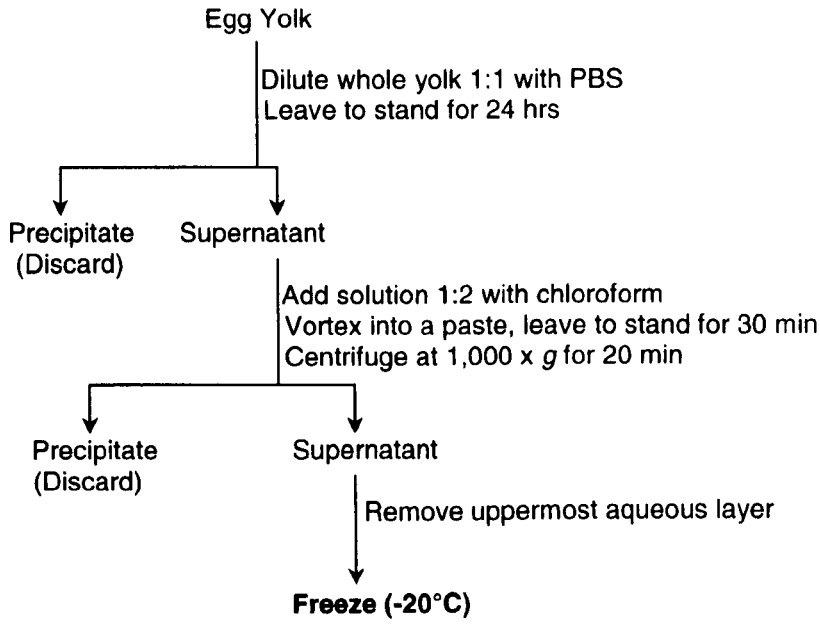
Figure 3.16 *Schematic diagram of ammonium sulfate precipitation of yolk IgY*



3.5.2.2 PBS/Chloroform extraction of egg yolk IgY

This method of antibody extraction was taken from the method developed by Mohammed *et al.* (1986). As before, egg yolk was separated from the white, washed in distilled water and rolled on Whatman filter paper to remove adhering albumen. The membrane was then punctured using a glass Pasteur pipette and the yolk allowed to flow into a graduated centrifuge tube. The yolk was then mixed with an equal volume of PBS, agitated in a vortex shaker and held overnight at 4°C. After settling, the solution was then mixed with chloroform (2:1), vortexed until a thick paste formed and left at room temperature for 30 min. Following this, the homogenate was centrifuged at 1,000 x *g* for 20 min. The resulting solution comprised of three layers, of which the upper immunoglobulin containing layer was removed and frozen at -20°C for subsequent use (see Figure 3.17). As with ammonium sulfate precipitation, this method of extraction was performed on 10 replicate egg yolk samples.

Figure 3.17 *Schematic diagram of PBS/Chloroform antibody extraction of yolk IgY*



3.5.2.3 Total IgY estimation

Concentrations of total IgY were determined for both ammonium sulfate precipitated and PBS/chloroform extracted proteins by a Bradford assay according to the manufacturer's instructions (Sigma, St. Louis, US). The sample absorbance values were read for all 20 samples using an eppendorf Biophotometer (Eppendorf, Hamburg, Germany). The protein concentration of each sample was determined from a standard curve generated by a range of bovine serum albumin (BSA) concentrations (Sigma, St. Louis, US) from 0.2 to 1.0 mg/ml. Sample and BSA dilution were prepared in phosphate-buffered saline (PBS).

3.5.2.4 Determination of IgY purity

The purity of IgY extracts was compared using SDS-PAGE, as described previously (see Section 3.3). Briefly, 20 µg of denatured yolk extract in sample buffer were loaded into NuPAGE® Novex Bis-Tris mini-gel lanes and run in NuPAGE® MOPS running buffer (Invitrogen, Paisley, UK) under reducing conditions for approximately 1 hour at 200 V and room temperature. Gels were then stained in Coomassie Blue and destained in deionised water to allow visual determination of protein profiles.

3.5.3 Results

3.5.3.1 Total IgY estimation

The concentration of protein generated using both ammonium sulfate precipitation and PBS/Chloroform extraction was calculated using the Bradford method. Results demonstrated that proteins extracted using the PBS/chloroform method consistently generated higher yields, with the mean concentration being almost 5 times greater when employing extraction via chloroform. The individual sample variation in terms of the S.E. Mean, however, was greater after PBS/chloroform extraction (Table 3.15).

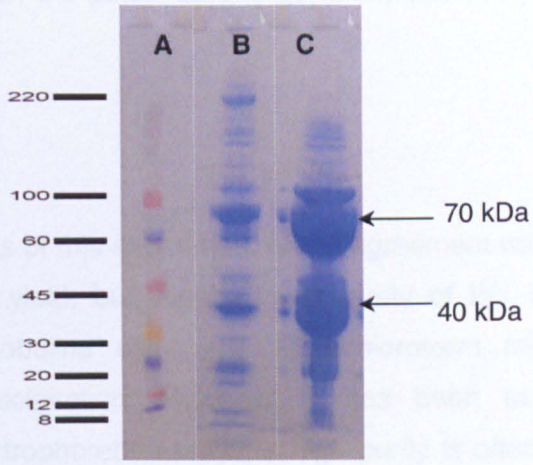
Table 3.15 *Comparison of protein concentrations of IgY extracts using the Bradford assay*

Egg No.	Extraction	
	Ammonium sulfate (mg/ml)	**PBS/Chloroform (mg/ml)**
1	1.1	5.7
2	0.7	5.1
3	0.4	3.7
4	1.0	4.3
5	1.5	4.0
6	0.7	4.2
7	0.5	4.6
8	1.3	3.5
9	0.9	3.8
10	1.0	4.6
Mean	**0.9**	**4.4**
S.E. Mean	**0.11**	**0.21**

3.5.3.2 Determination of IgY purity

Immunoglobulin extraction was compared by SDS-PAGE and subsequently visualised using Coomassie Blue stain. When analysing protein bands on stained gels it was apparent that the two extraction methods produced similar profiles. With bands on both gels visualised at around 20, 40 and 70 kDa. However, it appeared that precipitation using ammonium sulfate resulted in the elimination of several additional bands at heavier molecular weights, in particular an intense band at approximately 220 kDa (see Figure 3.18).

Figure 3.18 *SDS-PAGE comparison of IgY extraction methods (5 µl per lane); Lane A: Molecular weight marker (kDa); Lane B: PBS/Chloroform extraction; Lane C: Ammonium sulfate extraction*



3.5.4 Discussion

Since yolk-derived IgY is an accessible and abundant source of avian antibody, the aim of this experiment was to compare two commonly practiced extraction protocols for subsequent use in immunological assays. Optimum parameters for extraction were determined via the concentration and purity of protein extracts.

Mean IgY concentrations were observed to be higher for the PBS/chloroform extraction than for the ammonium sulfate method (4.4 mg/ml and 0.9 mg/ml, respectively). This remains consistent with previous studies where levels have been recorded at between 1.6 and 4.0 mg/ml for the respective extraction methods (Bizhanov *et al.,* 2004). However, higher IgY levels have also been observed in other studies, particularly for chloroform extraction with levels reaching between 7-9 mg/ml (Verdoliva *et al.,* 2000; Bizhanov and Vyshniauskis, 2000). Nonetheless, the current study observed almost a 5-fold increase in total IgY yield when using chloroform over ammonium sulfate precipitation.

In order to determine the purity of IgY extracts, SDS-PAGE was carried out. The two methods of extraction produced a similar SDS-PAGE profile and both showed major bands at 20, 40 and 70 kDa. These large bands are frequently observed at these approximate weights and are regarded as the major bands representing IgY proteins (Guang-Ping *et al.,* 2005; Bizhanov *et al.,* 2004; Bizhanov and Vyshniauskis, 2000). A number of minor protein bands, between 8 and 220 kDa were also present in the

current study, particularly when extracting using the PBS/chloroform method. This has been identified as a common problem when precipitating with chloroform, which often results in up to 20 % protein impurities (Guang-Ping *et al.*, 2005). Precipitation via ammonium sulfate on the other hand is generally seen to yield fewer impurities (Bizhanov *et al.*, 2004).

3.5.5 Conclusion

In conclusion, the results of this experiment are in agreement with previous research, in that a much higher yield, but slightly lower purity of IgY was observed when precipitating immunoglobulins using the PBS/chloroform method compared to ammonium sulfate precipitation. However, it has been suggested that when conducting immunoelectrophoretic assays, a high purity is often less important than specificity (Bizhanov *et al.*, 2004). In addition, relative labour intensity and cost of the two extraction protocols favours that of chloroform extraction which is inexpensive and simple to perform. Therefore this method will be used for the extraction of IgY for subsequent immunological analysis.

3.6 *In vitro* red mite feeding

3.6.1 Introduction

In vitro feeding systems have been successfully developed across a variety of arthropod species (Crystal, 1986; Carroll *et al.,* 1992; Waladde, 1996; Patarroyo *et al.,* 2002). There are numerous advantages associated with the development of such systems, not least the ethical perspective of reducing the number of animals required for experiments. They also allow reliable and reproducible testing of therapeutic agents, the chance to establish dynamics of pathogen/disease, as well as the possibility of biological studies allowing determination of parasite life cycle parameters.

A number of previous studies have attempted to maintain a population of red mite under *in vitro* conditions, with varying degrees of success (Kirkwood, 1971; Zeman, 1988; Bruneau *et al.,* 2001; McDevitt *et al.,* 2006a and McDevitt *et al.,* 2006b). In these studies feeding rates were seen to range from 30-70 %, using similar conditions (day-old chick skin, 40°C, 60-95 % RH, and heparinized blood). It was therefore essential that optimal and repeatable conditions for feeding and fecundity were established in the current study for red mite allowing the rapid comparison of the effectiveness of subsequent treatments.

3.6.2 Materials and methods

3.6.2.1 Source of poultry red mite

Samples of red mite used were collected from commercial poultry houses using established trapping methodology (Section 4.2.3). Red mite were brought back to the laboratory and maintained in an incubator at 30°C, approximately 60-95 % RH and total darkness. They were stored under these conditions for a minimum of 7 days to allow them to reach an unfed status, characterised by their change of colour from red to grey, which allowed elimination of the effects of any previous blood-meals (Bruneau *et al.,* 2001).

3.6.2.2 Feeding membrane

The feeding membranes used were obtained from a range of sources. Firstly the skin of 60 week-old laying hens and one day-old chicks was used. In order to obtain these membranes birds were dispatched via cervical dislocation and the desired area of

skin plucked and carefully removed using a scalpel and surgical scissors. All subcutaneous fat and tissue debris was removed from the underside of the skin, washed in saline and dried on paper towel. Skins were then stored at either 4°C or frozen at -20°C for subsequent application. Secondly, Parafilm® M (SPI Supplies, West Chester, US) was tested as a potential skin replacement. In order to mimic chicken skin as closely as possible Parafilm® M was placed in a sealed polythene bag and refrigerated overnight at 4°C with chicken skin and used the following day. This allowed the potential impregnation of some of the pheromones and skin surface lipids which have been demonstrated to play a vital role in host recognition (Zeman, 1988).

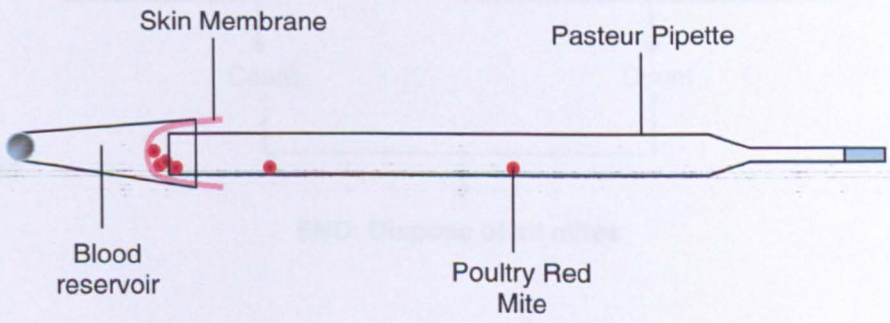
3.6.2.3 Source of blood

Blood was taken from broiler birds, which had previously not been exposed to red mite. Whole blood was drawn directly from the heart once death had been confirmed (dispatched via cervical dislocation) and immediately placed in vacutainers coated with lithium heparin (Becton Dickinson vacutainer systems, Oxford, UK). This blood was used either immediately or stored at 4°C for use in subsequent feedings.

3.6.2.4 Feeding system

The feeding device used was previously described by Bruneau *et al.* (2001), and was composed of a glass Pasteur pipette, blocked at one end with a fine weave gauze (to prevent red mite escaping) into which mites were aspirated. The narrow pipette end was then blocked using cotton wool. To allow red mite to feed, the gauze covering the large opening was removed and replaced with a substitute membrane, which was stretched over the end and secured using a pipette tip. The pipette tip acted dually as a blood reservoir and a seal (see Figure 3.19).

Figure 3.19 In vitro *feeding device for the poultry red mite*

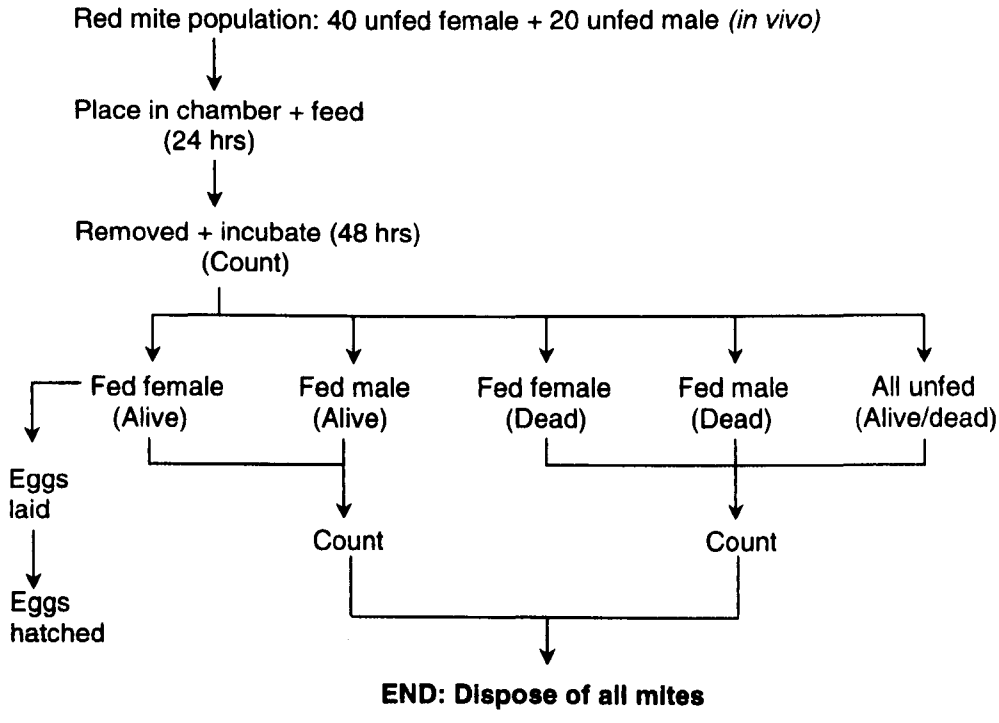


3.6.2.5 Feeding trial

For the purpose of determining optimal feeding and fecundity rates, the three different feeding membranes were tested with blood from three separate birds naïve to red mite. These were replicated three times per bird, giving 27 replicates overall.

A total of 60 red mite (40 female and 20 male) were placed inside each feeding device and the blood reservoir filled. The feeding devices were then placed on an orbital shaker at 30°C in a dark room for 12 hours, in order to simulate conditions for red mite, mimicking those in poultry housing systems. Relative humidity was not controlled, but casual readings indicated that it ranged from 65-90 % during this experiment. After this feeding period, blood reservoirs were removed, replaced with gauze and the ratio of fed to unfed red mite was established, along with total mortality of mites. Unfed and dead red mite were removed, whilst fed female mites were maintained in an incubator for a further 48 hours to establish oviposition rates and eggs were monitored to determine hatching rate (Figure 3.20). The relative levels of survival and mortality observed for each feeding device were calculated as percentages for separate male/female and fed/unfed mite populations.

Figure 3.20 *Schematic diagram of counting schedule for* in vitro *feeding of red mite*



3.6.3 Results

No poultry red mite fed on the skin from either 60 week-old chickens or the Parafilm® M. Mites did, however, engorge on day-old chick skin membranes. This feeding membrane was therefore used to investigate survival and fecundity of red mite, results for which are given in Tables 3.16-3.18. Table 3.16 presents data on the number of females recovered and illustrates that 60 % of the female mites fed, with individual variation between feeding devices ranging from 46-84 %. Out of the 60 % of female red mite which fed, 17 % died, with the total combined mortality of both fed and unfed mites of 37 %.

Table 3.17 on the other hand gives data for male red mite numbers for the duration of the feeding trial. Fewer males, 54 %, were observed to feed, in comparison to female, with a larger variation (33-71 %), giving an standard error of 13.45 (n = 9), compared to 4.52 (n = 9) for females. 20 % of these fed male mites died, generating an accumulated mortality the same as for females (37 %), although once again with a larger S.E. mean (n = 9) than for females (13.18 and 3.89, respectively).

Table 3.18 displays results for total red mite survival and fecundity for male and female mites. Overall mean engorgement was 58 % (49-80 %), whilst mortality was 37 % (20-51 %). Full recovery of mites was not observed as some were lost during membrane removal and counting procedures, consequently recovery rates were varied from 67-100 %, with a mean of 89 %. Finally, mean oviposition rate was 0.5 per mite, therefore, in theory half of all females laid at least one egg, all of which subsequently hatched into larvae.

Table 3.16 In vitro *survival rates for **female** poultry red mite 48 hours after engorgement (numbers shown are percentages of the total female population)*

Bird No.	Live Fed	Live Unfed	Dead Fed	Dead Unfed	Total Fed (Alive + Dead)	Total Unfed (Alive + Dead)	Total Alive (Fed + Unfed)	Total Dead (Fed + Unfed)
1	23	31	23	23	46	54	54	46
1	38	8	15	38	54	46	46	54
1	45	5	16	34	61	39	50	50
2	38	28	18	15	56	44	67	33
2	33	44	14	8	47	53	78	22
2	38	25	13	25	50	50	63	38
3	66	13	19	3	84	16	78	22
3	42	19	22	17	64	36	61	39
3	64	8	15	13	79	21	72	28
Mean:	**43**	**21**	**17**	**19**	**60**	**40**	**63**	**37**
S.E. Mean	**4.64**	**4.33**	**1.18**	**3.85**	**4.52**	**4.52**	**3.89**	**3.89**

Table 3.17 In vitro *survival rates for **male** poultry red mite 48 hours after engorgement (numbers shown are percentages of the total male population)*

Bird No.	Live Fed	Live Unfed	Dead Fed	Dead Unfed	Total Fed (Alive + Dead)	Total Unfed (Alive + Dead)	Total Alive (Fed + Unfed)	Total Dead (Fed + Unfed)
1	30	25	25	20	55	45	55	45
1	50	14	21	14	71	29	64	36
1	26	21	21	32	47	53	47	53
2	17	56	17	11	33	67	72	28
2	21	29	29	21	50	50	50	50
2	35	15	20	30	55	45	50	50
3	53	29	18	0	71	29	82	18
3	29	47	12	12	41	59	76	24
3	53	21	16	11	68	32	74	26
Mean:	**35**	**28**	**20**	**17**	**54**	**46**	**63**	**37**
S.E. Mean	**4.62**	**4.74**	**1.67**	**3.36**	**13.45**	**13.45**	**13.18**	**13.18**

Table 3.18 In *vitro survival and fecundity rates for* ***all*** *(female plus male) poultry red mite 48 hours after engorgement (numbers shown are percentages of the total population)*

Bird No.	% Recovered	All Fed	All Unfed	Total Alive	Total Dead	Eggs/female	% Hatched
1	98	49	51	54	46	0.8	100
1	67	60	40	53	48	1.3	100
1	95	56	44	49	51	0.2	100
2	95	49	51	68	32	0.4	100
2	83	48	52	70	30	0.8	100
2	100	52	48	58	42	0.5	100
3	82	80	20	80	20	0.4	100
3	88	57	43	66	34	0.1	100
3	97	76	24	72	28	0.5	100
Mean:	**89**	**58**	**42**	**63**	**37**	**0.5**	**100**
S.E. Mean	**3.17**	**3.52**	**3.52**	**3.08**	**3.12**	**0.36**	-

3.6.4 Discussion

Preliminary experiments using an artificial feeding device revealed that the poultry red mite were observed to feed only on day-old chick skin membranes and not on skin from 60 week-old chickens or on Parafilm® M. This corresponds with what has been previously documented by Bruneau *et al.* (2001) and Zeman (1988). Zeman (1988) suggested that in order for red mite to recognise and feed on particular membranes certain surface skin lipids must be present. These lipids are produced in the uropygial gland and frequently play a role in pathogen recognition, not only with mites, but also bacteria, fungi and worms (Bandyopadhyay and Bhattacharyya, 1996; Bandyopadhyay and Bhattacharyya, 1999; Haas and Van de Roemer, 2004). Other membrane characteristics, such as texture, elasticity, affinity to lipids, amongst others, may also have a role in mite feeding (Zeman, 1988). This might potentially explain the failure of red mite to feed on skin from 60 week-old chickens, but successful engorgement on day-old chick skin (Bruneau *et al.*, 2001).

Once the most appropriate skin membrane was established, survival and fecundity were assessed. Feeding success varied slightly between replicates, however, on average about 58 % of mites were seen to feed (females, 60 % and males, 54 %). This rate is higher than those previously recorded where rates of feeding were 39.5 % and 47.5 % in Zeman (1988) and Bruneau *et al.* (2001), respectively. However, in a recent paper, red mite feeding rates were increased to 70 % following 4°C refrigeration of mites for 30 days (McDevitt *et al.*, 2006b).

In the current study, the mean natural mortality rate was 37 % for all mites, both fed and unfed (18.5 % when corrected for only those red mite which had fed). These rates are slightly higher than those from previous studies, where mortality was observed at only 13 % for all red mite (McDevitt *et al.*, 2006b) and 5-15 % when adjusted for engorgement (Bruneau *et al.*, 2001; McDevitt *et al.*, 2006b).

Oviposition rate is a parameter which has been rarely documented for the poultry red mite. The number of eggs laid per female in the current study was 0.5, which was slightly lower than that observed previously of 0.7-2.9 eggs per female by Bruneau *et al.* (2001). However, the same authors reported that the higher levels of egg laying were due to repeated blood-meals offered 4 times at 24 hour intervals.

In conclusion, a reliable *in vitro* feeding system for the poultry red mite has been developed. A baseline of red mite survival and fecundity has been determined, against which subsequent treatments can be compared, including the screening of potential antigens for use in vaccine development.

3.7 Taqman RT-PCR

TaqMan reverse transcription polymerase chain reaction (RT-PCR) is a recently developed technique which allows the measurement of an accumulating PCR product in real time (Medhurst *et al.*, 2000). It can be used to determine the level of expression of any gene provided that both RNA and suitable primers are available. Taqman RT-PCR has been used to assess the vector capacity of ectoparasites, including the red mite (Mumcuoglu *et al.*, 2006), and also investigate host immune response to parasitic invasion (Goodridge *et al.*, 2001). As such it was used in the current study to evaluate the levels of specific cytokines expressed in spleen tissue of poultry exposed to red mite antigens.

3.7.1 Introduction

TaqMan PCR assays were performed in triplicate on cDNA samples in 96-well optical plates using an ABI PRISM^{TM} 7700 sequence selection system (Applied Biosystems). The ABI Prism 7700 directly detects RT-PCR products by monitoring the fluorescence of a target-specific dye-labelled probe. The probe anneals to a target sequence amplified by forward and reverse primers during the PCR. As the probe is displaced during the PCR when the 5' end is cleaved by the 5' nuclease activity of DNA polymerase, causing separation of a reporter and quencher dye, resulting in increased fluorescence. The fluorescent accumulation of PCR product is expressed as a cycle threshold value (Ct), which is a value assigned manually to a level above the baseline in the exponential phase of PCR. The Ct value sets the point at which the sample amplification plot crosses the threshold. These Ct values are subsequently correlated with the initial amount of specific template (Barrachina *et al.*, 2006).

3.7.2 Materials and methods

3.7.2.1 RNA extraction

RNA was extracted from RNA*later* (QIAGEN, West Sussex UK) stabilised spleens from red mite exposed poultry using the RNeasy mini kit (QIAGEN, West Sussex UK). Frozen spleens were thawed for 30 min at room temperature and 30 mg of each spleen sample removed and placed in a sterile vile where RNA extraction would take place. Spleens were homogenised in a 2 ml safe-lock eppendorf tube (sterile and

RNase-free) with 600 µl RLT lysis buffer (containing 2-mercaptoethanol) using a bead mill (Retsch MM 300), for 4 min at 20 Hz.

The lysate was then centrifuged at 14,000 x *g* for 10 min and the supernatant carefully removed by pipetting and transferred to a new microcentrifuge tube. One volume of 70 % ethanol was added to the lysate and mixed immediately by pipetting. The sample was then transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged at 8,000 x *g* for 15 seconds, and the flow through discarded.

The next step was to add 700 µl wash buffer to the spin column and centrifuge for 15 s at 8,000 x *g*, to wash the spin column membrane and the flow through was discarded. Next, 500 µl of wash buffer containing ethanol was added to the RNeasy spin column and centrifuged for 15 s at 8,000 x *g* to once again wash the spin column membrane and the flow through was discarded, this process was repeated twice.

Finally RNA was eluted by placing the RNeasy spin column in a new 1.5 ml collection tube and then adding 50 µl RNase-free water directly to the spin column membrane. This was then centrifuged for 1 min at 8,000 x *g* to elute the RNA, which was immediately used in a TaqMan PCR assay. It should be noted that all reactions were carried out on ice to prevent RNA degradation.

3.7.2.2 TaqMan PCR assay

Firstly, the reaction mix was prepared containing 12.5 µl PCR master mix (Eurogentec, Hampshire, UK), 1 µl reverse transcriptase qPCR primer mix (gene-specific primers, 100 µM in DEPC-H_20; and fluorescent-labelled probe, 5 µM in DEPC-H_20), 0.5 µl probe, 0.125 µl "Euroscript" enzyme kit (Eurogentec, Hampshire, UK) and 5.9 µl DEPC H_20. Primers used were specific to cytokines expressed during both Th1- (IL-12α, IFNγ) and Th2-type immune responses (IL-4, IL-13, IL-5).

A thermofast 96-well plate was then placed in a rack on ice and 5.0 µl DEPC-H_20 was added per control well, followed by 20 µl master mix and then capped off to prevent contamination. Previously extracted RNA from spleen samples was then thawed on ice and diluted 1:10 for cytokine expression analysis. 15 µl (5 µl/well, triplicate wells) of each unknown sample of diluted RNA was required, therefore for the 1:10 dilution 1.6 µl RNA was pipetted into 14.4 µl DEPC H_20. This was then

mixed by vortexing and placed on ice. Positive RNA standards were then thawed and placed on ice and serially diluted. The first dilution was 1:100 and followed by 5 successive 10-fold dilutions. This was achieved by pipetting 0.6 µl RNA into 5.4 µl DEPC H_20, and then mixed thoroughly by vortexing. 0.6 µl of this dilution was then transferred to a new tube containing 5.4 µl DEPC H_20, and repeated by mixing serial dilutions in the same way. All tubes were then placed on ice.

20 µl of the master mix and 5.0 µl vortexed RNA was then added to all wells being used. These wells were then capped off and the plate spun down briefly before being loaded into the Taqman machine. Once loaded, samples underwent the following thermal cycling patterns. The initial cycle was 1 x 50°C, 2 min; 96°C, 5 min; 60°C, 30 min; 95°C, 5 min followed by melting and annealing/extension cycles (40 x 94°C, 20 s; 59°C 1 min). Results were interpreted by comparing unknown samples against standard curves generated with a 28S housekeeping gene to give a quantitative result.

Figure 3.21 *Example of Taqman output after typical amplification of samples from a dilution series (reporter signal against cycle number)*

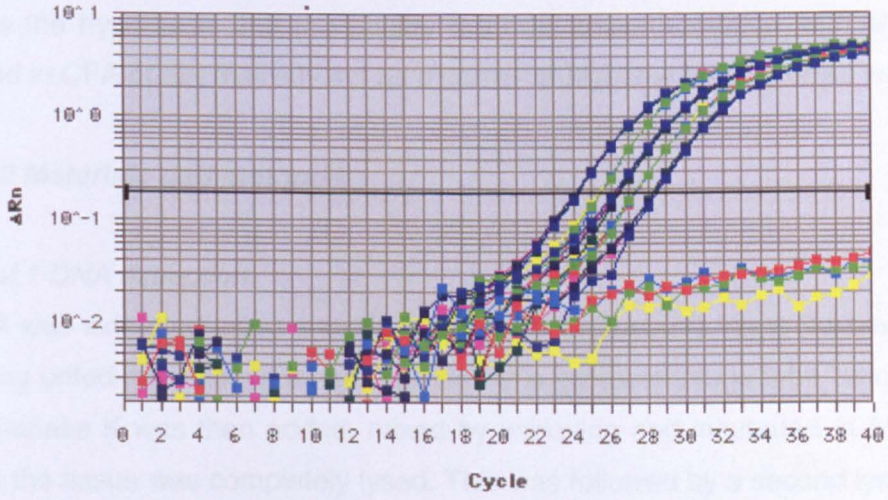


Figure 3.21 shows the typical output from a Taqman assay whereby fluorescence of reporter signal is plotted against cycle number. The cycle threshold value is marked by the dark horizontal line and any value detected above this threshold is significant.

In conclusion, TaqMan RT-PCR assays provide a rapid and reliable method for semi-quantitative analysis of gene expression of specific cytokines (Medhurst *et al.*, 2000) and as such were used to determine the levels of specific cytokines expressed in spleen tissue of either naturally infested or red mite immunised poultry.

3.8 Determination of Mycobacterial presence

3.8.1 Introduction

The experiments outlined in this section were performed following the initial immunisation trial (Chapter 6) and provide a link to the second immunisation trial (Chapter 7). It was concluded in Chapter 6 that perhaps the presence of *Mycobacterium* in both Complete Freund's adjuvant (CFA) and poultry red mite extracts was the reason that a large degree of non-specific binding was observed in both treatment groups, thus resulting in no apparent differences in western blot profiles between control and antigen immunised birds.

Therefore, in this section PCR and DNA sequencing were carried out to determine the possible presence of *Mycobacterium* species or their bacterial enzymes within the poultry red mite. Also, in order to determine whether that bacteria were alive by verification of the expression of these bacterial genes, RNA was extracted from red mite and subject to reverse-transcription PCR analysis.

Thus the hypothesis that the poultry red mite share homology with *Mycobacterium* found in CFA or alternatively act as a carrier of Mycobacterial species was tested.

3.8.2 Materials and methods

3.8.2.1 DNA extraction

DNA was extracted using a tissue extraction kit (QIAGEN, West Sussex, UK). Firstly, 25 mg unfed red mite were homogenized in a microcentrifuge tube, using lysis buffer. Proteinase K was then added, mixed by vortexing and incubated at 55°C overnight until the tissue was completely lysed. This was followed by a second lysis step by the addition of 200 µl more lysis buffer to the sample, which was mixed by vortexing and incubated at 70°C for 10 min. 200 µl ethanol (99 %) was then added to the sample and mixed thoroughly by vortexing. The mixture was then pippetted into a mini-spin column, placed inside a 2 ml collection tube and centrifuged for 1 min at 6,000 x *g*. The flow through and collection tube was discarded as the DNA was retained on the silica membrane inside the mini-spin column.

The spin column was placed in a new 2 ml collection tube, and 500 µl wash buffer was added and centrifuged for 1 min at 6,000 x *g*. The flow through and collection tube was discarded.

Again the mini-spin column was placed in a new 2 ml collection tube and 500 µl wash buffer added and centrifuged for 3 min at 20,000 x *g* to dry the membrane. The flow through and collection tube was discarded.

Finally, the column was placed in a clean 2 ml microcentrifuge tube and 200 µl of elution buffer was added directly onto the membrane. This was incubated at room temperature for 1 min and then centrifuged at 6,000 x *g* to elute. This elution step was repeated again into the same tube and the final product was then stored at -20°C until used in PCR.

3.8.2.2 Polymerase Chain Reaction (PCR)

(i) Gene specific primers

Primers were used for the detection of two genes, the first were for a 1,030 base pair 16S *Mycobacterium* genus gene. Primers were designed from those described by Parra *et al.* (2006). The sequence for the forward primer was 5'-AGA GTT TGA TCC TGG CTC AG-3' (Tm = 56.0°C) and for the reverse primer was 5'-TGC ACA CAG GCC ACA AGG GA-3' (Tm = 72.5°C).

The second set of primers coded for a 13.8 kDa bacteriolytic enzyme according to Mathaba *et al.* (2002). Two separate forward primers were used, one coding for 411 base pairs 5'-CT ATT ATG AAA TTC TTC TTC ACT TTA GCT-3' (Tm = 60.4°C) and the second coding for 345 base pairs 5'-AAT GGT GCC GCT ATT GTA TCG GCT-3' (Tm= 71.5°C). One reverse primer was used, with the sequence 5'-TTA CCA ACA TCG TGC AAC ATT AGC-3' (Tm= 66.5°C). Samples were compared for size against a 50 base pair (bp) DNA ladder (Amersham Biosciences, UK).

(ii) PCR reaction

Following standard manufacturers protocols the PCR samples were amplified as 50 µl volumes in 500 µl microcentrifuge tubes. Each reaction tube contained 25 pMole of each primer (0.25 µl), 25 µl of REDTaq ReadyMix with 3 mM $MgCl_2$ (Sigma, St Louis, USA), 19.5 µl of molecular water and 5 µl of DNA extract (to be used as a DNA template). The PCR reactions were run in a thermocycler (Eppendorf

mastercycler) under a denaturation, annealing and extension program of 35 cycles. Annealing steps were run at 5°C below the lowest primer melting temperature (Tm) of the primers involved in each particular reaction. The specific PCR cycle included 1 cycle at 94°C for 10 min, followed by 35 cycles of 30 seconds at 94°C for denaturation, 30 seconds at 55°C +- 5°C for primers annealing and 60 seconds at 72°C for DNA extension. The final extension step was run at 72°C for 5 min, after which samples were held at 4°C until being loaded into agarose gel lanes for electrophoresis.

The PCR products were detected on a 1 % agarose gel (1.25 g of agarose in 125 ml of TAE buffer) mixed with ethidium bromide (1.5 µl of ethidium). Gels were run at 105 V for approximately 1 hour and were observed under UV light for potential bands.

3.8.2.3 DNA sequencing

Subsequent to migration and detection of positive bands through electrophoresis, DNA was excised from agarose gels using a gel extraction kit (Sigma, St Louis, USA). These specific fragments were subsequently sent for DNA sequencing (Macrogen, Korea). Upon receipt of DNA sequences, these were aligned against available genes in the National Centre for Biotechnology Information Nucleotide Database (NCBI; http://www.ncbi.nlm.nih.gov).

3.8.2.4 RNA extraction

Bacterial RNA was extracted from poultry red mite using the GenElute™ Total RNA Purification Kit (Sigma, St Louis, USA). 30 mg of unfed red mite were aspirated into a Pasteur pipette and transferred to a sterile vile where RNA extraction took place. Red mite were homogenised in 200 µl working bacterial digestion solution in order to lyse cell walls (180 µl bacterial lysis solution, plus 20 µl bacterial enzyme stock). The homogenate was incubated for 10 min at room temperature and then mixed with a cellular lysis and RNAse 2-mercaptoethanol solution. The remaining lysate was then pipetted into a 2 ml GenElute filtration column and centrifuged at 14,000 x *g* for 2 min, a process used to remove cellular debris and shear the DNA. The supernatant was retained and mixed with 350 µl 99 % ethanol by vortexing. This lysate/ethanol mixture was then bound to a GenElute binding column, washed and centrifuged at 14,000 x *g* for 2 min three times in order to remove ethanol to prepare for elution. RNA elution was achieved by pipetting 50 µl elution solution into the binding column and centrifuged at 14,000 x *g* for 1 min. The resulting RNA containing elution was

either used immediately or stored at -20°C. All steps in this process were carried out on ice as RNA is very unstable at higher temperatures.

3.8.2.5 RT-PCR

Reverse transcription was performed using an Omniscript® Reverse Transcription kit (QIAGEN, West Sussex, UK). A master mix was prepared on ice according to Table 3.19.

Table 3.19 *Reverse-transcription reaction components*

Master Mix Component	Volume/reaction	Final concentration
10 x Buffer RT	2µl	1 x
dNTP Master Mix	2µl	0.5 mM each dNTP
Mycobacterium genus primer	2µl	1 µM
Omniscript Reverse Transcription	1µl	4 units (per 20 µl reaction)
RNase-free water	8µl	-
Template RNA	5µl	-
Total volume	**20µl**	-

Following preparation of the reaction master mix components were mixed by vortexing and then incubated for 60 min at 37°C. An aliquot of this reverse-transcription mix was subsequently added to the PCR mix and underwent PCR as described earlier in this section.

3.8.2.6 Comparison of red mite samples for the presence of Mycobacterium

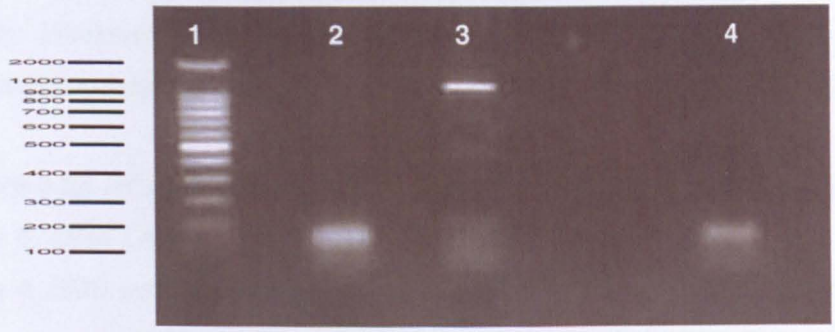
In order to avoid contamination from DNA in the previous blood-meals of red mite, PCR was run on unfed mite DNA samples only. The presence of *Mycobacterium* in red mite across different farms (n = 3) and also red mite life-stages (adults, larvae and eggs) was also investigated. In addition, to determine whether *Mycobacterium* was either contained internally or on external surfaces, red mite were washed extensively in PBS or 10 % SDS and PCR was conducted on both washed mites and the used wash solution.

3.8.3 Results

3.8.3.1 PCR

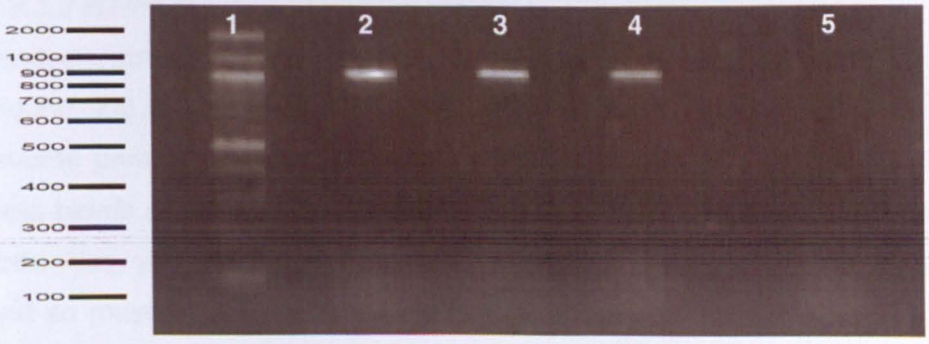
Initial PCR showed that DNA extracted from unfed poultry red mite gave a positive band at approximately 1,000 base pairs when using *Mycobacterium* genus primers (Figure 3.22). However, this was not the case after PCR using primers coding for bacteriolytic enzymes, since no bands were seen. Therefore, subsequent analysis was performed using *Mycobacterium* genus primers only.

Figure 3.22 *PCR of unfed poultry red mite using gene specific primers. Lane 1, DNA Ladder (bp); Lane 2, bacteriolytic enzyme primer; Lane 3,* Mycobacterium *genus primer; Lane 4, Negative control*



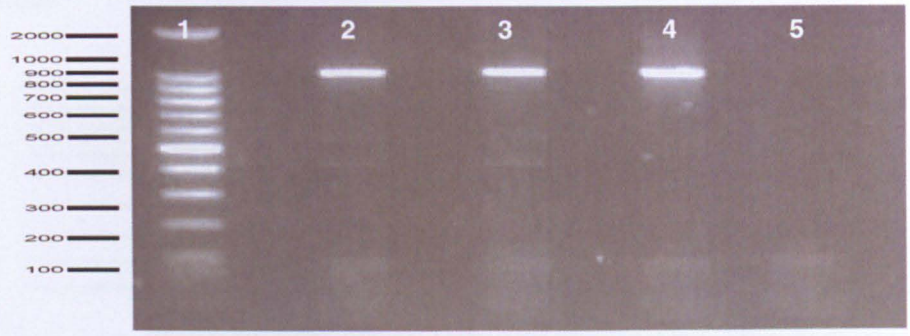
PCR using *Mycobacterium* genus primers on DNA extracted from unfed mites on three independent poultry farms showed that *Mycobacterium* was present in all three red mite populations (Figure 3.23).

Figure 3.23 *PCR of unfed red mite collected on three poultry farms using primers for* Mycobacterium *genus. Lane 1, DNA Ladder (bp); Lane 2, farm 1; Lane 3, farm 2; Lane 4, farm 3; Lane 5, Negative control*



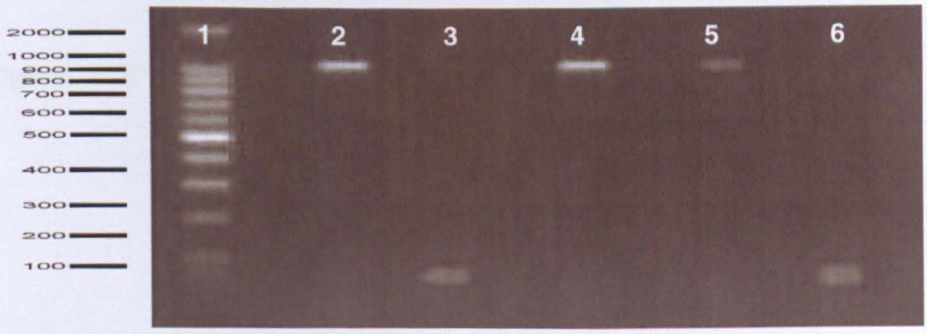
Similarly, red mite adults, larvae and eggs all possessed *Mycobacterium* (Figure 3.24), confirmed by the presence of a band at around 1,000 base pairs.

Figure 3.24 *PCR of poultry red mite stages using primers for* Mycobacterium *genus. Lane 1, DNA Ladder (bp); Lane 2, Unfed adult mite; Lane 3, Red mite larvae; Lane 4, Red mite eggs; Lane 5, Negative control*



It would appear from Figure 3.25 that after washing in PBS alone, Mycobacteria are present solely inside the mite, as no band was detected from the spent PBS wash buffer. However, after using a more aggressive washing agent (10 % SDS), Mycobacteria appear to be both present on both the surface and also inside the mite.

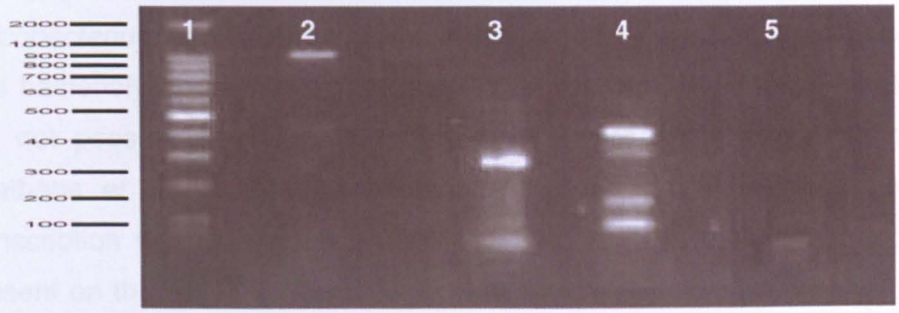
Figure 3.25 *PCR of washed poultry red mite using primers for* Mycobacterium *genus. Lane 1, DNA Ladder (bp); Lane 2, PBS washed red mite; Lane 3, Used PBS buffer; Lane 4, SDS washed red mite; Lane 5, Used SDS buffer; Lane 6, Negative control*



3.8.3.2 RT-PCR

Products amplified by RT-PCR for the *Mycobacterium* genus gave rise to bands at around 300 base pairs for the forward primer and a band at 400 base pairs for the reverse primer. This was unexpected since the presence of *Mycobacterium* should yield bands at 100 bp as no splicing occurs during translation of DNA in prokaryotic cells. Also, you would not expect both forward and reverse primers to produce bands, and so must be put down to non-specific reactions. Therefore, care must be taken when interpreting these results.

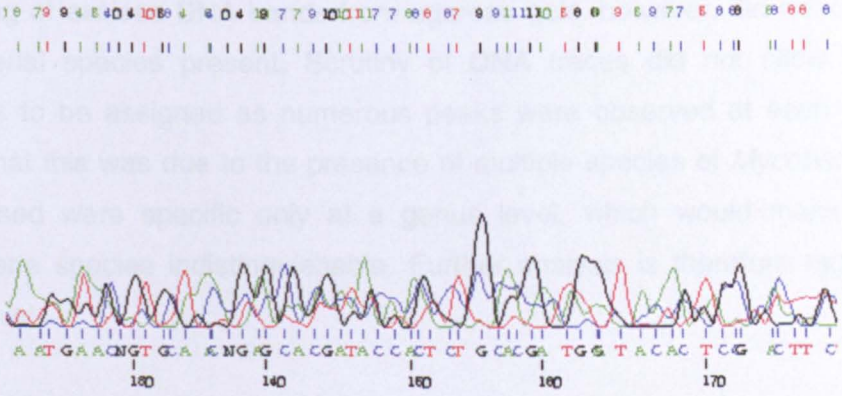
Figure 3.26 *PCR and RT-PCR using primers for* Mycobacterium *genus for adult unfed poultry red mite. Lane 1, DNA Ladder (bp); Lane 2, Unfed mite PCR products; Lane 3, RT-PCR forward primer; Lane 4, RT-PCR reverse primer; Lane 5, Negative control*



3.8.3.3 DNA sequencing

Following amplification and electrophoresis, bands generated from PCR products were sequenced by an external laboratory. Alignment of results of this sequencing within GenBank (www.ncbi.nlm.nih.gov/Genbank) failed to produce sequence homology with previously entered genes. It is apparent from DNA traces (Figure 3.27) that multiple bands are observed at every position along the length of the sequence, therefore making it difficult to assign individual nucleotides.

Figure 3.27 *Example of the DNA trace readout*



3.8.4 Discussion

PCR was conducted to test the hypothesis that the poultry red mite shares genetic homology or acts as a carrier of *Mycobacterium* found in CFA. Thus explain why control birds injected with CFA showed an increase in specific red mite antibody levels and also generated non-specific bands on western blots in Chapter 6. Bacterial homology to red mite was assessed using two sets of primers. The first set for the

Mycobacterium genus and the second for a bacteriolytic enzyme gene previously found in mite species (Mathaba *et al.,* 2002).

The results indicated that red mite DNA was homologous to Mycobacteria, as a strong positive band was observed when red mite DNA was amplified using *Mycobacterium* genus primers. However, homology was not seen between red mite and bacteriolytic enzyme primers, as no bands were detected. Therefore, red mite did not possess bacteriolytic enzymes as previously observed in other species (Mathaba *et al.,* 2002). *Mycobacterium* expression was confirmed when reverse-transcription was performed on mRNA, thus verifying that live Mycobacteria were present on the red mite. Their origin was also assessed by extensively washing red mite and then performing PCR on both washed mites and used wash buffer. It appeared from results that after washing in SDS, but not PBS, Mycobacteria actually reside both on the surface and also inside the mite as PCR was positive for both fragments. This was not surprising as the poultry red mite has been implicated previously as a vector for several pathogens (Chauve, 1998). It is likely that washing in SDS was more successful at removing *Mycobacterium* as it is an ionic surfactant which works by disrupting non-covalent bonds in the proteins. This is a process which has been previously exploited for the removal of debris bound to the surface of the sheep scab mite (Pettit *et al.,* 2000; Huntley *et al.,* 2004; Smith and Pettit, 2004).

Sequencing of excised DNA bands from agarose gels, however, did not confirm the Mycobacterial species present. Scrutiny of DNA traces did not allow nucleotide sequences to be assigned as numerous peaks were observed at each point. It is possible that this was due to the presence of multiple species of *Mycobacterium,* as primers used were specific only at a genus level, which would make individual Mycobacteria species indistinguishable. Further analysis is therefore required at a species level.

3.8.5 Conclusion

The discovery of *Mycobacterium* in red mite obtained from commercial farms means that care must be taken when interpreting future immunological results. It may indeed be advisable to use an alternative to CFA in immunisation trials to avoid the occurrence of false-positive results. Alternatively, future research should concentrate on the possibility of eliminating Mycobacterial protein from poultry red mite protein antigens using different protein extraction procedures.

Chapter 4

Poultry red mite population dynamics in relation to acaricide application and egg production on a commercial free-range laying unit

4.1 Introduction

The poultry red mite is a ubiquitous ectoparasite of laying hens, causing numerous problems for welfare and production, which were outlined in Chapter 2. The predominant methods of control for red mite are to use a combination of house sanitisation along with application of a range of acaricides (McDevitt *et al.*, 2006a). However, acaricide application is an expensive process and has been estimated to cost the UK poultry industry approximately of £3.7 m per annum (Anon, 2003a).

Control of the poultry red mite is difficult for a number of reasons. Firstly, typical poultry layer housing consists of a wide range of furniture including, perches, nest boxes, egg belts, feeders, drinkers, amongst others, which provide ideal harbourages for red mite and render them almost completely inaccessible to both birds and acaricides (Nordenfors and Höglund, 2000). Extensive free-range and barn systems offer a greater array of these hiding places and therefore typically have higher levels of mite infestation when compared to caged birds (Hamscher *et al.*, 2003).

There is also evidence that the poultry red mite has developed heritable resistance to a number of acaricides, including pyrethroids, DTT, permethrin, tetramethrin, trichlorifon and organophosphates, which exacerbates the problem of control further (Beugnet *et al.*, 1997). Resistance to chemical acaricides has been observed across a range of other parasitic species and is often associated with the prolonged and high level use of chemicals (Beugnet and Chardonnet, 1995; Chauve, 1998).

Therefore, the optimal chemical for use as an acaricide would overcome both the innate biology (i.e. withdrawing into harbourages after feeding) and resistant capacity of the red mite. In addition, it would not compromise the health of the bird or result in harmful residues (Hamscher *et al.*, 2003).

Amongst chemicals currently approved by the UK government, carbamates, and in particular Bendiocarb, have proved to be effective at controlling a range of parasites

(Scharf *et al.*, 1997; Villatte *et al.*, 1999; Yu, 2006; Fiddes *et al.*, 2006; Vatandoost *et al.*, 2006). The effectiveness of Bendiocarb stems from its properties as a cholinesterase inhibitor, which prevents the breakdown of the neurotransmitter, acetylcholine. This inhibition causes prolonged and repeated synaptic stimulation resulting in muscle spasm, paralysis and eventually death (Vatandoost *et al.*, 2006)

The aim of this study was to gain an insight into the effectiveness of regular spraying of a Bendiocarb based acaricide (Ficam® W, AgrEvo, Berlin) on controlling poultry red mite populations in a commercial free-range system for laying hens, whilst evaluating the impact of red mite populations on a series of production parameters.

4.2 Materials and methods

4.2.1 Animals and housing

The trial took place on a commercial free-range laying flock. The site consisted of a single building, housing 4,000 laying hens of a commercial genotype. At the beginning of the trial the birds were 51 weeks of age and were monitored over a 17 week period. Stocking density, feed and water provisions were in line with standard commercial practice (Rose, 2001). Stocking density inside the building was 11 birds/ m^2, whereas outside it was 1,000 birds per hectare. Feed provisions were of a standard commercial ration (15 % protein, 3.5 % oil, 3.5 % fibre, 13.3 % ash, 0.34 % methionine, 6,000 IU/kg vitamin A, 3,000 iu/kg vitamin D3, 6.00 iu/kg vitamin E and 15 mg/kg copper) and birds were fed hourly using a chain driven system. Water was supplied *ad libitum* in bell-shaped drinkers. Birds were on a 16:8 L:D illumination programme and the building was naturally ventilated through Yorkshire boarding. Eggs were collected via an automated belt system. The trial was conducted in 2003, from August to December.

4.2.2 Acaricide spraying

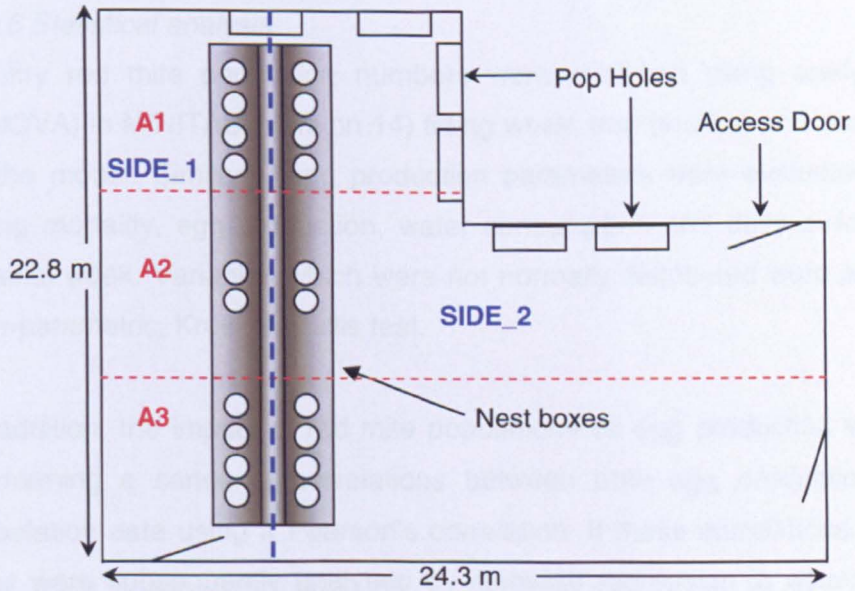
The poultry house was sprayed with the carbamate acaricide Ficam® W, (80 % w/w Bendiocarb, AgrEvo, Berlin). This is a commercially available product and comes in the form of a water dispersible powder, with inclusion at 3 grams per 1 litre water. The solution was sprayed on all exposed surfaces within the house, using a knapsack sprayer. Spraying was carried out once every six weeks, starting from week 4 of the trial when the birds were 54 weeks of age. Spraying was performed over the 17 week trial duration and was repeated twice when birds were 60 and 66 weeks of age.

4.2.3 Trap design and placement

A simple trap design, modified from Nordenfors and Chirico (2001) was employed. This consisted of a rectangular section of cardboard (30 x 100 mm) to which two drinking straws were fixed (to act as a harbourage). The traps were secured to the underside of the perches using Velcro and plastic cable-ties. A total of 20 traps were secured under the perches at intervals along the length of the building, in Areas 1, 2 and 3, respectively. Traps were numbered 1 to 20 and placed in a specific region of the shed. 10 traps were secured to perches along one side of the building where pop holes gave access to pasture and 10 traps fastened to perches along the side adjacent to a wall with no external access (Figure 4.1).

On a weekly basis the traps were removed, placed into individual sealable polythene bags to prevent mites from escaping and transported to the laboratory. New traps were placed at each of the same 20 positions, whilst old traps were subject to filtration and counting of red mite life-stages

Figure 4.1 *Diagram of house layout and trap placement*



○ = Poultry Red Mite Trap

A1 = Area 1; **A2** = Area 2; **A3** = Area 3

4.2.4 Red mite filtration and counting

In the laboratory, individual bags containing live mites were submerged in approximately 25 ml of 70 % ethanol, which killed and preserved the red mite. Mites

remained in alcohol for approximately 2-3 hours to ensure that they were all dead, after which traps were dismantled and washed with distilled water through a water driven vacuum pump and Buckner funnel. Red mite collected on the filter paper were then transferred to a plastic storage container by washing with a further 25 ml of 70 % ethanol.

Red mite numbers were estimated by withdrawing 500 µl sub-samples of the ethanol/mite suspension, counting under a light microscope and then multiplying up to estimate the total population in the 25 ml volume. A series of life-stages were recorded, including adult and nymph (both fed and unfed), larvae, egg and total mite numbers. Each sample taken was counted and recorded by two separate individuals.

4.2.5 Production data

A number of production parameters were recorded on a weekly basis in order to assess the influence of the poultry red mite on egg production. The production parameters which were recorded included, mortality, egg production, water consumption and daily building temperature.

4.2.6 Statistical analysis

Poultry red mite population numbers were analysed using analysis of variance (ANOVA) in MINITAB (Version 14) fitting week, trap position and observer as factors in the model. Similarly, egg production parameters were evaluated using ANOVA fitting mortality, egg production, water consumption and daily building temperature against week. Variables which were not normally distributed were assessed using a non-parametric, Kruskal-Wallis test.

In addition, the impact of red mite populations on egg production was assessed by performing a series of correlations between both egg production and red mite population data using a Pearson's correlation. If these correlations were significant, they were subsequently analysed by stepwise regression to establish the greatest determinant of variability within that model, i.e. the most important predictor affecting a particular response variable.

4.3 Results

4.3.1 Population of poultry red mite over time

It is evident form Table 4.1 that there were significant differences in red mite populations based on mean weekly count results. A gradual decline in red mite populations was seen over the course of the experiment and by the end of the experiment numbers were extremely low.

Spraying of the acaricide resulted in a reduction in red mite population levels in the following weeks, with the exception of the initial spray (week 4) where red mite numbers increased directly after spraying and was in fact higher than that observed in the weeks prior to spraying (see Table 4.1).

Table 4.1 *Mean weekly red mite population of different life-stages (mean per trap, rows in bold were when bendiocarb was applied)*

Week	Age (weeks)	Fed Adult and Nymph	Unfed Adult and Nymph	Larvae No.	Mite egg No.	Total Mite Population
1	51	880.0^{a}	244.8^{a}	123.6^{ace}	380.7^{ac}	1628.9^{a}
2	52	660.2^{acd}	158.4^{ac}	80.7^{ad}	377.3^{c}	1276.5^{ac}
3	53	373.6^{bc}	95.3^{bc}	70.4^{ac}	139.9^{acd}	779.1^{bc}
4	**54**	**209.0^{b}**	**53.5^{bd}**	**58.5^{ac}**	**163.3^{acd}**	**484.1^{bde}**
5	55	487.8^{ad}	97.3^{bcd}	178.1^{e}	285.8^{acd}	1048.8^{aef}
6	56	364.1^{bc}	62.8^{bd}	29.1^{bd}	253.8^{acd}	709.9^{bcdf}
7	57	170.9^{b}	79.0^{bcd}	18.3^{bd}	168.6^{acd}	436.8^{bdf}
8	58	211.0^{b}	19.8^{bd}	8.5^{b}	91.9^{ad}	331.2^{bdf}
9	59	367.6^{b}	16.4^{bd}	12.5^{b}	203.7^{acd}	600.1^{bcdf}
10	**60**	**338.0^{bc}**	**7.3^{bd}**	**1.8^{b}**	**103.7^{ad}**	**450.8^{bdf}**
11	61	117.3^{bc}	13.3^{bd}	0.2^{b}	67.7^{bd}	198.4^{bdf}
12	62	236.6^{b}	0.1^{d}	9.3^{b}	24.1^{bd}	270.1^{bdf}
13	63	349.6^{bc}	7.6^{bd}	22.0^{bd}	241.5^{ac}	620.6^{bcdf}
14	64	94.5^{b}	2.6^{bd}	0.2^{b}	22.1^{bd}	119.3^{bdf}
15	65	48.2^{b}	2.6^{bd}	2.7^{b}	12.2^{bd}	65.7^{bdf}
16	**66**	**49.9^{b}**	**5.9^{bd}**	**3.0^{b}**	**6.2^{bd}**	**65.0^{bdf}**
17	67	15.0^{b}	0.1^{d}	0.2^{b}	8.2^{bd}	23.4^{d}
P-Value	-	***	***	***	***	***
SE Mean	-	23.60	5.48	3.68	14.80	41.80

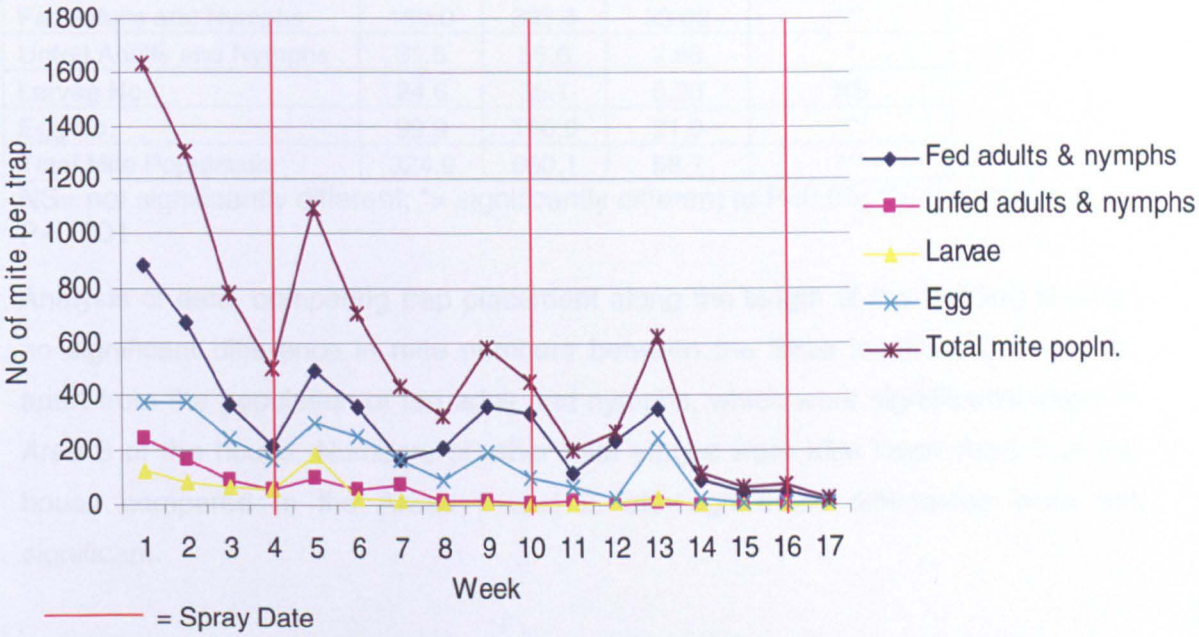
***= P<0.001

Means within a column followed by a different superscript letter are significantly different at P<0.05.

Red mite populations also fluctuated between weeks, irrespective of acaricide application. Prior to both the first and second spray dates red mite numbers were

declining, therefore suggesting that other factors are involved in red mite survival and proliferation. This is made particularly apparent in Figure 4.2.

Figure 4.2 *Mean weekly red mite population of different life-stages per trap*



4.3.2 Consistency of counting between observers

Apart from mean egg number, there was no significant difference between the two observers in the estimation of the number of red mite found per trap (Table 4.2).

Table 4.2 *Comparison of mean red mite populations between observers*

	Observer 1	Observer 2	SE Mean	Significance
Fed Adults and Nymphs	242.7	321.2	33.56	NS
Unfed Adults and Nymphs	60.0	27.3	7.65	NS
Larvae No.	27.1	32.8	5.25	NS
Egg No.	140.8	146.1	21.11	*
Total Mite Population	470.5	518.2	59.52	NS

NS= not significantly different; *= significantly different at P<0.05

4.3.3 Distribution of mean red mite population

Table 4.3 shows the distribution of red mite between the two different sides of the house. With the exception of larvae number, traps situated on the side of the house with pop holes, enabling access to pasture had significantly higher numbers of each of the red mite life-stages.

Table 4.3 *Distribution of mean red mite population per house side (mean population per trap)*

	Side 1	Side 2	SE Mean	Significance
Fed Adults and Nymphs	169.0	383.4	33.02	***
Unfed Adults and Nymphs	31.5	55.6	7.68	*
Larvae No.	24.6	35.1	5.20	NS
Egg No.	99.9	186.0	21.0	**
Total Mite Population	324.9	660.1	58.7	***

NS= not significantly different; *= significantly different at P<0.05; **= P<0.01; ***= P<0.001

Analysis of data, comparing trap placement along the length of the building showed no significant difference in mite numbers between the three locations (Table 4.4), apart from the population of fed adult and nymphs, which were significantly lower in Area 3 of the house. Numbers of other mite stages were also lower Area 3 of the house compared to the Areas 1 and 2, although these differences were not significant.

Table 4.4 *Distribution of mean red mite populations in the poultry house*

	Area 1	Area 2	Area 3	SE Mean	Significance
Fed Adults and Nymphs	348.08^{a}	286.98^{ab}	205.16^{b}	49.132	*
Unfed Adults and Nymphs	48.31	51.41	35.39	9.787	NS
Larvae No.	39.94	20.92	24.83	6.617	NS
Egg No.	149.41	153.01	132.90	26.827	NS
Total Mite Population	585.71	512.30	398.25	75.398	NS

*= P<0.001; NS = not significantly different
Means within a row followed by a different superscript letter are significantly different at P<0.05.

4.3.4 Egg production and temperature parameters

Table 4.5 gives mean values for a number of production parameters and shows a steady decline in mean egg output per hen, which is significantly lower in week 16 (P<0.05). There was a small reduction in water consumption as the birds' aged, although this was not significant. There was also a significant drop in mean building temperature over the course of the experiment from approximately 22°C to 12°C (P<0.001). Conversely, a steady and significant increase (P<0.001) in bird mortality was observed.

Table 4.5 *Mean weekly egg production, mortality and building temperature*

Week	Age (weeks)	Egg/bird/ week	Water /bird	Hen Mortality %	Mean Temp. (°C)
1	51	0.77^{a}	0.24	0.06^{a}	22.21^{a}
2	52	0.75^{ab}	0.24	0.12^{a}	21.21^{a}
3	53	0.77^{a}	0.25	0.30^{ab}	20.86^{ab}
4	54	0.77^{a}	0.24	0.41^{b}	19.86^{acd}
5	55	0.74^{ab}	0.25	0.52^{b}	21.00^{a}
6	56	0.76^{ab}	0.28	0.84^{c}	18.50^{bce}
7	57	0.72^{ab}	0.28	1.19^{d}	17.79^{de}
8	58	0.74^{ab}	0.23	1.43^{e}	16.14^{ef}
9	59	0.73^{ab}	0.25	1.63^{e}	14.64^{f}
10	60	0.72^{ab}	0.24	1.89^{f}	14.57^{fg}
11	61	0.73^{ab}	0.23	2.07^{fg}	14.14^{fg}
12	62	0.73^{ab}	0.23	2.17^{g}	14.43^{fg}
13	63	0.74^{ab}	0.27	2.22^{gh}	14.00^{fg}
14	64	0.73^{ab}	0.26	2.29^{gi}	14.50^{fg}
15	65	0.71^{ab}	0.21	2.44^{hi}	12.43^{g}
16	66	0.67^{b}	0.21	2.70^{j}	13.43^{fg}
17	67	0.70^{ab}	0.24	3.91^{k}	13.86^{fg}
P Value	-	*	NS	***	***
SE Mean	-	0.019	0.018	0.0960	0.478

*= P<0.05; ***= P<0.001, NS= not significantly different
Means within a column followed by a different superscript letter are significantly different at P<0.05.

4.3.5 Correlations of red mite population and production parameters

Pearson correlations were performed using both mean weekly red mite populations and production parameters as response variables (Table 4.6). Most parameters were significantly correlated with each other, with the exception of water consumption which was only found to be significantly correlated to egg production. Mean daily building temperature had a strong negative correlation with hen mortality ($r = -0.91$; $P<0.001$). Mean building temperature also had a significant relationship with egg output, although on this occasion it was positive ($r = 0.74$; $P<0.001$). Correlations between production and red mite population variables produced a series of significant results. For example, total red mite population was positively correlated with both daily building temperature ($r = 0.86$; $P<0.001$) and mean egg production ($r = 0.70$; $P<0.01$), but negatively correlated with hen mortality ($r = -0.82$; $P<0.001$).

Table 4.6 *Correlations between production parameters and mite population (P-value, followed by r)*

	Age	Egg/bird/ week	Water/bird	Mean temp. (°C)	Hen mortality (%)	Fed adult and nymph	Unfed adult and nymph	Larvae no.	Mite egg no.
Egg/bird/week	0.000 (-0.821)								
Water/bird	0.192 (-0.333)	0.040 (0.502)							
Mean temp. (°C)	0.000 (-0.938)	0.001 (0.741)	0.197 (0.329)						
Hen mortality (%)	0.000 (0.974)	0.000 (-0.805)	0.220 (-0.314)	0.000 (-0.909)					
Fed adult and nymph	0.000 (-0.795)	0.004 (0.666)	0.319 (0.257)	0.000 (0.855)	0.000 (-0.763)				
Unfed adult and nymph	0.000 (-0.830)	0.013 (0.590)	0.469 (0.188)	0.000 (0.883)	0.000 (-0.791)	0.000 (0.872)			
Larvae no.	0.001 (-0.707)	0.027 (0.535)	0.556 (0.154)	0.000 (0.831)	0.001 (-0.715)	0.001 (0.730)	0.000 (0.761)		
Mite egg no.	0.000 (-0.852)	0.001 (0.722)	0.064 (0.458)	0.000 (0.843)	0.000 (-0.833)	0.000 (0.912)	0.000 (0.847)	0.000 (0.763)	
Total mite population	0.000 (-0.850)	0.002 (0.697)	0.222 (0.313)	0.000 (0.855)	0.000 (-0.824)	0.000 (0.979)	0.000 (0.918)	0.000 (0.820)	0.000 (0.958)

4.3.6 Stepwise regression

Stepwise regression was used to determine the proportion of variation of a particular production or red mite population parameter which could be accounted for by a number of predicting variables. Table 4.7 shows that the most predominant predictor for egg production parameters was age, which explains most of the variation for egg output, mean daily building temperature and mortality (r^2 = 67.2, 87.3 and 94.5, respectively). The population of red mite larvae were seen to have a lesser, but still significant relationship with building temperature (r^2 = 5.5)

Table 4.7 *Stepwise regression showing factors affecting production parameters*

Response	Predictor	Significance	r^2
Eggs/bird	age	***	67.2
Total:			67.2
Water/Bird	Eggs/bird	*	20.2
Total:			20.2
Mean Temp. (°C)	age	***	87.3
	Larvae No.	**	5.5
Total:			92.7
Hen mortality %	age	***	94.5
Total:			94.5

*= significant predictor at P<0.05; **= P<0.01; ***= P<0.001

When considering factors affecting the population of different red mite life-stages, relationships between those different stages were the largest predisposing factors (Table 4.8). However, after this the second greatest explanatory factor of variation was frequently the effect of building temperature. Excluding red mite eggs, building temperature had a significant effect on all life-stages, with the largest effect being with that of larval numbers (r^2 = 68.1).

Table 4.8 *Stepwise regression showing factors affecting red mite populations*

Response	Predictor	Significance	r^2
Total Mite Population	Fed Adult and Nymph	***	95.6
	Mean Temp. (°C)	***	2.8
	Mite egg No.	**	0.7
	Larvae No.	**	0.5
	Unfed Adult and Nymph	*	0.4
Total:			99.9
Fed Adult and Nymph	Total Mite Population	***	95.6
	Mean Temp. (°C)	**	1.9
	Larvae No.	NS	0.5
	Mite egg No.	*	0.9
	Unfed Adult and Nymph	***	0.8
Total:			99.6
Unfed Adult and Nymph	Total Mite Population	***	83.2
	Mean Temp. (°C)	*	3.6
	Mite egg No.	NS	1.2
Total:			88.1
Larvae No.	Mean Temp. (°C)	***	68.1
	Total Mite Population	*	7.3
	age	NS	3.3
Total:			78.7
Mite egg No.	Total Mite Population		91.3
Total:			91.3

NS= not significantly different; *= significant predictor at P<0.05; **= P<0.01; ***= P<0.001

4.4 Discussion

The current situation in Europe regarding the control of poultry red mite is very limited as the range of registered or recommended compounds available is severely restricted (Chauve, 1998). Many of the most effective chemicals previously available have been, or are soon to be, withdrawn from the commercial use due to concerns over toxicity to both animal and man (Chirico and Tauson, 2002). This is particularly apparent in several of the Scandinavian countries, where there are currently no acaricides registered for the control of red mite in poultry systems (Chirico *et al.*, 2003). Few alternative control methods have been documented and those available have been seen to have limited success in eradicating red mite populations (Downing *et al.*, 1990; Carroll, 1994; Costa *et al.*, 1994; Kim *et al.*, 2004). Therefore the aim of this study was to gain an insight into the effectiveness of the regular application of a commercially available acaricide (Bendiocarb) on poultry red mite populations in a commercial free-range system for laying hens, whilst evaluating the impact of red mite populations on a series of production parameters.

4.4.1 Red mite population

Monitoring red mite populations using cardboard traps showed that there was significant reduction in their size over the course of the experiment, with almost a complete suppression of red mite population by the end of the study. However, the cause of this reduction is not obvious, particularly with the lack of a control hen population. Therefore, it is uncertain whether the reduced mite population was as a direct result of the application of Bendiocarb or alternatively related to inherent population dynamics of red mite in response to environmental variables. It is commonly accepted that temperature has a profound impact on, and is often the predominant driving force behind red mite survival and proliferation, with optimal temperatures for mite survival and reproduction at approximately 20-25°C (Maurer and Baumgartner, 1994; Nordenfors *et al.,* 1999). This trial was initiated in the summer months (August) and continued until winter (December), over which time a significant ($P<0.001$) fall in mean daily building temperature was seen, from approximately 22°C to a low of 12°C. Therefore, it is highly probable that ambient temperature had a significant influence on the population of poultry red in this housing system, as has been previously demonstrated by Nordenfors and Höglund (2000).

Alternatively, it is possible that acaricide spraying had a direct impact on red mite numbers, since after spraying in the weeks 10 and 16 reductions in red mite numbers were seen. The product used (Ficam® W, AgrEvo, Berlin) contains a carbamate derivative, Bendiocarb. The high toxicity of Bendiocarb and particularly that of carbamate itself has been well documented, in some cases showing complete eradication of parasites (Zeman and Zelezny, 1985; Fletcher and Axtell, 1991; Fiddes *et al.,* 2006). Reduced red mite populations following the application of acaricides has also previously been observed, although it is suggested that these are only temporal effects, and resurgence of red mite populations after such application is common (Nordenfors and Höglund, 2000). This was seen to some extent in the current experiment, with red mite numbers fluctuating between spray dates.

However, the site used in this experiment did not have a particularly heavy red mite burden in relation to previous studies. Several experiments by a group in Sweden, involving the trapping of red mite observed populations to range from approximately 3,700 to 67,600 per trap (Nordenfors and Höglund, 2000; Nordenfors *et al.,* 2001; Nordenfors and Chirico, 2001; Chirico and Tauson, 2002). In this study initial total mite infestation levels per trap started at just over 1,500, between 2.5 and 45 times

lower than that of previous research. Therefore, the potential for successful red mite eradication in the current study was much greater, requiring less time to reduce the parasite population (Chirico and Tauson, 2002).

4.4.2 Distribution of mean red mite population

Trapping of red mite is frequently carried out using the technique outlined here and in previous research e.g. Höglund *et al.* 1995 and Nordenfors and Chirico, 2001. However, there is limited information available regarding the influence of placement of traps within a housing system on the estimation of mite population. In the current experiment, the red mite population was significantly higher on the side of the house located adjacent to pasture access. This effect is possibly due to the unequal distribution of birds within the system. Unfortunately, no formal records of spatial distribution of hens were made in this study. However, casual observations did see that upon re-entry into the house from the pasture, birds were drawn to the feeders or nest boxes within closest proximity and as a result red mite would aggregate in this area to increase the probability of obtaining a blood-meal. Similar unequal red mite distribution was reported by Nordenfors and Höglund (2000), where the spread of red mite was seen to mimic the perching behaviour of the poultry breeds.

As for horizontal distribution of red mite along the length of the house, little significant difference was seen in the present study. However, higher populations of red mite were observed to accumulate towards the left and middle of the building, although this was not a significant effect. Again, this is likely to be due to both bird characteristics and building design, as towards the left/middle of the house there was access to pasture and a large open feeding region, where a higher number of birds were found. Previous research showed similar observations in horizontal distribution, with mites showing preference to certain regions within the house, which was interpreted as being linked to specific differences in the birds choice of perching places (Nordenfors and Höglund, 2000). In addition to variation in red mite populations within the poultry house, there was also observed to be large fluctuation between mite numbers in the traps themselves, as previously reported by Nordenfors *et al.* (2001).

Although it is not possible from trap data to estimate the actual population of red mite present in a poultry house these findings may be of use when considering experimental design for further research (Nordenfors and Chirico, 2001). It has been established that building design and hen genotype significantly affect distribution of

birds within a particular house (Abrahamsson and Tauson, 1995), which in turn is likely to determine the distribution of the red mite population (Nordenfors and Höglund, 2000).

4.4.3 Egg production and temperature parameters

Production figures demonstrated a steady decline in egg output per hen over the course of the experiment, which was significantly lower in week 16 ($P<0.05$). This reduced productivity is typical of laying hens as they move through their laying period and near the end of their commercial life. The reduction in mean building temperature recorded within the building can be accounted for by the change in ambient temperature, since the trial started in the summer months and terminated during the winter, which would in turn reduce water consumption (Nix, 2000; Rose, 2001).

4.4.4 Correlations of red mite population and production parameters

One of the objectives of this study was to investigate the relationship between mite population and production parameters. Correlation of variables resulted in a number of significant relationships, although several of these were unexpected. In previous studies, increased red mite numbers were seen to actively reduce production both in terms of eggs laid and increase in mortality of birds. For example, a fall in egg production of between 10-15 % and rise in hen mortality of 5-47 % were seen by Wojcik *et al.* (2000) and Cosoroaba (2001), respectively. However, in the present study the opposite effects were seen, namely a significant positive relationship between red mite population and egg output per bird. Also, there was a significant negative correlation between hen mortality and red mite population. The reasons for this are unclear, with the absence of a control hen population, although it might be explained once again by the relatively low infestation levels observed in the current study, meaning that the red mite were not able to impact significantly on natural levels of hen mortality and egg production.

Building temperature was one parameter which was closely linked to each of the red mite life-stages, as might be expected from results seen in previous research (Nordenfors *et al.,* 1999). Positive correlations with mean building temperature were observed for red mite adults, larvae and eggs. This remains consistent with previous investigations into the effect of temperature on red mite numbers, which have shown that increases in temperature within an optimum range (20-25°C) result in a simultaneous increase in red mite survival and reproduction (Maurer and Baumgartner, 1994; Kilpinen, 2001; Kilpinen, 2004). Temperatures in this study

gradually declined from 22°C to 12°C as the season passed from summer into winter, which was closely related to the decline in red mite numbers. Commercial egg producers with experience of red mite report this recurring seasonal pattern, with an increase in mite numbers in early summer and decrease in late autumn (Nordenfors and Höglund, 2000). Indeed the close relationship which exists between environmental temperature and red mite population may be considered as a future possible control method since extremes of hot or cold temperature (>45°C and <-20°C) are detrimental to red mite survival (Nordenfors *et al.*, 1999).

Stepwise regression was carried out on all egg production and red mite population parameters to determine how much of the variation observed could be counted for by one another. It was revealed that the majority of the variation observed within any particular red mite life-stage could, in fact, be attributed to other red mite stages. However, after mite parameters, mean building temperature had the next largest effect, with r^2 ranging between 1.9 and 68.1. Again, this is a frequently documented occurrence and has been seen to be a critical predicting determinant when modelling population dynamics of the poultry red mite (Maurer and Baumgartner, 1994). Egg production parameters on the other hand were solely explained by week, with the exception of mean building temperature which had a significant relationship with the population of red mite larvae ($r^2 = 5.5$).

4.5 Conclusions

In conclusion, this experiment observed the apparent effects of acaricide spraying on red mite populations in a commercial free-range system over a 17 week laying period. Despite there being no control population used, results observed a significant reduction, to almost complete eradication of red mite populations which appeared to be as a result of acaricide application.

The importance of trap placement when estimating red mite populations in poultry houses was also highlighted, establishing the need for careful consideration regarding house design and also genotype of the hen. However, this method of trapping merely provides an approximate guide into red mite population levels within a system and can by no means be used as an accurate measure to determine precise levels of infestation.

It has also shown a number of potentially important relationships between red mite and production variables, in particular the level of seasonal fluctuation as a result of changes in building temperature.

Despite the promising results seen with this carbamate-derived acaricide, as with most other compounds used for red mite control, it lacks sustainability due too limitations including, carbamate resistance (Chandre *et al.,* 1997; Oakeshott *et al.,* 2005; Fiddes *et al.,* 2006; Liming *et al.,* 2006;) and also the possibility of their link with human diseases (Zheng *et al.,* 2001). For these reasons it is recommended that this product should only be applied to an empty house and not in the presence of birds, as in the current study (ADAS, 2006).

Chapter 5

Effects of poultry red mite infestation on production and immunological parameters of laying hens

5.1 Introduction

As described in Chapter 2, the poultry red mite is generally accepted as the principal parasite affecting laying hens in European egg production (Höglund *et al.,* 1995; Kilpinen, 2000). Since current poultry production has progressed into large scale integrated systems, this has led to the evolution of very uniform and controlled environments. Consequently, the ecology of the poultry red mite has evolved from a wild type, nest dwelling parasite, to one which flourishes in this wholly synthetic environment (Axtell and Arends, 1990).

It is believed that the poultry red mite has a predilection for environments which offer the greatest number of potential hiding places (Höglund *et al.,* 1995). Hence red mite are seen in greater numbers in alternative free-range and barn systems, compared with conventional cage systems (Kilpinen, 1999; Guy and Edwards, 2006). Therefore, the impending ban on battery cages in the EU (European Council Directive 1999/74/EC) which takes effect in the UK in 2012 will result in a greater number of hens being housed in alternative, non-cage systems. This will indirectly reduce welfare of hens by promoting environments suitable for red mite proliferation (Höglund *et al.,* 1995).

Since the control of red mite using conventional methods of acaricide spraying is becoming increasingly difficult (see Section 2.4), it has been proposed that the development of alternative means of control are needed (Chauve, 1998). In order for this to be achieved, it is important to broaden the understanding of the relationship between the red mite, the chicken and the poultry house environment.

Attempts have been made previously at formulating population models for the poultry red mite in order to identify gaps in the current ecological understanding. This information would form the solid basis for planning calculated and tactical control strategies by the manipulation of the poultry house environment (Maurer and Baumgartner, 1994). However, many of these studies have been constructed in an

artificial laboratory environment and not in a real, commercial setting (Maurer and Baumgartner, 1994; Nordenfors *et al.,* 1999; Kilpinen, 2001; Kilpinen, 2005).

An alternative control method for the poultry red mite which has been suggested is the development of a novel vaccine as described in Section 2.13, which has shown previous success in the cattle tick, *Boophilus microplus* (Willadsen *et al.,* 1997). However, little is known regarding the immune response of birds to natural red mite exposure, which is an essential first step for vaccine development.

The aim of this study was therefore to monitor a number of egg production, environmental and immunological parameters between laying hens and poultry red mite populations over the flock laying cycle of several commercial laying farms to distinguish relationships and potential areas for future control.

5.2 Materials and methods

5.2.1 Study sites

This experiment followed 7 different populations of laying hens located on various sites around the North of England and Scottish Borders, all of which were known to have a history of red mite infestation. All sites were managed according to commercial husbandry guidelines and the housing systems chosen were considered to be typical of commercial practice in the UK (Rose, 2001). Sites 1, 2 and 3 consisted of a cage, free-range and barn systems, respectively and provided samples of poultry red mite and hens' eggs for IgY extraction, as well as production data. Site 4 was a barn system and provided poultry red mite and yolk IgY samples only. Site 5 was a free-range unit, used to monitor poultry red mite population and also for serological analysis of blood serum and egg yolk. Sites 6 and 7 were both free-range units, from which blood serum, red mite and production data were obtained (see Table 5.1). Not all sites yielded the same data series due to inherent problems with obtaining the full data set, such as human error, changes to staff on sites and simple failure to record the required information.

Table 5.1 *Summary of sample collection from different study sites*

Site No.	Housing system	Red mite	Production Data					Immunological data		
			Feed Intake	Water Consumption	% Lay	Hen mortality	Temperature	Yolk IgY	Serum IgY	Cytokine
1	Cage	√	√	√	√	√	√	√		
2	Free-range	√		√	√	√	√	√		
3	Barn	√	√	√	√	√		√		
4	Barn	√		√			√	√		
5	Free-range	√	√		√	√		√	√	
6	Free-range	√		√	√	√	√		√	√
7	Free-range	√	√		√	√	√		√	√

5.2.2 Poultry red mite sampling

Samples of poultry red mite were collected at monthly intervals from the point of lay until subsequent depopulation of the flocks (approximately 75 weeks of age). Red mite were trapped using the procedure described in Section 4.2.3, this time using rectangular pieces of box-section plastic sheet (30 x 100 mm) to provide a red mite harbourage. On free-range and barn sites, 10 traps were placed at the front edge of nest boxes at regular intervals along the length of both sides of the building. In the cage system, traps were secured with plastic ties under the feed troughs where red mite had previously been observed in abundance. Traps were replaced with new ones every month, which would subsequently remain in position until the following sampling date. Upon removal, each trap was placed inside a separate plastic bag containing approximately 25 ml of 70 % ethanol, sealed and sent to the laboratory for quantification of the number of red mite per trap. The protocol for quantification of red mite population was previously described in Section 4.2.4.

5.2.3 IgY collection and extraction

Hen eggs were collected as a means of determining IgY concentration. On each separate sampling date, 20 eggs were collected and delivered overnight to the laboratory where the yolks were separated from albumen and processed using PBS-chloroform precipitation. IgY extraction was followed by ELISA, which was performed on each IgY sample separately, as described in Sections 3.5 and 3.2, respectively.

5.2.4 Blood serum sampling

Blood serum was also collected on a monthly basis (from Sites 5, 6 and 7) in order to determine IgY response to natural red mite exposure. On Site 5, blood serum was obtained from 5 randomly selected birds at the time of routine veterinary sampling by pricking the brachial/wing vein with a needle and allowing approximately 500 µl of blood to flow into a 1.5 ml Eppendorf tube. On both Sites 6 and 7, blood was collected directly from the heart of hens shortly after cervical dislocation removing a volume of up to 5 ml. On these two Sites 5 randomly selected birds were bled at each sampling date. Blood collected from all sites was allowed to clot for a minimum of 2 hours, after which samples were sent to the laboratory, centrifuged at 3,000 x *g* for 10 min and serum removed. After serum removal all samples were analysed separately using an IgY specific ELISA assay (see Section 3.2).

5.2.5 Spleen samples

In order to establish cytokine responses on Sites 6 and 7 the spleen of each of 5

randomly selected birds were removed after cervical dislocation and previous blood sample removal. Birds were abdominally dissected, the spleen removed and a section of approximately one third taken and immediately stored in RNA*Later*® (QIAGEN, West Sussex, UK) for subsequent RNA extraction and cytokine analysis at the Institute for Animal Health, Compton, UK.

In order to quantify the level of expression of specific cytokines in RNA extracted from spleen samples a Taqman assay was used, the protocol for which was outlined in Section 3.7. The presence of RNA from cytokines which are classical indicators of both Th1-type and Th2-type immune responses were investigated using gene specific primers. Th1-type immunity was determined by the expression of interleukin-12α (IL-12α) and interferon-γ (IFNγ), whilst Th2-type immunity was determined by the expression interleukin-4 (IL-4), interleukin-13 (IL-13) and interleukin-5 (IL-5).

5.2.6 Production data

A series of production parameters were recorded at intervals during the study, to take account of egg production, hen mortality, feed/water consumption and mean building temperature (see Table 5.1). These were recorded on either a daily or weekly basis by staff working on study sites. Mean monthly values were then calculated for dates corresponding to when samples of red mite and yolk- or serum-IgY were obtained.

5.2.7 Statistical analysis

Data were analysed by ANOVA in the statistical package MINITAB (V14) to determine the effect of site on red mite population, egg production parameters and IgY levels. Also, values for Pearson's correlation were calculated to measure the relationships between different parameters including yolk- and serum-IgY levels, poultry red mite population and production variables. Significant correlations were subsequently assessed by stepwise regression in order to establish the greatest determinant of variability within that model. Analysis was initially performed on data from individual sites and later carried out on the overall mean values for all sites.

5.3 Results

5.3.1 Production parameters, poultry red mite population and immune response for all sites over the entire laying period

Mean egg production, red mite population and IgY levels are shown in Table 5.2, which illustrates the variability in the data collected. With uneven red mite and hen

populations in each category it is inappropriate to make generalisations about one particular housing system, so instead comments will be restricted to observations about the range of data presented. Also, as this data was seen to be particularly variable in the previous chapter, care must be taken when interpreting them as overall means. Nonetheless the average number of birds in a flock was considerably larger in the cage system compared to the other two systems. However, even within the free-range system the population of hens varied considerably, from 5,000 in Site 5 to 27,000 in Site 2. Feed intake (for the four sites for which data were available) varied from 98 g/bird to 129 g/day, water consumption from 153.1 ml/bird to 218.7 ml/bird and mean building temperature from 18 to 22 °C. Mean egg production also showed a range in performance, with % lay values from 71.6 to 88.1 %. There were significant differences between sites for hen mortality which ranged from a low of 1.3 % in Site 1 to a high of 6.7 % in Site 2.

Significant differences were also observed for mean poultry red mite population and immunoglobulin levels. The lowest values for both red mite population and yolk IgY optical density (means = 1 and 0.36, respectively) were observed on Site 1, with the highest values seen on Site 4 (mean = 10365 and 0.78, respectively). Mean red mite population on other sites was intermediate but nonetheless appeared to have a degree of correlation to mean yolk IgY levels, with the exception of Site 5 which had a comparatively high red mite infestation, but low yolk IgY level. Variation observed in mean red mite population between sites was considerable, with an overall mean of 5,843 and a coefficient of variation (C.V.) of 137 %. Mean yolk IgY optical density also showed a relatively high variation between sites (mean 0.66; C.V. 55 %). With regard to serum IgY levels, Site 5 had a significantly lower antibody level ($P<0.001$) in comparison to Sites 6 and 7. Variability between mean serum IgY levels was also relatively high (mean = 0.69; C.V. of 56 %).

Data for individual sites is displayed in Tables 5.3-5.9, showing how mean parameters change during the course of the laying period. Several general observations can be made. Firstly, a low, yet persistent level of hen mortality was apparent across all farms. Secondly, there was a common tendency for production levels to reduce, whilst feed intakes progressively increased. On each of the sites the population of red mite was seen to fluctuate considerably during the laying period, whilst maintaining an endemic red mite level relative to each site. However, there were no obvious trends in either yolk or serum IgY levels over the course of the experiment.

Table 5.2 *Summary of production parameters, poultry red mite population and IgY level for all sites*

	Site							S.E. Mean	Significance
	1	2	3	4	5	6	7		
Housing system	Cage	Free-range	Barn	Barn	Free-range	Free-range	Free-range	-	-
Hen population (birds)	44608	27000	6528	5000	5000	11923	6000	-	-
Feed intake (g/bird/day)	119.5	-	128.7	-	119.3	-	98.0	11.80	NS
Water consumption (ml/bird/day)	213.2	218.7	185.4	153.1	-	215.5	-	6.96	NS
% Lay	88.1	76.5	71.6	-	75.1	84.8	78.3	1.96	NS
Hen mortality %	1.3^{a}	6.7^{b}	1.5^{a}	-	3.6^{ab}	5.4^{bd}	2.4^{ad}	0.40	***
Mean house temperature (°C)	22^{a}	19^{bd}	-	21^{ac}	-	20^{bc}	18^{d}	0.21	***
Total poultry red mite (per trap)	1^{a}	2333^{a}	644^{a}	10365^{bc}	4644^{ac}	4203^{ad}	9444^{bcd}	641	***
Yolk IgY (Optical Density)	0.36^{b}	0.70^{ab}	0.52^{ab}	0.78^{a}	0.44^{ab}	-	-	0.057	**
Serum IgY (Optical Density)	-	-	-	-	0.28^{a}	0.99^{b}	0.76^{b}	0.068	***

*= P<0.05; **= P<0.01; ***= P<0.001; NS= no significant difference; - = Missing value
Means within a row followed by a different superscript letter are significantly different at P<0.05

Table 5.3 *Mean production and poultry red mite data over the laying period for Site 1*

Age (weeks)	Poultry Red Mite Parameters					Immunoglobulin		Production Parameters				
	Egg no.	Larvae no.	Adult and nymph fed	Adult and nymph unfed	Total Population	Yolk IgY	Serum IgY	% Lay	Hen mortality (%)	Feed intake (g/b/d)	Water Consumption (ml/b/d)	Temp. (°C)
20	0	0	7	1	7	-	-	73	0.17	88	172	21
22	0	0	0	0	0	-	-	93	0.32	108	214	21
23	0	0	0	0	0	-	-	94	0.38	107	231	21
25	0	0	0	0	0	0.17	-	95	0.54	129	217	21
28	0	0	0	0	0	0.25	-	95	0.65	130	238	23
30	0	0	0	0	0	0.33	-	95	0.73	121	221	21
32	0	0	1	0	2	0.18	-	95	0.83	133	222	21
35	0	0	1	0	2	0.11	-	93	0.96	123	251	23
39	0	0	0	0	0	0.01	-	92	1.18	126	217	21
43	0	0	0	1	0	0.28	-	87	1.45	124	226	20
47	0	0	0	0	2	0.42	-	85	1.75	118	211	20
51	0	0	0	0	1	0.73	-	83	2.14	118	211	20
55	0	0	0	1	1	0.68	-	84	2.52	126	219	20
62	0	0	0	0	1	0.96	-	81	3.02	131	219	20
65	0	0	1	0	1	0.16	-	76	3.23	110	212	20

Table 5.4 *Mean production and poultry red mite data over the laying period for Site 2*

Age (weeks)	Poultry Red Mite Parameters					Immunoglobulin		Production Parameters				
	Egg no.	Larvae no.	Adult and nymph fed	Adult and nymph unfed	Total Population	Yolk IgY	Serum IgY	% Lay	Hen mortality (%)	Feed intake (g/b/d)	Water Consumption (ml/b/d)	Temp. (°C)
25	0	0	1	0	1	-	-	66	1.64	-	195	18
26	43	3	49	3	98	1.26	-	90	2.51	-	225	20
28	398	330	342	307	1377	0.69	-	91	2.77	-	220	19
31	2991	1370	3778	348	8487	0.87	-	89	3.27	-	229	18
34	428	29	338	30	825	0.39	-	80	4.25	-	220	20
38	102	153	85	27	366	0.85	-	70	4.81	-	201	21
42	2126	1834	1399	954	6313	0.71	-	75	6.26	-	200	21
46	1719	1738	1203	613	5272	0.29	-	77	7.61	-	210	19
50	426	310	370	46	1153	0.58	-	68	8	-	199	17
54	515	678	640	58	1891	0.08	-	74	9.19	-	220	20
61	809	341	679	93	1921	1.10	-	77	10.27	-	202	18
64	23	815	425	70	1333	0.98	-	71	12.77	-	225	18
67	645	125	386	140	1297	0.64	-	67	14.3	-	225	18

Table 5.5 *Mean production and poultry red mite data over the laying period for Site 3*

Age (weeks)	Poultry Red Mite Parameters					Immunoglobulin		Production Parameters				
	Egg no.	Larvae no.	Adult and nymph fed	Adult and nymph unfed	Total Population	Yolk IgY	Serum IgY	% Lay	Hen mortality (%)	Feed intake (g/b/d)	Water Consumption (ml/b/d)	Temp. (°C)
20	152	20	377	5	553	-	-	28	0.28	89	162	-
21	195	16	124	151	486	-	-	65	0.31	99	194	-
23	108	68	194	38	408	0.42	-	83	0.35	132	193	-
26	129	237	203	80	648	0.28	-	85	0.44	131	214	-
28	387	391	305	21	1104	0.75	-	85	0.54	132	179	-
30	26	7	48	2	83	0.48	-	86	0.83	139	171	-
33	232	230	321	51	834	0.31	-	85	1.55	122	219	-
42	565	136	625	110	1436	0.77	-	83	2.16	119	200	-
45	1	1	2	0	4	0.62	-	38	2.34	113	135	-
54	256	767	572	55	1650	0.83	-	75	2.77	188	192	-
63	53	15	106	13	186	0.68	-	74	3.28	156	192	-
72	40	76	181	35	333	0.08	-	72	3.69	124	174	-

Table 5.6 *Mean production and poultry red mite data over the laying period for Site 4*

Age (weeks)	Poultry Red Mite Parameters					Immunoglobulin		Production Parameters				
	Egg no.	Larvae no.	Adult and nymph fed	Adult and nymph unfed	Total Population	Yolk IgY	Serum IgY	% Lay	Hen mortality (%)	Feed intake (g/b/d)	Water Consumption (ml/b/d)	Temp. (°C)
20	12	4	2	13	32	1.11	-	-	-	-	124	23
23	51	16	18	37	122	1.02	-	-	-	-	126	24
25	*	-	-	-	-	1.09	-	-	-	-	129	24
27	722	358	251	1697	3029	1.07	-	-	-	-	132	23
28	*	-	-	-	-	0.48	-	-	-	-	134	19
29	*	-	-	-	-	0.80	-	-	-	-	136	21
31	1485	710	713	2808	5715	-	-	-	-	-	138	20
32	1663	844	506	3325	6338	1.01	-	-	-	-	140	19
34	1683	1100	808	3514	7106	0.80	-	-	-	-	142	19
50	2616	1484	2425	1981	8506	1.18	-	-	-	-	-	20
55	3458	2825	7608	3128	17019	1.08	-	-	-	-	-	21
61	4242	1842	5936	3478	15497	0.05	-	-	-	-	180	20
62	*	-	-	-	-	-	-	-	-	-	182	22
66	4165	3508	10380	15490	33543	0.04	-	-	-	-	187	27
70	3478	2083	8935	2615	17110	0.77	-	-	-	-	193	21
75	*	-	-	-	-	0.45	-	-	-	-	199	22

Table 5.7 *Mean production and poultry red mite data over the laying period for Site 5*

Age (weeks)	Poultry Red Mite Parameters					Immunoglobulin		Production Parameters				
	Egg no.	Larvae no.	Adult and nymph fed	Adult and nymph unfed	Total Population	Yolk IgY	Serum IgY	% Lay	Hen mortality (%)	Feed intake (g/b/d)	Water Consumption (ml/b/d)	Temp. (°C)
21	21	171	148	58	398	1.04	0.05	68.45	0.54	87.1	-	-
25	90	258	350	23	721	0.63	0.04	87.33	0.88	126.4	-	-
29	470	1527	1177	196	3370	0.53	0.15	88.89	1.68	114.5	-	-
33	1273	1573	2486	273	5604	0.75	0.09	92.23	2.42	121.7	-	-
37	697	733	1421	83	2933	0.23	0.12	91.46	2.88	109.9	-	-
41	1404	2161	2536	246	6347	0.20	0.10	90.06	3.32	114.1	-	-
45	1344	1012	2311	89	4756	0.16	0.13	86.36	4.14	117.5	-	-
49	1878	1739	3050	167	6833	0.20	0.35	78.33	5.82	130.4	-	-
53	3052	1870	3620	2295	10839	0.21	0.32	67.38	14.72	126.3	-	-

Table 5.8 *Mean production and poultry red mite data over the laying period for Site 6*

Age (weeks)	Poultry Red Mite Parameters					Immunoglobulin		Production Parameters				
	Egg no.	Larvae no.	Adult and nymph fed	Adult and nymph unfed	Total Population	Yolk IgY	Serum IgY	% Lay	Hen mortality (%)	Feed intake (g/b/d)	Water Consumption (ml/b/d)	Temp. (°C)
27	2	1	3	2	8	0.85	-	91	1.88	-	196	19
32	275	278	1012	33	1597	1.03	-	79	2.54	-	328	19
37	686	724	3179	240	4829	0.99	-	92	3.34	-	233	19
41	1005	735	4639	124	6502	0.72	-	92	3.74	-	215	18
45	1735	667	3362	25	5789	1.22	-	90	4.09	-	195	19
49	33	68	276	6	383	1.31	-	82	4.72	-	212	20
54	60	110	190	11	371	0.80	-	96	5.29	-	195	19
58	1673	776	2284	26	4759	0.47	-	87	6.08	-	212	19
62	2130	836	2871	32	5869	0.77	-	82	7.06	-	197	20
67	1001	676	2200	22	3900	1.34	-	74	9.08	-	204	20
71	3583	3153	5229	261	12226	1.43	-	68	11.63	-	183	22

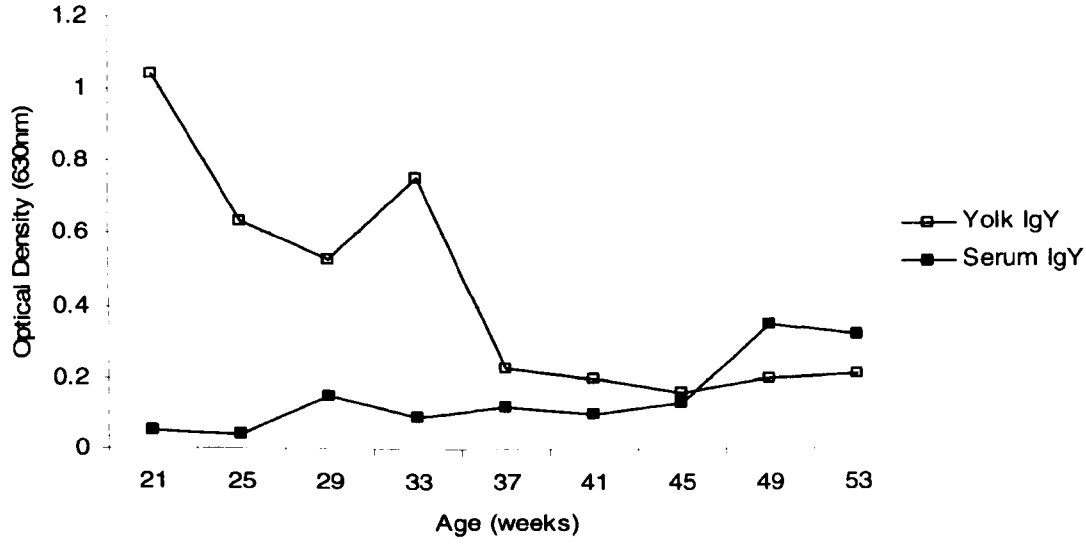
Table 5.9 *Mean production and poultry red mite data over the laying period for Site 7*

Age (weeks)	Poultry Red Mite Parameters					Immunoglobulin		Production Parameters				
	Egg no.	Larvae no.	Adult and nymph fed	Adult and nymph unfed	Total Population	Yolk IgY	Serum IgY	% Lay	Hen mortality (%)	Feed intake (g/b/d)	Water Consumption (ml/b/d)	Temp. (°C)
22	2549	490	4029	112	7180	0.99	-	25.04	0.03	-	-	18.7
27	1125	1291	3449	318	6183	0.82	-	81.67	0.3	105.32	-	18.6
31	1509	1509	5093	67	8178	0.32	-	-	0.62	103.64	-	18
36	5588	2520	10174	255	18537	0.63	-	83.01	0.99	-	-	17.8
40	2869	2244	11679	288	17080	0.94	-	81.69	1.21	102.9	-	18.2
45	2364	1361	7090	36	10850	0.90	-	81.93	2.01	86.03	-	17.6
48	574	485	3523	163	4744	0.76	-	81.02	2.37	102.37	-	17
53	2475	1668	6007	371	10521	0.76	-	83.08	2.9	103.24	-	17.8
58	1095	1275	4935	215	7520	0.88	-	74.79	3.5	105.57	-	17.6
62	3346	3321	9433	296	16396	1.22	-	82.99	4.08	103.38	-	-
67	663	625	2041	75	3403	0.42	-	83.47	4.68	69.09	-	-
72	1071	343	1136	186	2736	0.48	-	82.03	5.56	-	-	-

5.3.2 Comparison of yolk and serum IgY levels

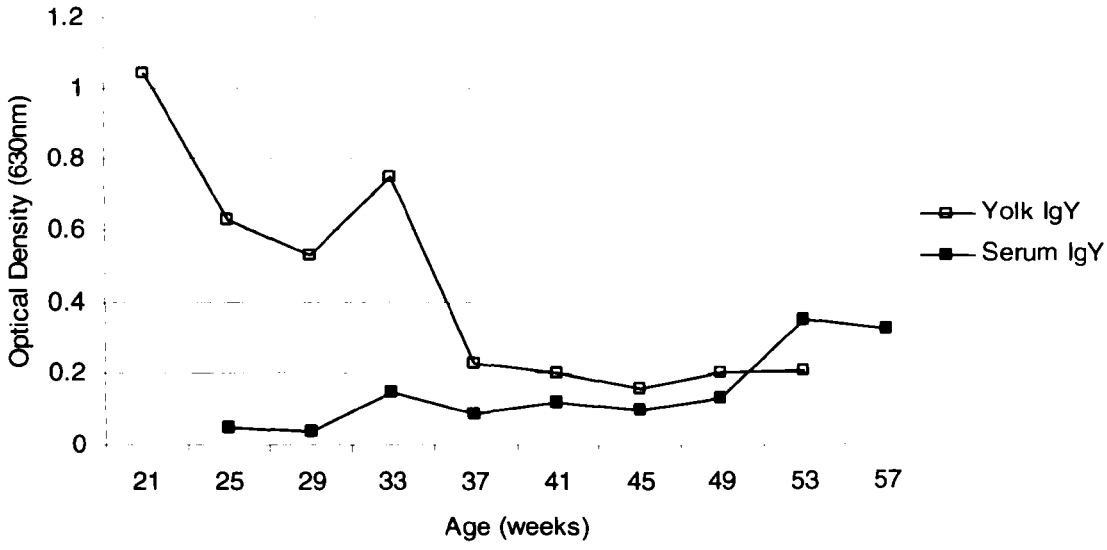
On Site 5 samples of both yolk and serum IgY were taken in order to establish the association between the level of IgY in these two sampling methods. There were significant differences in the level of antibody detected. Mean serum IgY had a significantly lower level compared to mean yolk IgY level (mean = 0.15 vs. 0.44; $P<0.05$). When plotted over the course of the experiment, it also appears that there was no obvious relationship between serum and yolk IgY levels (Figure 5.1). The Pearson's correlation value confirms this ($r = -0.58$; $P = 0.10$).

Figure 5.1 *Comparison of yolk and serum IgY levels (optical density) during the laying period (Site 5)*



However, immunoglobulins are deposited in the egg over a period of time (about 21 days). Therefore, if the effects of IgY accumulation are removed from yolk IgY so that the value for serum IgY is plotted against the value for yolk IgY 4 weeks prior to that point, some degree of association between serum and yolk IgY levels is shown (Figure 5.2). Fluctuations between weeks follow a similar trend i.e. at each sampling point, as the value for serum IgY increases/decreases so does that of yolk IgY and vice versa.

Figure 5.2 *Comparison of yolk and serum IgY levels during the laying period, corrected for lag effect of IgY deposition (Site 5)*



5.3.3 Relationships between egg production, red mite population and IgY levels.

(i) Site 1

Despite having a very low mite infestation level, several significant relationships were found for Site 1 (Table 5.10). Red mite population was observed to have negative correlations with feed and water consumption ($r = -0.63$ and -0.68, respectively) and egg production ($r = -0.59$, $P<0.05$). On this site it was also found that the hen mortality was positively correlated with mean yolk IgY level ($r = 0.60$, $P<0.05$).

(ii) Site 2

On Site 2 there were no significant correlations, apart from those within the red mite life-stage estimates (Table 5.11).

(iii) Site 3

Site 3 also showed a comparatively low level of red mite infestation, but still generated a number of significant correlations amongst the data collected (Table 5.12). These were observed between both larval numbers and feed intake, and also unfed adults/nymphs and water consumption. These correlations were both positive, suggesting that increases in red mite levels resulted in greater feed/water consumption, although these associations were only moderate ($r = 0.68$ and 0.59, respectively).

(iv) Site 4

Site 4 was observed to have the highest red mite population of all the units sampled. However, the only production data available for this site was water consumption, which was observed to have a significant positive relationship with all red mite variables (Table 5.13). Yolk IgY level was also negatively correlated to all of the red mite population parameters, with the exception of larvae.

(v) Site 5

Site 5 was sampled for both yolk and serum IgY and showed a number of significant correlations (Table 5.14). Interestingly both yolk and serum IgY levels were significantly correlated with age of the birds, although this correlation was negative for yolk IgY ($r = -0.80$; $P<0.01$) levels and positive for serum IgY levels ($r = 0.86$; $P<0.001$). Levels of both yolk and serum IgY were also correlated with a number of the red mite life-stage estimates. Site 5 was the only site observed to demonstrate this positive correlation between humoral immunity and red mite population ($r = 0.78$; $P<0.01$). Significant correlations were also observed between various red mite life-stages and hen mortality (e.g. total red mite $r = 0.88$; $P<0.01$), suggesting that an increase in mortality of hens was observed with a concurrent increase in red mite population.

(vi) Site 6

On Site 6, there was a significant relationship between age of the flock and red mite egg ($r = 0.67$; $P<0.05$) and larvae ($r = 0.60$; $P<0.05$) estimates (Table 5.15). These two indicators of the level of red mite population were in turn positively correlated to hen mortality ($r = 0.76$ and 0.78, respectively). In addition, the number of red mite eggs and larvae also showed a significant relationship with building temperature ($r = 0.66$ and 0.77, respectively). Egg production of birds also had a negative relationship with building temperature ($r = -0.82$; $P<0.01$) and larval numbers ($r = -0.64$; $P<0.05$). Several correlations within production parameters were also seen, such as an increase in hen mortality with age ($r = 0.95$; $P<0.001$) and a decrease in egg output with increased hen mortality ($r = -0.74$; $P<0.01$).

(vii) Site 7

On site 7, no significant correlations were observed between any of the egg production and red mite parameters (Table 5.16). However, significant correlations were observed between building temperature and hen mortality ($r = 0.81$; $P<0.01$), where reductions in building temperature caused increases in hen mortality.

*(viii) Relationships between egg production, red mite population and **yolk** IgY levels from all sites*

When the data from those sites providing yolk IgY were combined (n = 5), significant relationships observed were between hen mortality and red mite population estimates (Table 5.17). Here, rises in red mite infestation caused a significant rise in the proportion of bird deaths (e.g. total red mite r = 0.48; P<0.001). Few other significant correlations were observed, with the exception of a positive relationship between increased bird age and that of all red mite life-stages, excluding larvae and eggs.

*(ix) Relationships between egg production, red mite population and **serum** IgY levels from all sites*

Combining data from three sites sampled for blood serum showed hen mortality was significantly correlated to both building temperature and also the number of unfed red mite adults and nymphs (Table 5.18). Serum IgY levels on the other hand were seen to be significantly correlated to both hen mortality (r = 0.36; P<0.01) and building temperature (r = 0.61; P<0.01).

(x) Relationships between egg production and red mite population from all sites.

Finally, data from all sites were combined in one dataset to investigate the relationship between production parameters and red mite populations, with the exclusion of immunological data. Significant correlations found here confirmed those previously observed on individual sites with hen mortality, feed intake and building temperature all showing significant and positive interactions with various red mite life-stages (Table 5.19).

Table 5.10 *Correlations between poultry red mite population, production parameters and immune response for Site 1 (P-value, followed by r)*

	Age (weeks)	Hen mortality (%)	% Lay	Feed intake (g/b/d)	Water consumption (ml/b/d)	Temperature (°C)	Mite egg number	Larvae number	Fed adult and nymph	Unfed adult and nymph	Total mite Population
Hen mortality (%)	**0.000(0.993)**										
% Lay	**0.037(-0.541)**	**0.027(-0.569)**									
Feed intake (g/b/d)	0.284(0.296)	0.351(0.259)	**0.048(0.518)**								
Water consumption	0.902(0.035)	0.997(0.001)	**0.005(0.684)**	**0.009(0.646)**							
Temperature (°C)	**0.022(-0.587)**	**0.019(-0.597)**	**0.035(0.547)**	0.702(0.108)	0.057(0.501)						
Mite egg number	0.936(-0.023)	0.824(0.063)	0.214(-340)	0.206(-0.346)	0.478(-0.199)	0.873(0.045)					
Larvae number	0.846(0.055)	0.984(0.006)	0.307(-0.283)	0.888(-0.040)	0.725(-0.990)	0.957(0.015)	**0.002(0.738)**				
Fed adult and nymph	0.279(-0.299)	0.307(-0.283)	**0.041(-0.533)**	**0.011(-0.636)**	**0.007(-0.667)**	0.648(0.128)	0.087(0.457)	0.074(0.475)			
Unfed adult and nymph	0.992(-0.003)	0.954(0.016)	**0.048(-0.518)**	0.105(-0.435)	0.087(-0.457)	0.307(-0.283)	0.392(0.238)	0.208(0.345)	0.079(0.468)		
Total mite population	0.465(-0.204)	0.481(-0.197)	**0.022(-0.586)**	**0.012(-0.626)**	**0.006(-0.675)**	0.848(0.054)	**0.045(0.524)**	**0.034(0.034)**	**0.000(0.958)**	0.083(0.462)	
Yolk IgY	**0.048(0.580)**	**0.038(0.603)**	0.074(-0.534)	0.826(0.071)	0.300(-0.327)	0.090(-0.511)	0.648(0.147)	0.648(0.147)	0.467(-233)	0.555(0.190)	0.787(0.087)

Table 5.11 *Correlations between poultry red mite population, production parameters and immune response for Site 2 (P-value, followed by r)*

	Age (weeks)	Hen mortality (%)	% Lay	Temperature (°C)	Water consumption (ml/b/d)	Mite egg number	Larvae number	Fed adult and nymph	Unfed adult and nymph	Total mite Population
Hen mortality (%)	**0.000(0.997)**									
% Lay	0.067(-0.522)	0.070(-0.518)								
Temperature (°C)	0.276(-0.327)	0.284(-0.322)	0.498(0.207)							
Water consumption	0.687(0.124)	0.618(0.153)	0.058(0.537)	0.905(-0.037)						
Mite egg number	0.783(-0.085)	0.812(-0.073)	0.285(0.321)	0.978(0.009)	0.714(0.113)					
Larvae number	0.758(0.095)	0.737(0.103)	0.668(0.132)	0.650(0.139)	0.991(0.003)	**0.001(0.815)**				
Fed adult and nymph	0.710(-0.114)	0.717(-0.112)	0.185(0.393)	0.672(-0.130)	0.332(0.293)	**0.000(0.928)**	**0.010(0.683)**			
Unfed adult and nymph	0.848(-0.059)	0.909(-0.035)	0.561(0.178)	0.328(0.295)	0.656(-0.137)	**0.004(0.743)**	**0.000(0.870)**	0.086(0.494)		
Total mite population	0.856(-0.056)	0.881(-0.046)	0.298(0.313)	0.948(0.020)	0.657(0.137)	**0.000(0.985)**	**0.000(0.887)**	**0.000(0.927)**	**0.002(0.772)**	
Yolk IgY	0.703(-0.123)	0.696(-0.126)	0.367(0.287)	0.701(-0.124)	0.877(0.050)	0.742(-0.106)	0.470(-0.231)	0.997(0.001)	0.593(-0.172)	0.727(-0.113)

Table 5.12 *Correlations between poultry red mite population, production parameters and immune response for Site 3 (P-value, followed by r)*

	Age (weeks)	Hen mortality (%)	% Lay	Feed Intake (g/b/d)	Water consumption (ml/b/d)	Mite egg number	Larvae number	Fed adult and nymph	Unfed adult and nymph	Total mite Population
Hen mortality (%)	**0.000(0.983)**									
% Lay	0.874(0.051)	0.994(0.002)								
Feed intake (g/b/d)	0.107(0.489)	0.139(0.453)	0.090(0.510)							
Water consumption	0.680(-0.133)	0.692(-0.128)	**0.009(0.712)**	0.429(0.252)						
Mite egg number	0.579(-0.178)	0.713(-0.119)	0.313(0.319)	0.991(-0.004)	0.181(0.414)					
Larvae number	0.663(0.140)	0.671(0.137)	0.300(0.326)	**0.014(0.684)**	0.311(0.320)	0.165(0.428)				
Fed adult and nymph	0.938(0.025)	0.789(0.087)	0.645(0.148)	0.504(0.214)	0.216(0.386)	**0.001(0.815)**	**0.029(0.627)**			
Unfed adult and nymph	0.508(-0.212)	0.576(-0.180)	0.378(0.280)	0.601(-0.168)	**0.045(0.587)**	0.095(0.504)	0.745(0.105)	0.310(0.320)		
Total mite population	0.988(-0.005)	0.906(0.038)	0.307(0.322)	0.252(0.359)	0.128(0.465)	**0.000(0.849)**	**0.001(-0.809)**	**0.000(0.929)**	0.182(0.413)	
Yolk IgY	0.954(-0.021)	0.897(0.047)	0.722(-0.129)	0.206(0.438)	0.628(-0.175)	0.139(0.502)	0.248(0.403)	0.220(0.426)	0.972(0.013)	0.160(0.480)

Table 5.13 *Correlations between poultry red mite population, production parameters and immune response for Site 4 (P-value, followed by r)*

	Age (weeks)	Water consumption (ml/b/d)	Mite egg number	Larvae number	Fed adult and nymph	Unfed adult and nymph	Total mite Population
Water consumption	**0.000(1.000)**						
Mite egg number	**0.000(0.959)**	**0.000(0.957)**					
Larvae number	**0.000(0.899)**	**0.001(0.913)**	**0.000(0.920)**				
Fed adult and nymph	**0.000(0.940)**	**0.000(0.966)**	**0.000(0.888)**	**0.000(0.935)**			
Unfed adult and nymph	0.074(0.559)	**0.081(0.610)**	**0.046(0.610)**	**0.007(0.759)**	**0.027(0.660)**		
Total mite population	**0.000(0.873)**	**0.002(0.885)**	**0.000(0.883)**	**0.000(0.961)**	**0.000(0.933)**	**0.000(0.876)**	
Yolk IgY	0.121(-0.434)	0.064(-0.549)	**0.030(-0.681)**	0.073(-0.590)	**0.052(-0.628)**	**0.018(-0.722)**	**0.015(-0.736)**

Table 5.14 *Correlations between poultry red mite population, production parameters and immune response for Site 5 (P-value, followed by r)*

	Age (weeks)	Hen mortality (%)	% Lay	Feed Intake (g/b/d)	Mite egg number	Larvae number	Fed adult and nymph	Unfed adult and nymph	Total mite Population	Yolk IgY
Hen mortality (%)	**0.001(0.881)**									
% Lay	0.333(0.342)	0.754(0.114)								
Feed intake (g/b/d)	0.801(0.099)	0.895(0.052)	0.467(0.279)							
Mite egg number	**0.000(0.954)**	**0.000(0.927)**	0.364(-0.344)	0.662(-0.184)						
Larvae number	**0.045(0.678)**	0.141(0.531)	0.803(0.097)	0.526(-0.265)	**0.022(0.743)**					
Fed adult and nymph	**0.000(0.931)**	**0.013(0.783)**	0.771(-0.113)	0.609(-0.265)	**0.000(0.957)**	**0.004(0.844)**				
Unfed adult and nymph	**0.053(0.659)**	**0.000(0.934)**	0.103(-0.578)	0.347(-0.385)	**0.011(0.792)**	0.242(0.435)	0.085(0.604)			
Total mite population	**0.001(0.917)**	**0.002(0.883)**	0.517(-0.250)	0.570(-0.238)	**0.000(-0.983)**	**0.004(0.844)**	**0.000(0.967)**	**0.015(0.772)**		
Yolk IgY	**0.010(-0.797)**	0.132(-0.542)	0.654(-0.174)	0.873(-0.068)	0.065(-0.636)	0.112(-0.566)	**0.041(-0.687)**	0.454(-0.287)	0.071(-0.627)	
Serum IgY	**0.001(0.858)**	**0.005(0.806)**	0.764(0.109)	0.624(-0.190)	**0.009(0.806)**	0.094(0.591)	**0.019(0.755)**	0.090(0.596)	**0.014(0.775)**	0.102(-0.580)

Table 5.15 *Correlations between poultry red mite population, production parameters and immune response for Site 6 (P-value, followed by r)*

	Age (weeks)	Hen mortality (%)	% lay	Temperature (°C)	Water consumption (ml/b/d)	Mite egg number	Larvae number	Fed adult and nymph	Unfed adult and nymph	Total mite Population
Hen mortality (%)	**0.000(0.949)**									
% Lay	0.060(-0.583)	**0.010(-0.735)**								
Temperature (°C)	**0.024(0.671)**	**0.002(0.814)**	**0.002(-0.815)**							
Water consumption	0.111(-0.507)	0.153(-0.461)	0.820(-0.078)	0.451(-0.254)						
Mite egg number	**0.023(0.0674)**	**0.007(0.756)**	0.075(-0.557)	**0.028(0.656)**	0.248(-0.380)					
Larvae number	**0.050(0.603)**	**0.005(0.776)**	**0.035(0-0.638)**	**0.006(0.765)**	0.381(-0.294)	**0.000(0.905)**				
Fed adult and nymph	0.246(0.383)	0.133(0.482)	0.375(-0.297)	0.453(0.253)	0.496(-0.230)	**0.003(0.805)**	**0.005(0780)**			
Unfed adult and nymph	0.670(0.145)	0.309(0.338)	0.471(-0.243)	0.258(0.373)	0.853(-0.064)	0.101(0.521)	**0.009(0.743)**	**0.011(0.729)**		
Total mite population	0.083(0.545)	**0.025(0.667)**	0.137(-0.478)	0.100(0.521)	0.365(-0.303)	**0.000(0.938))**	**0.000(0.925)**	**0.000(0.950)**	**0.011(0.726)**	
Serum IgY	0.475(0.241)	0.221(0.401)	**0.034(-0.640)**	**0.044(0.615)**	0.898(-0.044)	0.531(0.212)	0.253(0.377)	0.641(0.159)	0.434(0.263)	0.475(0.241)

Table 5.16 *Correlations between poultry red mite population, production parameters and immune response for Site 7 (P-value, followed by r)*

	Age (weeks)	Hen mortality (%)	% lay	Feed Intake (g/b/d)	Temperature (°C)	Mite egg number	Larvae number	Fed adult and nymph	Unfed adult and nymph	Total mite Population
Hen mortality (%)	**0.000(0.991)**									
% Lay	0.222(0.491)	0.343(0.317)								
Feed intake (g/b/d)	0.124(-0.552)	0.111(-0.568)	0.375(-0.365)							
Temperature (°C)	**0.013(-0.781)**	**0.017(-0.763)**	0.212(-0.495)	0.401(0.379)						
Mite egg number	0.367(-0.286)	0.306(-0.323)	0.541(0.207)	0.536(0.239)	0.722(0.139)					
Larvae number	0.869(-0.054)	0.706(-0.122)	0.181(0.435)	0.329(0.368)	0.880(0.059)	**0.008(0.725)**				
Fed adult and nymph	0.463(-0.235)	0.304(-0.324)	0.367(0.302)	0.379(0.334)	0.991(-0.005)	**0.002(0.795)**	**0.000(0.868)**			
Unfed adult and nymph	0.897(0.042)	0.985(0.006)	0.330(0.325)	0.098(0.585)	0.710(0.145)	0.305(0.324)	0.086(0.517)	0.225(0.378)		
Total mite population	0.475(-0.229)	0.334(-0.305)	0.337(0.320)	0.364(0.345)	0.894(0.052)	**0.000(0.884)**	**0.000(0.906)**	**0.000(0.981)**	0.167(0.427)	
Serum IgY	0.700(-0.124)	0.658(-0.143)	0.288(-0.352)	0.371(0.340)	0.619(0.193)	0.329(0.309)	0.145(0.447)	0.108(0.487)	0.210(0.390)	0.128(0.465)

Table 5.17 *Correlations between poultry red mite population, production parameters and* ***yolk*** *IgY immune response for Sites 1-5 (P-value, followed by r)*

	Age (weeks)	Hen mortality (%)	% Lay	Feed Intake (g/b/d)	Mite egg number	Larvae number	Fed adult and nymph	Unfed adult and nymph	Total mite Population
Hen mortality (%)	**0.000(0.665)**								
% Lay	0.979(0.004)	0.380(-0.127)							
Feed intake (g/b/d)	0.532(0.106)	0.628(0.082)	0.204(0.214)						
Mite egg number	0.064(0.266)	**0.000(0.499)**	0.949(-0.009))	0.669(0.074)					
Larvae number	0.059(0.272)	**0.002(0.432)**	0.716(0.053)	0.637(0.081)	**0.000(0.896)**				
Fed adult and nymph	**0.051(0.281)**	**0.005(0.395)**	0.728(0.051)	0.612(0.087)	**0.000(0.895)**	**0.000(0.872)**			
Unfed adult and nymph	**0.048(0.284)**	**0.000(0.536)**	0.417(-0.119)	0.853(-0.032)	**0.000(0.678)**	**0.000(0.665)**	**0.000(0.735)**		
Total mite population	**0.039(0.295)**	**0.000(0.484)**	0.926(0.014)	0.642(0.080)	**0.000(0.917)**	**0.000(0.898)**	**0.000(0.954)**	**0.000(0.882)**	
Yolk IgY	0.511(-0.511)	0.461(0.115)	0.129(-0.235)	0.944(0.013)	0.885(-0.020)	0.653(-0.063)	0.545(-0.085)	0.477(-0.100)	0.556(-0.083)

Table 5.18 *Correlations between poultry red mite population, production parameters and* ***blood serum*** *IgY immune response for Sites 5-7 (P-value, followed by r)*

	Age (weeks)	Hen mortality (%)	% lay	Feed Intake (g/b/d)	Temperature (°C)	Mite egg number	Larvae number	Fed adult and nymph	Unfed adult and nymph	Total mite Population
Hen mortality (%)	**0.000(0.829)**									
% Lay	0.169(0.249)	0.652(0.083)								
Feed intake (g/b/d)	0.901(-0.031)	0.942(0.019)	0.362(0.236)							
Temperature (°C)	0.071(0.412)	**0.000(0.762)**	0.527(-0.155)	0.573(0.260)						
Mite egg number	0.147(0.262)	0.228(0.219)	0.502(-0.125)	0.409(-0.214)	0.865(-0.041)					
Larvae number	0.115(0.284)	0.246(0.211)	0.840(0.038)	0.426(-0.207)	0.840(0.048)	**0.000(0.782)**				
Fed adult and nymph	0.636(0.087)	0.613(-0.093)	0.963(-0.009)	0.351(-0.241)	0.071(-0.412)	**0.000(0.814)**	**0.000(0.743)**			
Unfed adult and nymph	0.079(0.315	**0.001(0.563)**	0.318(-0.186)	0.325(-0.254)	0.301(-0.243)	**0.049(0.351)**	**0.054(0.344)**	0.359(0.168)		
Total mite population	0.278(0.198)	0.640(0.083)	0.804(-0.046)	0.343(-0.245)	0.272(-0.258)	**0.000(0.920)**	**0.000(0.850)**	**0.000(0.961)**	0.059(0.337)	
Serum IgY	0.060(0.331)	**0.037(0.364)**	0.943(0.013)	0.308(-0.255)	**0.005(0.605)**	0.147(0.262)	0.441(0.141)	0.086(0.308)	0.994(0.001)	0.122(0.279)

Table 5.19 *Correlations between poultry red mite population and production parameters for all sites (P-value, followed by r)*

	Age (weeks)	Hen mortality (%)	% lay	Feed Intake (g/b/d)	Temperature (°C)	Mite egg number	Larvae number	Fed adult and nymph	Unfed adult and nymph
Hen mortality (%)	**0.000(0.695)**								
% Lay	0.648(0.055)	0.488(-0.083)							
Feed intake (g/b/d)	0.777(0.043)	0.722(0.054)	0.185(0.201)						
Temperature (°C)	0.258(-0.166)	0.197(-0.190)	0.120(0.230)	**0.019(0.484)**					
Mite egg number	**0.030(0.256)**	**0.022(0.271)**	0.926(-0.011)	0.547(-0.092)	0.355(-0.132)				
Larvae number	**0.010(0.302)**	**0.010(0.302)**	0.651(0.055)	0.733(-0.052)	0.720(-0.051)	**0.000(0.880)**			
Fed adult and nymph	0.093(0.199)	0.597(0.063)	0.652(0.054)	0.423(-0.123)	0.120(-0.220)	**0.000(0.866)**	**0.000(0.846)**		
Unfed adult and nymph	0.056(0.277)	**0.000(0.444)**	0.433(-0.095)	0.461(-0.133)	**0.012(0.349)**	**0.000(0.481)**	**0.000(0.496)**	**0.000(0.420)**	
Total mite population	**0.033(0.252)**	0.106(0.192)	0.792(0.032)	0.484(-0.107)	0.883(-0.021)	**0.000(0.916)**	**0.000(0.904)**	**0.000(0.926)**	**0.000(699)**

5.3.4 Stepwise regression

Production, red mite and immunological data which were seen to be significantly correlated with one another, were subsequently analysed by stepwise regression in order to establish the greatest determinant of variability within that model, i.e. the most important predictor affecting a particular response variable (Table 5.20).

Table 5.20 *Stepwise regression showing factors affecting red mite populations, production parameters and IgY levels*

Response	Predictor	Significance	r^2	Site	Source table in Appendix I
Mite egg number	Age (weeks)	***	90.8	4	8
Unfed adult and nymph	% Lay	*	21.2	1	2
	Water consumption	*	27.9	2	4
	Hen mortality (%)	***	85.4	5	10
	Age (weeks)	***	12.1	5	10
	Total mite population	**	1.95	5	10
	Larvae number.	***	23.4	All	20
	Hen mortality (%)	**	8.7	All	20
Fed adult and nymph	Total mite population	***	92.6	5	10
	Hen mortality (%)	NS	1.8	5	10
	Mite egg number	**	4.4	5	10
Yolk IgY	Total mite population	*	48.5	4	7
Hen mortality (%)	Unfed adult and nymph	***	85.4	5	9
	Age (weeks)	***	13.6	5	9
	Total mite population	***	0.8	5	9
	Mite egg number	*	0.1	5	9
	Age (weeks)	***	89.0	6	11
	Larvae number.	**	6.8	6	11
	% Lay	*	1.57	6	11
	Age (weeks)	***	46.9	6	11
	Unfed adult and nymph	***	8.2	All	19
Water consumption	% Lay	**	42.7	1	1
	Fed adult and nymph	NS	10.1	1	1
	% Lay	**	45.8	2	3
	Unfed adult and nymph	*	13.9	2	3
Feed intake (g/b/d)	Larvae number	*	41.5	2	3
Temperature (°C)	Unfed adult and nymph	***	40.4	All	19

The values displayed in Table 5.20 are those which were considered important when investigating relationships between red mite populations, production parameters and IgY levels, the remaining data is presented in Appendix I.

For red mite stages the predominant determinants of variability in the vast number of cases were other red mite stages (Appendix I), although on several occasions these predicting variables were in fact production parameters, as illustrated in Table 5.20. The production variable most frequently seen to be accountable for a significant proportion of variation of red mite stages was hen mortality (r^2 = 1.8 to 85.4), with the bird age also having a significant effect (r^2 = 12.1 to 90.8), as did hen egg production on one occasion (r^2 = 21.2).

Similarly, the greatest significant determinants of variability for individual production parameters were largely seen to be other production parameters (Appendix I). However, occasionally variability could be accounted for by red mite stages. Unfed red mite adult and nymph numbers was the most common parameter to account significantly for productive variability and did so for hen mortality (r^2 = 8.2 to 85.4), water consumption (r^2 = 13.9) and were also related to building temperature (r^2 = 40.4). Fed adult and nymph red mite (r^2 = 10.1), larvae (r^2 = 41.5) and mite eggs (r^2 = 0.1) also significantly predicted some of the variability seen in water consumption, fed intake and hen mortality, respectively.

Correlation analysis showed few relationships between IgY and other variables and as such was entered rarely into stepwise regression. However, total mite population was observed to account for some of the variability in IgY on Site 3 (r^2 = 48.5).

5.3.5 Relationship between red mite populations and cytokine expression

Table 5.21 gives data for total red mite population against serum IgY, humoral (IL-4, Il-5 and IL-13) and cellular (IL-12α and IFNγ) cytokine expression for Site 6. No significant correlations were observed between any of the parameters recorded. However, trends were observed between serum IgY levels, IL-4 and IL-5 (see Figures 5.3 and 5.4), with fluctuations in serum IgY levels being closely followed by changes in both IL-4 and IL-5 levels. Table 5.21 also highlights the large variability observed for mean red mite population (Mean 3,345; C.V. 81%) and each of the immune parameters measured.

Table 5.21 *Mean red mite population, serum IgY and cytokine levels for Site 6*

Age (weeks)	Total Mite Population	Serum IgY	Cytokines				
			IL-12α	IFNγ	IL-4	IL-5	IL-13
27	8	0.847	0.000	9.010	11.470	18.656	1.792
32	1597	1.031	0.000	9.738	13.518	20.595	3.655
37	4829	0.999	0.000	10.221	14.101	20.272	3.254
41	6502	0.717	1.598	9.718	11.120	19.433	2.757
45	5789	1.220	1.452	11.799	15.161	20.921	1.516
49	383	1.313	1.082	9.959	13.474	20.831	3.119
54	371	0.752	3.844	10.966	14.303	21.364	4.646
58	4759	0.608	0.753	11.203	13.540	21.023	3.751
62	5869	0.770	4.410	11.769	15.020	21.735	5.242
Mean:	3345	0.9174	1.460	10.487	13.523	20.537	3.304
S.E. Mean:	900	0.0797	0.546	0.329	0.469	0.321	0.403
C.V. (%):	80.72	26.07	112.13	9.41	10.41	4.69	36.60

Figure 5.3 *Plot of serum IgY and IL-4 over time for Site 6*

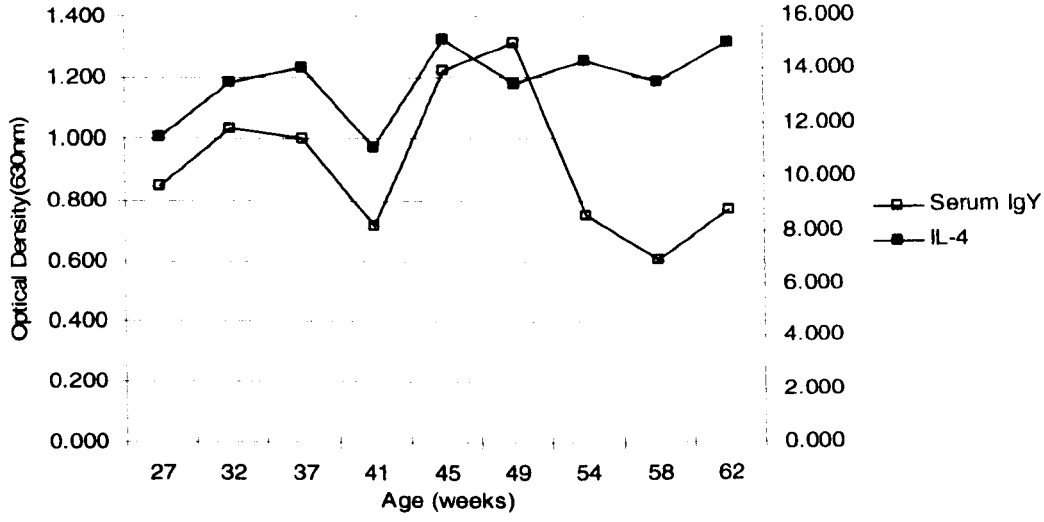
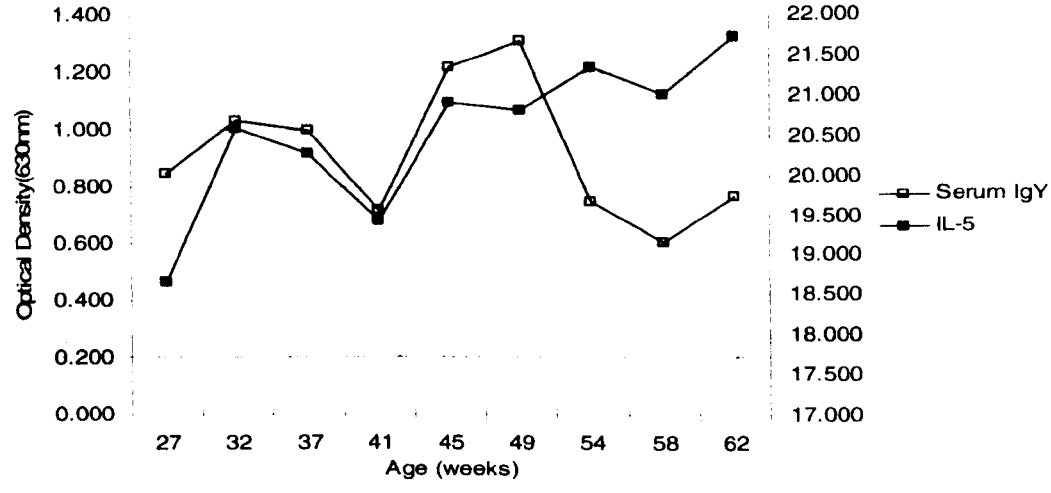


Figure 5.4 *Plot of serum IgY and IL-5 over time for Site 6*

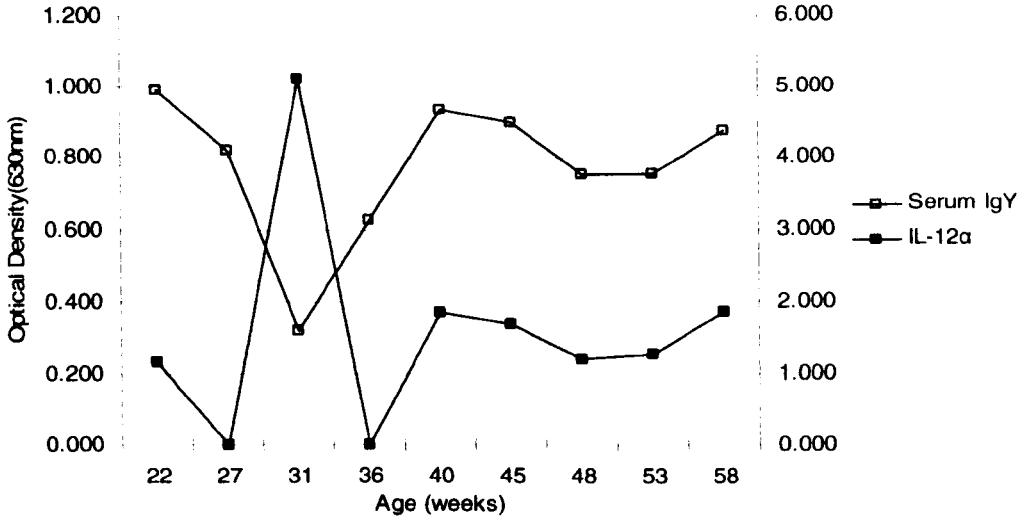


As with the previous table, Table 5.22 presents data for red mite population, serum IgY levels and cytokine expression, but this time for Site 7. On this occasion, a significant negative relationship between IL-4 and serum IgY levels was observed (P= 0.032; r = -0.705). None of the other correlations were significant, although a trend was observed between serum IgY levels and IL-12α (Figure 5.5), with fluctuations in both serum IgY levels and IL-12α mimicking one another over time.

Table 5.22 *Mean red mite population, serum IgY and cytokine levels for Site 7*

Age (weeks)	Total Mite Population	Serum IgY	Cytokines				
			IL-12α	IFNγ	IL-4	IL-5	IL-13
22	7180	0.992	1.147	8.617	12.783	19.703	0.000
27	6183	0.820	0.000	10.470	14.700	21.143	2.487
31	8178	0.318	5.117	11.519	17.006	22.767	1.831
36	18537	0.627	0.000	9.144	13.282	20.434	3.715
40	17080	0.940	1.859	10.757	13.508	20.233	2.014
45	10850	0.903	1.702	10.526	13.676	21.087	4.779
48	4744	0.755	1.201	10.797	14.989	21.837	3.092
53	10521	0.755	1.272	11.311	12.333	19.605	5.283
58	7520	0.877	1.847	10.099	13.979	21.280	2.434
Mean:	10088	0.7763	1.572	10.360	14.028	20.899	2.848
S.E. Mean:	1597	0.0682	0.501	0.317	0.466	0.341	0.535
C.V. (%):	47.48	26.35	95.66	9.17	9.96	4.90	56.36

Figure 5.5 *Plot of serum IgY and IL-12α over time for Site 7*



5.4 Discussion

The aim of this study was to monitor a number of egg production, environmental and immunological parameters between laying hens and poultry red mite populations over the flock laying cycle of several commercial laying farms to distinguish relationships and potential areas for future control.

5.4.1 Productive performance and red mite infestation levels

The productive performance of laying hens used in this study was equivalent to commercial egg production levels in the UK, in terms of feed/water consumption, hen mortality and eggs output (e.g. Nix, 2005). Similarly, the level of poultry red mite infestation observed across the three different housing systems was comparable to that previously recorded at between approximately 3,700 to 67,600 red mite per trap (Nordenfors and Höglund, 2000; Nordenfors *et al.*, 2001; Nordenfors and Chirico, 2001; Chirico and Tauson, 2002). Generally free-range systems had the largest red mite populations, followed by barn and finally cage systems. The exception to this was the barn unit, Site 4, which had the highest red mite population of any of the sites recorded. This distribution of mites between systems is frequently reported within Europe and is attributed to the greater number of potential mite hiding places in the barn and free-range systems (Chauve, 1998; Kilpinen, 2001). However, from this experiment it is difficult to make any conclusions about system differences due to the relatively small number of sites sampled per system. However, elucidating differences between housing systems was not the primary aim of the current study.

5.4.2 Relationship between production parameters and poultry red mite population

In this experiment, at some stage across every site, all production parameters were seen to have a significant relationship with poultry red mite population. The most frequent association seen was the significant positive relationship between poultry red mite population and mortality of hens, showing that an increased poultry red mite burden led to a rise in bird mortality. It is likely that this mortality is due, in part, to blood loss from feeding mites, since infestation by the poultry red mite often leads to hens becoming anaemic (Kirkwood, 1967; Kilpinen, 2001). This strengthens the argument that predation by this parasite is the real economic and welfare threat. Wojcik *et al.* (2000) for example, estimated that poultry red mite infestation could increase hen mortality by between 4 and 50 %.

It has also previously been reported that the poultry red mite can be responsible for reduced egg production, by as much as 20 % (Cosoroaba, 2001). Given this background, it is perhaps surprising that significant correlations between egg production and poultry red mite populations were detected on only 2 sites in the current study.

Existing research has also shown that the optimal temperatures for development of the poultry red mite ranges between 20-37°C, with lethal temperatures at extremes of

-20°C to 45°C (Maurer and Baumgartner, 1992; Nordenfors *et al.*, 1999; Nordenfors and Höglund, 2000). In the present study, building temperatures fluctuated between 16-26°C across all sites over the laying period, thus providing almost ideal conditions for survival and proliferation of red mite. Since survival was not challenged by extreme fluctuations in building temperature in this study, it is perhaps not surprising that red mite was only correlated to building temperature on one site. However, it may also be that the large variability seen in red mite populations per trap resulted in a loss of sensitivity to detect small changes in population size in response to external variables such as building temperature.

As with egg production and building temperature described above, both feed and water consumption demonstrated only weak associations to red mite population levels. Significant positive and negative relationships were observed between both feed/water consumption and red mite population levels, although explanations for both exist. In commercial poultry production, feed and water are both seen to increase steadily with hen age, until they reach a peak which coincides at approximately the same time as peak lay and then they slowly decline (Rose, 2001). Therefore, if red mite population levels increased/decreased simultaneously with egg production, as birds age then positive/negative correlations may be observed, although these would not be directly caused by red mite infestation. It has also been suggested that infestation by red mite increases stress levels of birds, which leads to increased movement/energy usage and in order to compensate for this birds increase their feed consumption, providing that their feed supply allows for this (Axtel and Arends, 1990; Kilpinen *et al.*, 2005).

In addition, both feed/water consumption and red mite populations are also said to bear a close relationship with environmental temperature (Maurer and Baumgartner, 1992; Teeter, 1996). Therefore if both feed/water consumption and red mite populations increased in response to a rise in temperature then this would lead to the occurrence of indirect correlations between parameters. Indirect relationships of this type were observed in the current study between all the production parameters and red mite populations on individual sites throughout this study. Perhaps the reason why these not detected as direct correlations was due to large variation in sampling.

Since variability between production parameters and red mite populations in any laying system is naturally high, it is difficult to attribute productive losses to changes in red mite populations, as Wojcik *et al.* (2000) assumed previously. Poultry red mite

can only realistically be associated with reduced production where all external parameters are controlled and variability limited, thus creating an artificial environment. One such study by Kilpinen (2005) on small groups of birds (n = 15) housed in 3.4 x 1.5 m pens allowed red mite infestations to be held directly responsible for both reductions in weight gain and an increase in behavioural stereotypes (self-grooming/head scratching). However, using artificial environments limits the usefulness of data, as in natural circumstances there is input from multiple integrated variables.

In the current study, stepwise regression was used to help determine which were the predominant factors contributing to the variation in both production performance of hens and red mite populations. Generally, the predictors of these parameters were as might be expected from the literature, i.e. production parameters were predominantly responsible for other productive variables (such as temperature, which was responsible for 64 % of the variation in egg out put per bird). On a number of occasions a significant proportion of the variability in red mite population could be accounted for by production parameters. The most common predictor was hen mortality, although building temperature, egg output and water consumption all contributed significantly to red mite population. These findings are in agreement with other research which has highlighted the relationship between red mite population and production performance of laying hens.

5.4.3 Relationship between IgY and poultry red mite populations

Prior to statistical analysis it appeared that there was a trend between the overall total means for IgY levels and poultry red mite populations, a trend previously documented by Sam-Sun *et al.* (2002). However, subsequent correlation of data showed that these relationships were not significant, with the exception of Site 5 where a significant, positive correlation between serum IgY levels and red mite population was seen. In addition, two significant, yet negative, correlations were observed between yolk IgY levels and red mite population. This is perhaps not an obvious correlation, since a negative relationship would imply that as the red mite population levels increase the yolk IgY levels fall suggesting the occurrence of immunosuppression. However, immunosuppression is entirely plausible since haematophagous parasites often use this as a means of obtaining a blood-meal (Gillespie *et al.*, 2000; Schoeler and Wikel, 2001). Ectoparasites have evolved several methods to induce immunosuppression in host species. The predominant mode is by reducing antigen presentation capabilities by the modulation of immune

components, such as macrophages or natural killer cells through secretion of compounds in the saliva during feeding (Authie *et al.*, 2001; Earnhart *et al.*, 2003; Okoko *et al.*, 2003). However, since the negative correlation found in the current study between IgY levels and red mite populations was observed on only one site, it remains difficult to draw definitive conclusions.

This lack of correlation is again perhaps not surprising since blood and yolk IgY samples were taken on a flock basis. In general, successful correlations between antibody responses and ectoparasite infestation levels were reported previously after repeated sampling of the same animals, thus reducing sampling variation (Sam-Sun *et al.*, 2002; Pruett *et al.*, 2006). However, as in the current study, Maurer (1993) also observed no difference in IgY level (mean O.D. = 0.50 and 0.51) to natural exposure of hens (approximately 2,500 to 75,000 mites per hen) to different levels of red mite. Maurer (1993) suggested that there may have been failure to generate any direct humoral response to red mite antigens, something which has been reported in other parasite species (Heller-Haupt *et al.*, 1996; Khokhlova *et al.*, 2004). It may also be that the ELISA assay used was not sensitive enough to detect small differences in antibody levels since even on sites where very low level mite infestation was observed, a relatively high level of IgY was observed.

5.4.4 Relationship between serum and yolk IgY levels

An additional aim of this study was to evaluate the relationship between yolk and serum IgY levels. It has previously been documented that yolk IgY can be used as a tool to determine serum immunoglobulin levels in response to exposure to particular antigens (Woolley and Landen, 1995). Therefore both yolk and serum IgY were collected on Site 5, although repeat samples were not taken from the same individual birds.

Previous research by Mohammed *et al.* (1986) comparing yolk and serum immunoglobulin levels suggested that there was no significant difference between the levels of these two parameters i.e. a very close relationship. Results of the current study contradict this, as yolk IgY optical density levels were significantly higher than serum IgY levels ($P<0.05$). This may be a result of an increased concentration of yolk IgY through the extraction process or alternatively because antibodies in egg yolk are an accumulation of IgY from the hen over a 21 day period (the duration needed for the complete formation of an egg yolk, Mohammed *et al.*, 1986). Furthermore, ELISA is a combined measure of both concentration and affinity

which are likely to differ between the two samples despite assay optimisation with respective immunoglobulins (Butler *et al.*, 1978).

In addition to there being a significant difference between the levels of IgY obtained from serum and yolk antibodies, no significant correlation was observed between these two sources either. This is true even when taking into account the delay between the formation of IgY in the blood and its deposition in the egg yolk (Mohammed *et al.*, 1986), although a non-significant trend was observed. Previously, although Mohammed *et al.* (1986) reported no significant difference between yolk and serum IgY levels, they did not document the presence of any significant correlation between yolk and serum IgY. In the present study the reason for this failure to generate a significant correlation is once again likely to be due to a large variability between mean optical density values for serum and yolk IgY (Mean 0.69 and C.V. 56 %; Mean 0.75 and C.V. 48 %, respectively), which could be potentially reduced by increasing sample replication or by obtaining yolk and serum IgY from the same individual.

5.4.5 Relationship between red mite populations and cytokine expression

Previously, research has observed changes in cytokine expression as a result of engorgement by haematophagous ectoparasites (Gillespie *et al.*, 2000; Mbow *et al.*, 1994; Arlain *et al.*, 2003; Rohousova *et al.*, 2005). Depending on the parasitic infection, cytokine expression can be skewed towards either a Th1 (cellular) or Th2 (humoral) response (Maldonado *et al.*, 2005). For example, Th1-type responses were induced in both tick and sand fly infestations as a result of increases in IFNγ, IL-12 and T-cell proliferation (Gillespie *et al.*, 2000; Rohousova *et al.*, 2005). Whereas mosquitoes have been observed to shift immunity away from Th1-type and towards eliciting Th2-type immunity, characterised by high IL-5 and IL-10 cytokine levels (Foy *et al.*, 2003). However, this increased expression is not necessarily associated with protective immunity, as has been previously documented, where parasites exhibited no increase in mortality subsequent to feeding (Mbow *et al.*, 1994; Foy *et al.*, 2003). As a result of this increased host cytokine expression, many parasites have developed evasion strategies with the ability to modulate certain host immune mechanisms through salivary secretions, for improved feeding (Schoeler and Wikel, 2001).

In the current study, spleen samples were dissected from birds on Sites 6 and 7, to determine the impact of poultry red mite infestation on specific Th1/Th2-type cytokine

expression. As with other parameters there were no significant correlations between cytokine expression and red mite population, but there was a significant negative relationship between IL-4 and serum IgY levels on Site 7. This is surprising, since IL-4 is a key regulator in Th2-type humoral immunity, stimulating the proliferation of B-cells and thus antibody production, particularly against parasitic infection and a positive relationship might have been expected (Berger, 2000). However, on Site 6 IL-4 and IL-5 displayed a positive trend with IgY, as did IL-12α also on Site 7, which would not be unexpected since IL-5 also functions to stimulate Th2-type humoral immunity, increasing B-cell growth and immunoglobulin secretion. IL-12α on the other hand is involved in the differentiation of naive T-cells into Th1 cells, which is important in resistance against pathogens. However, these relationships were not significant and it would appear from the current study that there is little evidence of an association between red mite population levels and cytokine expression. This apparent lack of correlation may be due to the failure to stimulate the immune system to elicit cytokine responses to red mite antigens, as has previously been observed in other parasite species (Cross *et al.,* 1994). Alternatively, as with antibody determination, correlative failure may once again be as a result of the large variability observed between parameters.

5.5 Conclusion

In conclusion, a number of correlations were observed between red mite population levels, egg production and immunological parameters on individual sites. However, when data from all sites was analysed as one dataset these relationships generally failed to show consistency, with the exception of hen mortality which was related to red mite population. This failing was predominantly attributed to the large variation observed in each one of these parameters, both between separate sites and within individual birds in the same site. Red mite population estimates in particular showed a very high variation between sampling dates and sites.

In order to limit variability, sampling must be carried out repeatedly on the same individual, something which has been done previously (Kilpinen, 2005). However, using artificial environments limits the usefulness of data, as in natural circumstances there is input from multiple integrated variables. Thus it remains innately difficult to establish and attribute the cause of productive, immunological or red mite population variability to any one individual parameter within a flock and care should be taken when considering these relationships.

Also, irrespective of correlative absence between red mite populations and IgY levels, ELISA analysis observed that birds were capable of producing a response to red mite infestation which differed significantly between sites. In addition, cytokine expression was negatively correlated to IgY levels, which was indicative of immunosupression.

Finally, this study supports the present understanding that current poultry housing provides an ideal environment for red mite proliferation, as the climatic conditions observed across all sites sampled were within the optimal limits, as previously described (Axtel and Arends, 1990; Maurer and Baumgartner, 1994; Höglund *et al.*, 1995; Chauve, 1999; Kilpinen *et al.*, 2005).

Chapter 6

Effect of immunisation with whole poultry red mite antigen on immune response of laying hens and survival of poultry red mite

6.1 Introduction

Previously in Chapter 5 the immune response of poultry when faced with natural exposure to the red mite was explored. Despite birds on different sites mounting different levels of IgY response to red mite infestation, statistical analysis showed no apparent relationship between these two parameters at a mean flock level. As a result, it would appear that birds showed no natural immune resistance to the red mite. However, since the previous chapter looked at natural exposure, it lacked a degree of control and resulted in wide-scale variability between individual birds and red mite population estimates. Therefore, this experiment looked at artificial and controlled exposure of hens to red mite antigens using immunisation. Since there is little information available in the literature regarding the immunological response of domestic fowl to the poultry red mite, parallels were drawn from research conducted on related ectoparasitic species.

Progress has been made with isolating potential antigens which elicit host protection to a range of mite species following immunisation with soluble whole mite extracts. These include the house dust mite (*Dermatophagoides pteronyssinus and Dermatophagoides farinae*; Mathaba *et al.*, 2002), sheep scab mite (*Psoroptes ovis*; Smith *et al.*, 2002), scabies mite (*Sarcoptes scabiei*; Tarigan and Huntley, 2005) and northern fowl mite (*Ornithonyssus sylviarum*; Minnifield *et al.*, 1993), amongst others. Protective immunity developed against these ectoparasites is generally observed with an increase in the Th2-type immune response which drives humoral antibody production. However, it can also be associated with increases in Th1-type immunity, which is largely accountable for cellular responses, mediated by the expression of specific cytokines (Baguet and Bix 2004; Zhang *et al.*, 2006).

Using such vaccination strategies has a direct advantage over traditional controls, such as the application of acaricides, since they do not result in environmental contamination or residues in foodstuffs, they have no withdrawal periods or reduce the possibility of arthropod resistance, etc. (Dalton and Mulcahy, 2001; Nisbet and

Huntley, 2006). However, the development of a successful anti-parasite vaccine has several steps. The first of these steps is to evaluate the mechanisms involved in the host-parasite relationship, in terms of the host-immune response to immunisation with parasitic antigens and its subsequent effect on parasite survival. This is followed by the identification and characterisation of protective antigens, the production of those protective antigens as effective recombinant proteins, and finally the formulation of those recombinant antigens into a vaccine able to generate a sustainable and appropriate immunological response (Tarigan and Huntley, 2005).

The aim of this experiment therefore was to evaluate the effect of immunisation with whole poultry red mite antigens on the humoral and cellular immune response of laying hens and the effect of this on survival and fecundity of mites using both *in vitro* and *in vivo* feeding systems. The null hypothesis was that immunisation with poultry red mite antigen would lead to the development of elevated immunoglobulin and cytokine levels in the hen, which when ingested by feeding mites would be detrimental to their health resulting in protection for the hen via reduced mite survival and fecundity.

6.2 Materials and methods

6.2.1 Experimental treatments

There were two experimental treatment groups: a Control which received an immunisation with saline plus Complete Freund's adjuvant (CFA) which contains *Mycobacterium tuberculosis,* at 6 weeks of age, followed by two immunisations with saline plus Incomplete Freund's adjuvant (IFA), which is a simple oil-in-water emulsion, at 9 and 12 weeks of age. Recommendations for the volume of material subcutaneously injected were taken from Morton *et al.* (2001) and did not exceed 5 ml per kg bodyweight.

A second Antigen treatment received an immunisation with red mite antigen extract plus CFA for the initial immunisation at 6 weeks of age, replaced by IFA plus red mite antigen extract for two subsequent immunisations. Each immunisation consisted of 1 mg mite protein, which was administered with equal volumes of either CFA or IFA. The volumes injected in both Control and Antigen treatments were equal.

6.2.2 Animals and housing

A total of 39 female domestic fowl of a commercial egg-laying strain (Shaver-579) were used. The birds were reared from day-old in a group using a brooder lamp and floor pen with wood-shavings. At 5 weeks of age they were separated and placed in 2 floor pens, 1 pen per room in 2 adjacent rooms. Standard commercial starter and subsequently grower diets, as well as water were available *ad libitum*. Ambient temperature was gradually reduced from approximately 26°C for day-old chicks, to approximately 17°C by 20 weeks of age to meet birds' thermostatic requirements. The illumination program used followed the standard for commercial production (Nix, 2000), designed to bring birds to the point of lay at 20 weeks of age. Leg rings were used to identify individual birds within a treatment. Birds were monitored daily following immunisation for signs of ill health. During the *in vivo* mite feeding test, birds were also monitored for clinical signs of anaemia, which include pale mucous membranes of the conjunctiva or inside the mouth, pale tongue, gums, intolerance to exercise and, at a more extreme level, an increased respiratory rate (Morton *et al.*, 1993). Mean weekly body weight, feed intake and daily room temperature were recorded for the duration of the study. At the end of the experiment, remaining birds were killed by cervical dislocation.

6.2.3 Sampling and immunisation schedule

Immunisations were administered via the subcutaneous route and took place on three separate occasions at 6, 9 and 12 weeks of age (Table 6.1). Each treatment comprised of 18 birds, with 3 naive birds sacrificed at the start of the trial to establish baseline parameters.

Table 6.1 *Schedule for administration of substances and sampling*

Study Week	Age (Weeks)	Approximate Weight (kg)	Treatment	Sampling	
				Serum	Spleen
1	6	0.45	Immunise (1) (Complete Freund's)	All#	3 Birds#
4	9	0.69	Immunise (2) (Incomplete Freund's)	All#	6 Birds#
7	12	0.94	Immunise (3) (Incomplete Freund's)	All#	6 Birds#
10	15	1.20	Infestation	All#	6 Birds#
12	17	1.40	END	All	All

#Samples were taken 1 day prior to subsequent immunisation or infestation

Blood samples were taken at 6, 9, 12, 15 and 17 weeks of age, 1 day prior to immunisation or infestation, from the brachial (wing) vein, removing a volume of

approximately 1 ml. At the end of the experiment, the remaining birds were dispatched via cervical dislocation and a sample of blood (up to 5 ml) taken directly from the heart. A sub-sample of this blood (about 2.5 ml) was taken and allowed to clot to yield serum which was removed for subsequent IgY analysis. The remaining blood (about 2.5 ml) was placed in vacutainers coated with the anticoagulant lithium heparin (Becton Dickinson vacutainer systems, Oxford, UK) and subsequently tested in an *in vitro* mite feeding system to determine efficacy of the antigen.

Spleen samples were also taken 1 day prior to immunisation from randomly selected birds (3 per treatment), sacrificed at 6, 9, 12, and 15 weeks of age. At the end of the experiment (week 17) the spleens from all remaining birds were taken shortly after cervical dislocation and abdominal dissection. A proportion of the spleen was removed immediately, placed in RNA*Later*® (QIAGEN, West Sussex, UK) and subsequently used for cytokine analysis, using a Taqman assay described in Section 3.7, at the Institute for Animal Health, Compton, UK.

6.2.4 Preparation of red mite antigen

Soluble, unfed whole red mite antigens were extracted in a PBS buffer, as described previously (Section 3.1). On the day of immunisation, antigens were defrosted for approximately 30 min at room temperature and mixed with the appropriate volume of adjuvant before being administered.

6.2.5 ELISA, SDS-PAGE and western blotting

Serum samples obtained from birds were subject to an ELISA assay to determine IgY levels, using the protocol outlined in Section 3.2. The cut-off point threshold was calculated as the negative plus 1.645 S.D. as recommended by Mire-Sluis *et al.* (2004) and optical density values of samples were standardised using a normalisation factor set as the ratio between negative standards (see Section 3.2). Specific antibody binding to individual red mite antigens was also assessed via SDS-PAGE and western blotting (Section 3.3). This was performed on serum from the five most immunodominant birds in each treatment (i.e. birds which displayed the highest IgY levels in ELISA), both before immunisation and after three immunisations.

6.2.6 Cytokine analysis

The Taqman assay was used to determine specific cytokine levels being expressed in RNA extracted from spleen samples, the protocol for which was outlined in Section 3.7. The presence of RNA coding for a number of cytokines was investigated using

gene specific primers. The Th1-type response was determined using a primer for the classical Th1 cytokine, interferon-γ (IFNγ). Th2-type cytokine expression on the other hand was assessed by levels of interleukin-10 (IL10), a regulatory cytokine involved with anti-inflammatory responses; interleukin-4 (IL4) and interleukin-13 (IL13), which are primarily involved in anti-helminthic protection and finally, interleukin-5 (IL5), which causes eosinophil activation to allow parasitic protection and also mediates allergic reactions.

6.2.7 In vivo *red mite feeding challenge*

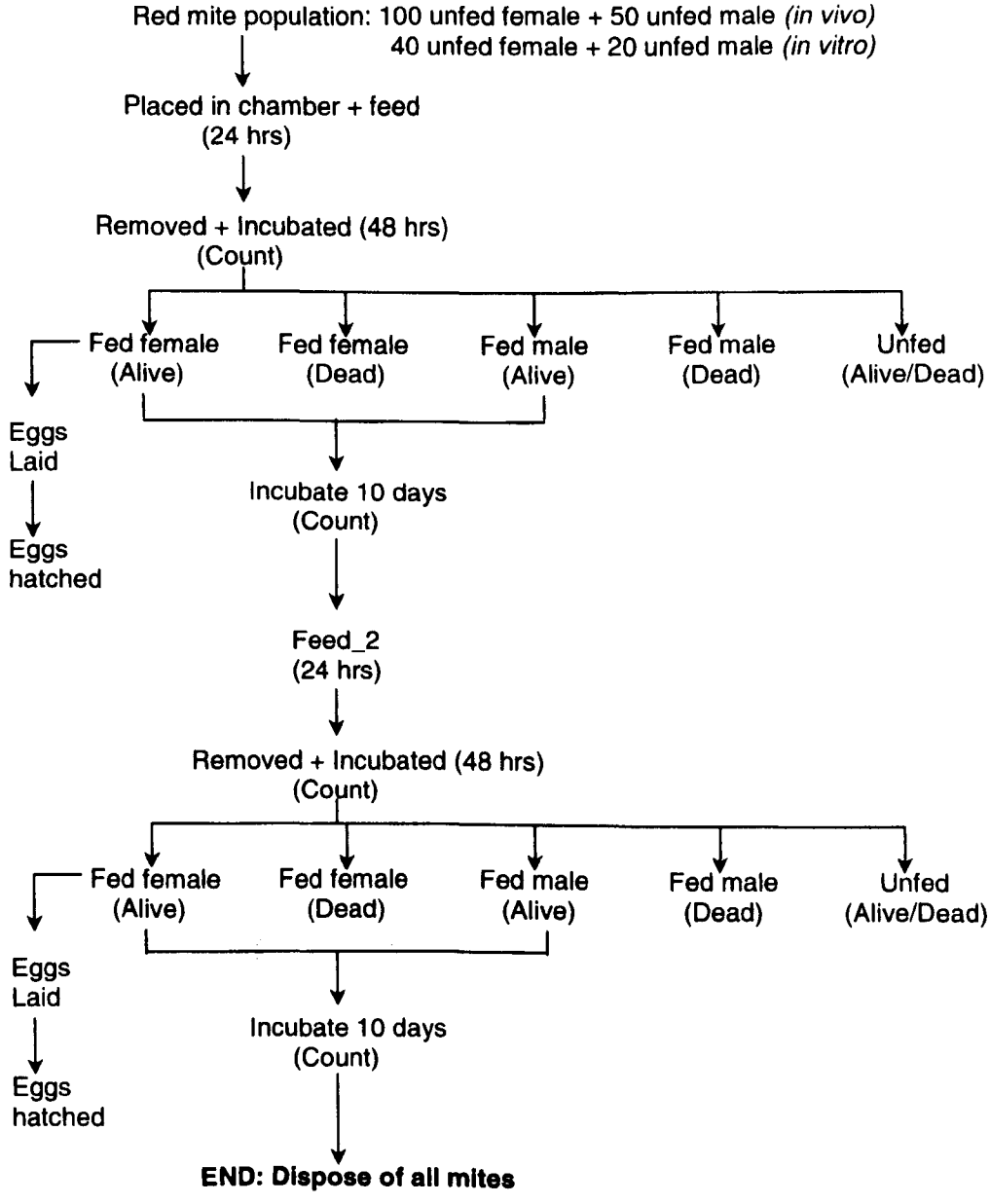
In order to establish the efficacy of the immunisation, an artificial infestation of red mite was created. This required the transfer of birds at 15 weeks of age to individual metabolism cages which contained a perch and troughs providing *ad libitum* feed and water. The red mite were exposed to the hens by placing the mites in a simple plastic chamber fixed to the back of the bird using tissue adhesive. The chamber contained 150 unfed red mite (100 female and 50 male) and a small trap for the red mite to reside in when not feeding. The chamber remained in place on the bird for a period of 24 hours, after which it was removed along with all mites which could be recovered. The chambers were incubated at room temperature in the dark for 48 hours to allow for egg deposition. The chambers were then emptied, the red mite counted and the number of fed mites established, along with total mortality and oviposition rates. Fed and unfed mites were separated and the unfed mites discarded. The fed mites (determined by their characteristic red appearance) were maintained in an incubator at 20°C for a further 10 days to establish further oviposition and survival rates and also allowing the fed red mite time to digest their blood-meal and return to an unfed status. Eggs were in turn monitored to determine hatching rate. After this period of 10 days, the remaining live adult mites were then placed back inside chambers which were secured on the same bird for a further period of 24 hours to allow a second feeding. After removing the chambers, red mite were then subject to the same procedure of incubation for 10 days, before identification and counting. At this point all remaining mites were disposed of. A summary of this procedure is given in Figure 6.1.

6.2.8 In vitro *red mite feeding challenge*

As a means of comparison with the *in vivo* feeding system, an *in vitro* method was devised to allow the rapid testing of red mite feeding on whole blood using a method modified from Bruneau *et al.* (2001). The feeding system is described in Section 3.6.

A similar protocol to the *in vivo* mite feeding system was used, except that only 60 red mite (40 female and 20 male) were placed inside the feeding systems and the blood reservoirs filled. These were then placed on an orbital shaker at 30°C in a dark room for 12 hours in order to simulate natural feeding conditions of the red mite. After this feeding period, the blood reservoirs were removed and the remaining red mite containing portion of each system placed in an incubator at 20°C, in the dark for a further 48 hours to allow for oviposition. The following part of the *in vitro* feeding was the same as described previously for *in vivo* feeding, where survival, oviposition and hatching of the relevant mite stages were recorded and fed red mite were subject to a second feed.

Figure 6.1 *Schedule for* in vivo *and* in vitro *feeding of poultry red mite*



6.2.9 Statistical analysis

Statistical analysis of the effect of treatment on hen parameters and optical density was performed using ANOVA in MINITAB (V14), with response variables including weekly hen bodyweight, IgY optical density and cytokine levels. The change in optical density over time for repeat samples on the same bird was also analysed using repeated measures analysis of variance (MANOVA) in GenStat®. Finally, all red mite survival and fecundity data for treatment (Control and Antigen), sampling point (2 and 10 days post 1st infestation and 2 days post 2nd infestation) and feeding system used (*in vivo* and *in vitro*) was entered into MINITAB as one dataset and analysed by ANOVA as a multi-factorial design to determine differences between each of these parameters.

6.3 Results

6.3.1 Bodyweight and feed intake

Mean weekly records for both Control and Antigen treatments showed a steady increase in both bird weight and feed consumption (Table 6.2). No significant differences were seen between treatments for mean bodyweight. However, since feed consumption was recorded on a mean pen basis ANOVA was not possible, although it appeared that there was no numeric difference between treatments.

Table 6.2 *Mean weekly bodyweight and feed records for both Control and Antigen treatments*

Bodyweight (g)					
	Control		**Antigen**		
Age (Week)	**Mean**	**SE Mean**	**Mean**	**SE Mean**	**Significance**
5	491.0	18.38	495.2	18.38	NS
6	584.2	21.59	585.7	21.59	NS
9	887.6	23.5	877	23.5	NS
12	1135	36.62	1106	36.62	NS
15	1359	39.09	1348	39.09	NS
17	1499	42.55	1513	42.55	NS
Feed consumption (g/bird/week)					
6	37.0	-	37.0	-	-
9	50.9	-	48.2	-	-
12	70.0	-	56.8	-	-
15	76.7	-	62.1	-	-
17	89.1	-	90.9	-	-

NS= values within rows not significantly different

-= Missing value

6.3.2 Immunological response

Prior to immunisation at 6 weeks of age, there was no difference in mean optical density values between treatments and the level for both Control and Antigen birds was below the negative cut-off point (Figure 6.2). However, after the first immunisation, both treatments showed a broad range of optical densities, with considerable variation between the responses of individual birds (see Table 6.3). At this point, 89 % of birds (8 out of 9 birds) in the Control treatment displayed IgY levels which were above that of the negative cut-off point, in comparison to 67 % (6 out of 9 birds) in the Antigen treatment (Figure 6.2). The mean optical density of the Control treatment was actually above that of the Antigen treatment at this point (1.02 vs. 0.63, respectively), although this difference was not statistically significant (see Table 6.4).

After the second immunisation, all but 4 birds (No's. 2, 5, 8 and 9) in the Control treatment had optical densities which were lower than those in the Antigen treatment. However, birds in both treatments had IgY levels which were above the negative cut-off point (Figure 6.2). Mean optical density in the Antigen treatment was greater than that of the Control treatment (1.73 vs. 1.50, respectively), although again this difference was not significant (Table 6.3).

After the third and final immunisation, mean IgY levels were maintained in the Control treatment but increased in the Antigen treatment so that the difference between treatments was significant (1.53 vs. 2.05, respectively)

Most birds showed a consistent increase in IgY level after successive immunisations, although post infestation there was a slight decrease in mean optical density levels. However, 44 % of birds (4 out of 9 birds) in the Antigen treatment and 22 % of birds (2 out of 9 birds) in the Control treatment showed a continued rise after red mite infestation (Table 6.3).

Table 6.3 *Mean ELISA optical density results for Control and Antigen treated birds*

Control					
Bird No.	**Pre-Immunisation**	**Post Immunise 1~**	**Post Immunise 2#**	**Post Immunise 3#**	**Post Infestation**
1	0.38	0.84	1.28	1.19	0.97
2	0.34	1.63	2.12	2.16	2.12
3	0.27	0.40	0.98	0.98	0.80
4	0.24	1.44	1.76	1.55	1.11
5	0.20	1.48	1.82	2.19	2.03
6	0.38	0.50	1.09	1.11	1.18
7	0.36	0.65	0.86	0.84	0.74
8	0.27	0.58	1.40	1.53	1.55
9	0.21	1.66	2.19	2.26	1.73
Mean	0.29	1.02	1.50	1.53	1.36
SE Mean	0.024	0.175	0.164	0.184	0.173
Antigen					
1	0.38	1.38	2.22	2.37	2.27
2	0.28	0.60	1.65	2.30	2.92
3	0.58	0.69	1.86	2.34	2.24
4	0.28	0.42	1.44	1.72	1.80
5	0.27	0.78	1.84	2.05	1.79
6	0.22	0.33	1.52	1.58	1.71
7	0.30	0.45	1.85	2.09	1.74
8	0.24	0.56	1.56	2.47	2.13
9	0.30	0.50	1.59	1.57	1.80
Mean	0.32	0.63	1.73	2.05	2.04
SE Mean	0.036	0.104	0.081	0.117	0.132

~Immunisation with CFA
#Immunisation with IFA

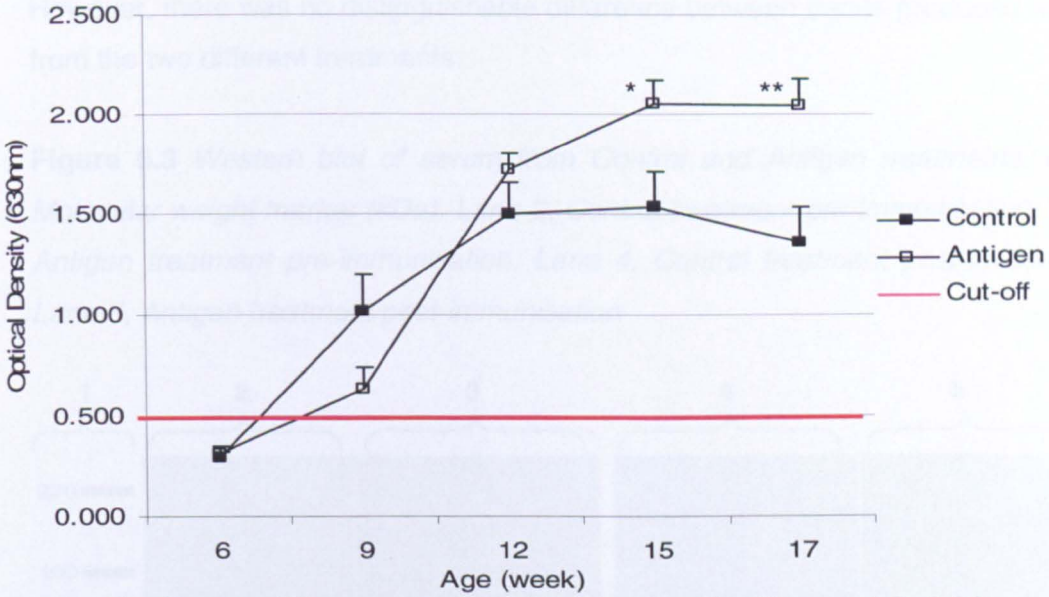
Table 6.4 *Comparison of mean optical density values between treatments*

Sampling point	**Replicates per group**	**Control**		**Antigen**		
		Mean	**SE Mean**	**Mean**	**SE Mean**	**Significance**
Pre-Immunisation	18	0.29	0.031	0.32	0.031	NS
Immunisation 1	15	1.02	0.144	0.63	0.144	NS
Immunisation 2	12	1.50	0.129	1.73	0.129	NS
Immunisation 3	9	1.53	0.154	2.05	0.154	*
Infestation	9	1.36	0.154	2.04	0.154	**

*= P<0.05; **= P<0.01; NS= no significant difference; - = Missing value
Means within a row followed by a different superscript letter are significantly different at P<0.05

Figure 6.2 illustrates the effect of immunisation on the IgY kinetics over the course of the experiment. Both treatments show a continual rise in IgY levels as a result of immunisation, with the initial response in Control birds being greater than the Antigen treatment. However, after the second and third immunisations the IgY level in the Antigen treatment surpasses that of the Control, but following infestation the IgY level plateaus in the Antigen treatment, and falls in the Control treatment.

Figure 6.2 *Comparison of mean optical density per treatment by ELISA*



*= Significantly different at P<0.05; **= Significantly different at P<0.01

A repeated measure function in GenStat® was used to assess the effect immunisation on IgY level over time and showed that there were weekly differences between treatments (see Table 6.5). For the Control treatment, mean IgY level was significantly increased after both the first and second immunisations but did not significantly increase after this point. In the Antigen treatment mean IgY level was similar between pre-immunisation and after the first immunisation. However, there was a significant increase in IgY level after the second immunisation, but again no significant change thereafter.

Table 6.5 *Comparison of mean weekly optical density within treatment*

Sampling point	Age (Weeks)	Control	Antigen
Pre-Immunisation	6	0.29^{a}	0.32^{a}
Immunisation 1	9	1.02^{b}	0.63^{a}
Immunisation 2	12	1.45^{c}	1.73^{b}
Immunisation 3	15	1.53^{c}	2.05^{b}
Infestation	17	1.36^{c}	2.04^{b}
P Value	-	***	***

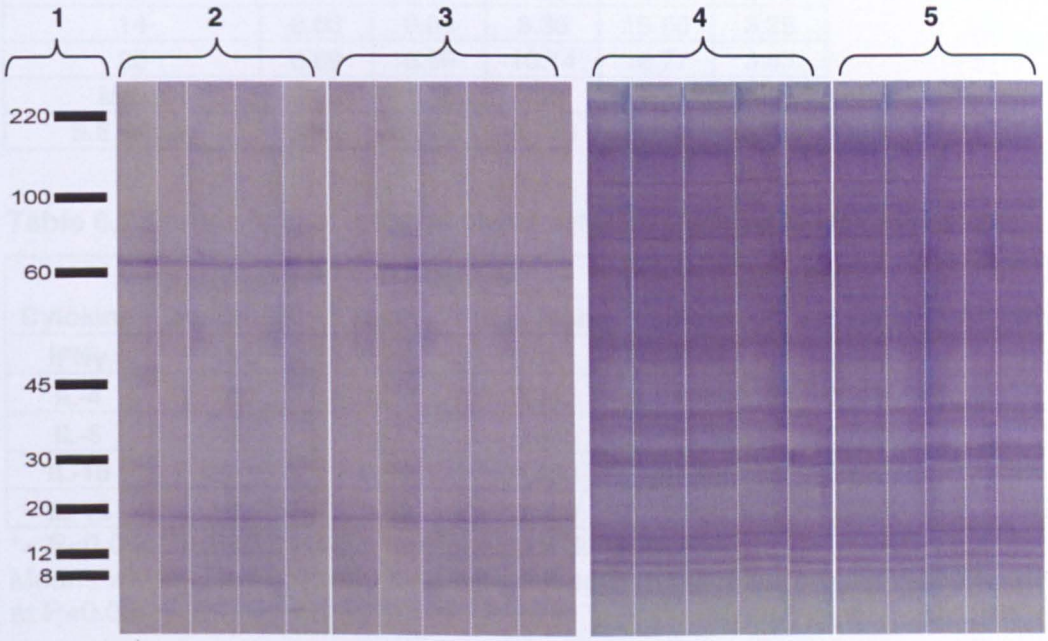
***= P<0.001; Means within a column followed by a different superscript letter are significantly different at P<0.05

6.3.3 Western blotting

Figure 6.3 illustrates that in both treatments there was some non-specific recognition of antigens prior to immunisation, as described in Section 3.4, with antibodies recognising proteins at approximately 20 and 60 kDa (Lanes 2 and 3). After three

immunisations, numerous additional bands were observed in both treatments. However, there was no distinguishable difference between bands produced with sera from the two different treatments.

Figure 6.3 *Western blot of serum from Control and Antigen treatments. Lane 1, Molecular weight marker (kDa). Lane 2, Control treatment pre-immunisation; Lane 3, Antigen treatment pre-immunisation. Lane 4, Control treatment post-immunisation; Lane 5, Antigen treatment post-immunisation*



6.3.4 Cytokine analysis

RT-PCR was carried out on spleen samples allow the determination of a number of cytokine responses involved in both Th1- and Th2-type responses. The results are shown Table 6.6, demonstrating that there is no real trend over time, with individual values fluctuating considerably. When looking at overall means it is possible to observe that, excluding IL-10, cytokine levels tend to be higher in the Control treatment.

Analysis of variance of cytokine levels was only performed on data following infestation, as prior to this insufficient sample numbers were available. Results are displayed in Table 6.7 and show significantly higher ($P<0.05$) levels of IL-10 present in the Antigen treatment. Conversely both IL-4 and IL-5 were seen to be significantly higher in the Control treatment, whilst IFN-γ and IL-13 did not display any significant differences between treatments.

Table 6.6 *Weekly mean cytokine levels between treatments*

Control					
Week of age	**IFNγ**	**IL-10**	**IL-4**	**IL-5**	**IL-13**
9	9.30	1.73	8.54	14.82	2.87
12	9.01	1.91	2.23	11.24	3.29
14	8.62	1.33	4.89	13.21	2.24
20	9.03	1.93	6.99	13.88	2.55
Mean	8.99	1.72	5.66	13.29	2.74
S.E. Mean	0.150	0.304	0.838	0.483	0.403
Antigen					
9	9.40	1.84	6.38	13.49	2.60
12	9.48	3.05	4.26	13.04	2.70
14	8.60	0.00	8.36	15.66	3.25
20	8.89	0.59	10.14	16.77	3.63
Mean	9.09	1.37	7.29	14.74	3.05
S.E. Mean	0.138	0.332	0.778	0.578	0.353

Table 6.7 *Comparison of cytokine levels between treatments after infestation*

Cytokine	Replicates per group	Control		Antigen		Significance
		Mean	S.E. Mean	Mean	S.E. Mean	
IFNγ	9	8.89	0.164	9.03	0.164	NS
IL-4	9	10.14	0.947	6.99	0.947	*
IL-5	9	16.77	0.680	13.88	0.680	**
IL-10	9	0.59	0.430	1.93	0.430	*
IL-13	9	3.63	0.669	2.55	0.669	NS

*= P<0.05; **= P<0.01; NS= no significant difference;
Means within a row followed by a different superscript letter are significantly different at P<0.05

6.3.5 Red mite feeding challenge

In order to standardise values between replicates and feeding system, red mite survival and fecundity were calculated as a percentage of the population within that replicate, rather than the actual number of red mite recovered. This was because different starting populations of red mite were used in the two feeding devices and there were also different recovery rates of red mite between replicates. It should also be noted that values expressed are for fed and not unfed mite, therefore data does not always total 100 % in Tables 6.8-6.10.

There were no significant differences in mite survival or fecundity rates between treatments (Table 6.8). However, there was a consistent trend in the data which suggested that there was a higher mortality of red mite in the Antigen treatment when compared to the Control treatment.

Reproductive success showed similar responses between treatments with no significant differences, although oviposition rates were higher in the Control treatment than in the Antigen treatment.

Table 6.8 *Effect of treatment on mean red mite populations*

	Treatment				
	Control		Antigen		
	Mean	S.E. Mean	Mean	S.E. Mean	Significance
Alive Female	47.1	3.20	44.6	3.26	NS
Alive Male	50.6	3.99	44.2	4.07	NS
Total Alive	48.1	2.67	44.3	2.72	NS
Dead Female	33.9	2.84	36.9	2.89	NS
Dead Male	31.9	3.96	36.9	4.04	NS
Total Mortality	33.4	2.42	37.1	2.46	NS
Eggs/Female	0.3	0.05	0.1	0.05	NS
% Eggs Hatched	98.3	1.48	98.3	1.68	NS

NS= values within rows are not significantly different

Significant differences between sampling point were seen for mortality, but not for survival rates of red mite populations (Table 6.9). It was observed that mortality for male, female and total red mite populations was significantly higher 10 days post first infestation than 2 days post first infestation, with male mortality not changing thereafter. However, both female and total red mite mortality was significantly higher 2 days post second infestation than 10 days post first infestation.

Table 6.9 *Effect of sampling point on mean red mite populations*

	Sampling point						
	2 days post 1st infestation		10 days post 1st infestation		2 days post 2nd infestation		
	Mean	S.E. Mean	Mean	S.E. Mean	Mean	S.E. Mean	Significance
Alive Female	51.8	3.92	41.7	3.92	44.1	4.03	NS
Alive Male	49.3	4.89	43.3	4.89	49.7	5.03	NS
Total Alive	51.2	3.26	42.1	3.26	45.2	3.36	NS
Dead Female	4.3^{a}	3.48	58.3^{b}	3.48	43.5^{c}	3.58	***
Dead Male	5.8^{a}	4.85	55.9^{b}	4.85	41.5^{b}	5.00	***
Total Mortality	4.8^{a}	2.96	57.9^{b}	2.96	43.1^{c}	3.05	***

***= P<0.001; NS= no significant difference;
Means within a row followed by a different superscript letter are significantly different at P<0.05

Comparing red mite population and fecundity values shows that there were no significant differences between the two different feeding systems (Table 6.10). There were also no apparent trends between red mite survival and mortality parameters. However, there did appear to be a higher hatching rate in the *in vitro* system, although this was not significant.

Table 6.10 *Effect of feeding system on mean red mite populations*

	Feeding system				
	In vivo		*In vitro*		
	Mean	**S.E. Mean**	**Mean**	**S.E. Mean**	**Significance**
Alive Female	44.6	3.23	47.2	3.23	NS
Alive Male	50.6	4.03	44.2	4.03	NS
Total Alive	46.1	2.69	46.3	2.69	NS
Dead Female	35.9	2.87	34.8	2.87	NS
Dead Male	33.9	4.00	34.8	4.00	NS
Total Mortality	35.6	2.44	34.9	2.44	NS
Eggs/Female	0.2	0.05	0.2	0.05	NS
% Eggs Hatched	96.6	1.61	100.0	1.55	NS

NS= values within rows are not significantly different

6.4 Discussion

The aim of this experiment was to evaluate the effect of immunisation with whole poultry red mite antigens on the humoral and cellular immune response of laying hens and the effect of this on survival and fecundity of mites using both *in vitro* and *in vivo* feeding systems.

6.4.1 Bodyweight and feed intake

The study used birds of a commercial genotype housing them under similar conditions to that of a commercial pullet rearing environment, except these birds were not vaccinated against any disease. Data on bodyweight and feed consumption showed that birds performed within commercial recommendations, reaching the appropriate weight for age (Nix, 2000; Rose, 2001). Birds were also observed to be in good health for the duration of the experiment with treatment having no significant effect on either bodyweight or feed intake.

6.4.2 Immune response-ELISA

The immune response of birds to vaccination was monitored using an IgY specific ELISA. Primary and secondary immunisations did not result in a significant difference in serum optical density between treatments. However, after the third immunisation the Antigen treatment had a significantly higher IgY level ($P<0.05$), a difference was maintained following infestation with red mite ($P<0.01$), suggesting that birds were manufacturing IgY directly against vaccine constituents. Increases in circulating IgY, in response to immunisation with both mite and other ectoparasitic antigens is an effect which has been widely documented (Devaney and Augustine; 1987; Wikel *et al.*, 1989; Minnifield *et al.*, 1993; Lee *et al.*, 2002).

There was also a significant effect of successive immunisations over time on optical density values. In both treatments, initial IgY levels were significantly lower than succeeding observations ($P<0.001$). This boosting effect induced by routine immunisation is as a result of the formation of memory B-cells which are synthesised against specific antigens following primary infection. Upon subsequent exposure to the same antigens, memory B-cells recognize them and quickly proliferate to enable an elevated and more rapid response, thus preventing recurrent infection (Smith *et al.,* 2004). As with the present study, it is not uncommon to find that at least three immunisations are necessary to elicit a response which causes a significant difference between treatments (Patarroyo *et al.,* 2002).

When considering IgY levels for individual birds, it was observed that prior to immunisation all birds were below the negative cut-off point i.e. were not producing antibodies against red mite antigens, as expected. However, following the initial immunisation all birds in the Control treatment reacted unexpectedly showing an increase in the production of IgY specific to red mite antigens. IgY levels were in fact higher in the Control treatment than in the Antigen treatment, although not significantly so. There was also a higher degree of variation observed between individual birds in the Control treatment in comparison to the Antigen treatment. This high variability remained apparent throughout, with several birds from the Control treatment maintaining IgY levels equivalent to those seen in the Antigen group. Control animals displaying an increased antibody production following immunisation is not altogether uncommon, as has been previously observed on numerous occasions, although these increases are generally not rationalized (Devaney and Augustine, 1987; Wikel *et al.,* 1989; Minnifield *et al.,* 1993; Sam-Sun *et al.,* 2002; Smith *et al.,* 2004; Hou *et al.,* 2006).

However, there are several potential explanations for this phenomenon. Firstly, it may be possible that there is a high degree of non-specific binding of antibodies in ELISA causing false positive results. For example, failure to distinguish self from non-self by the presence of serum anti-chicken IgY antibodies, equivalent to rheumatoid factor (RF) or human anti-mouse antibody (HAMA), would result in generation of non-specific binding (Johnson and Faulk, 1976; Larsson *et al.,* 1992). Alternatively, a false positive could arise from the presence of endogenous antibodies, which are non-specific polyclonal serum antibodies capable of recognising multiple epitopes on antigens (Ismail, 2005). Previous research suggests that serum concentrations of

endogenous antibodies are found to fluctuate widely from one individual to another, with differing affinity/avidity to various antigens. Endogenous antibodies are capable of mimicking antigens/antibodies and often have multiple paratopes (binding sites), so therefore can also disrupt reaction kinetics (Wingren *et al.*, 1995). The innate characteristics of endogenous antibodies cause various levels of interference and thus could potentially explain the wide variation observed between individual birds in the present study. However, such extensive non-specific and varied binding was not observed during assay validation (see Section 3.2).

An alternative suggestion to the occurrence of non-specific binding could be the maternal transfer of immunoglobulins (Fahey *et al.*, 1987; Al-Natour *et al.*, 2004). However, for this to be applicable one would expect to see birds mounting an immune response to red mite from the beginning of the experiment. Since this did not happen in the current experiment this explanation in unlikely.

A fourth and final explanation for the occurrence of non-specific binding concerns the constituents of the vaccine, which differed between the three immunisations. The first immunisation for each treatment was prepared using CFA which contains *Mycobacterium tuberculosis*, whereas subsequent immunisations used IFA, which is simply an oil-in-water emulsion. It was previously found by Mathaba *et al.* (2002) that the house dust mite (*Dermatophagoides farinae)* contains lytic enzymes which share sequence homology with specific prokaryotic proteins suggesting that they may be bacterially derived. Most interestingly for the purpose of the current study, this homology was seen with *Mycobacterium tuberculosis.* Mathaba *et al.* (2002) hypothesized that bacteriolytic enzymes may be utilized by mites for the digestion of bacteria, which form a part of their diet. It may therefore be possible to make the assumption that other mite species, including the poultry red mite, will show a similar sequence homology to bacteria by containing bacteriolytic enzymes. If this was the case then the red mite extracts administered in the current study might contain a series of proteins similar to those found in the *Mycobacterium* contained within the CFA. In the current study both Control and Antigen treatments received CFA in the first immunisation and IFA, which does not contain *Mycobacterium,* for second and third immunisations. Therefore, birds in both treatments would be expected too mount an initial antibody defence against *Mycobacterium.* However, after subsequent immunisation with IFA antibody production would slow in the Control treatment, whilst continuing to increase in the Antigen treatment and was in fact the case in the current study. The theory of mites coexisting with other pathogens (Chauve, 1998; Moro *et*

al., 2005), not only *Mycobacterium*, may be an additional source of non-specific binding in immunological studies. Since, if control birds are naturally exposed to similar environmental pathogens to those immunised in vaccines containing arthropod antigens, a similar immune response will be initiated between treatments. Purification and immunisation of specific red mite proteins may be one way to avoid this problem and has been shown to work previously (Willadsen *et al.,* 1999).

6.4.3 Western blotting

Results of the western blotting also produced unexpected results, antibody responses when using post immunisation sera revealed little difference between the Antigen and Control treatments, both displaying multiple bands and similar profiles. The same possible explanations for this binding can be applied as were discussed for the ELISA results. The suggestion that *Mycobacterium* shares sequence homology with mite protein is one possible theory, which assumes that protein extracted and fractionated would have a direct association with *Mycobacterium* in order to be recognised by antibodies from both treatments.

Additionally, as with the ELISA assay, there is the possibility of non-specific binding by endogenous antibodies, which increase with antigenic exposure, both with age and following vaccination (Wingren *et al.,* 1995).

Several other authors have observed that serum from birds which had never been exposed to mites produced multiple bands after western blotting, with similar profiles to those of birds which had faced either artificial or natural mite antigens (Devaney and Augustine, 1987; Wikel *et al.,* 1989; Minnifield *et al.,* 1993). Devaney and Augustine (1987) proposed that this was due to the secondary anti-chicken IgY antibody binding directly to chicken blood antigens present in the mite extract, rather than actual mite components. This was observed to some extent in the present study, where secondary anti-IgY antibody bound directly to fractionated mite extracts prior to immunisation (Section 3.4). However, this was only apparent to 20 and 60 kDa fragments and not the full spectrum. Wikel *et al.* (1989) simply attributed non-specific binding to the presence of cross-reactive natural immunoglobulins.

6.4.4 Cytokine analysis

Treatment appeared to have little impact upon the Th1-type immune response of birds, as no differences in IFN-γ levels were apparent between the Control or Antigen

treatments. Wakugawa *et al.* (2001) also observed no change in IFN-γ levels after spiking peripheral blood mononuclear cells with dust mite antigens. Lack of Th1-type response may also have been due to the inability of red mite antigen to penetrate into the spleen tissue, as it is more likely that any immune response would be much more localised (Wikel, 1982). In addition, the timing of the spleen sampling, three weeks post immunisation, may have missed any cytokine response that occurred, since optimal sampling time to detect Th2-type immune response is usually around 4-10 days after antigen exposure (Zeidner *et al.*, 1999).

Prior to red mite infestation there were significant changes to IL-10, IL-4 and IL-5 levels. IL-10 expression was significantly higher in the Antigen treatment when compared to the Controls. Since IL-10 is an anti-inflammatory cytokine, it is perhaps not surprising that increased levels were observed, since mite antigens have a history of evoking pro-inflammatory responses, although generally as a result of direct feeding (Van Den Broek *et al.*, 2000). IL-10 is also known as the cytokine synthesis inhibitory factor (CSIF) and has pleiotropic attributes, enabling it to inhibit the production of many other cytokines, in particular Th1-type stimulatory IFN-γ (Waal Malefyt *et al.*, 1991). This may explain why no increase in the level of IFN-γ was seen in the antigen treatment. Yang *et al.* (2001) reported that immunisation with dust mite antigens again stimulated a significant rise in IL-10 causing a significant drop in IFN-γ levels. These apparent differences between studies are likely to be as a result of the nature of the antigens immunised, in terms of structure (Schoeler and Wickel, 2001).

On the other hand, levels of both Th2-type cytokines IL-4, an anti-helminthic cytokine and IL-5, an anti-parasitic and allergen mediating cytokine were lower in the Antigen treatment after red mite infestation. The reasons for this are unclear, although it may possibly be as a result of immunosuppression in the Antigen treatment by a component of the red mite extract, as described in the previous chapter. Extracts from haematophagous arthropods have been associated with down-regulation of numerous cytokines, including both IL-4 and IL-5 (Schoeler and Wikel, 2001). The antigen elements causing this reduction are generally associated with salivary gland extracts and are important during the feeding process (Gillespie *et al.*, 2000). Therefore, if similar components were included in the antigen extract injected into birds in the present study, then this could explain the reduced cytokine expression. Contrary to this, other authors have reported arthropod saliva to up-regulate

cytokines associated with Th2-type response, whilst down-regulating the Th1-type response (Schoeler and Wikel, 2001).

With these conflicting views it is therefore difficult to draw definite conclusions about the cytokine response following immunisation. However, it would seem that the red mite extracts administered here have the capacity to manipulate the immune system in the favour of the feeding mite. Extracts appeared to avoid eliciting Th1-type IFN-γ, as well as up-regulating IL-10 cytokines which would assist feeding by reducing inflammation and also suppress Th1-type responses. Additionally, there was an apparent down-regulation of anti-parasitic/helminthic Th2-type responses. However, a significant increase in the levels of immunoglobulin-Y, which is driven by Th2-type immunity, was observed from ELISA assays. These findings remain consistent with results presented after natural red mite exposure in Chapter 5 and with numerous reports in the literature (Gillespie *et al.*, 2000). Ogden *et al.* (2002) demonstrated a similar response when low level infestation by ticks was met by protective Th1-type host immunity. However, an increase in the tick burden was seen to push immunity in the direction of a non-protective Th2-type response. Foy *et al.* (2003) also observed significant changes in cytokine expression away from those of the Th1-type and towards those of the Th2-type, with significantly higher antibody titres and IL-10 levels as a result of immunisation with mosquito midgut antigens.

6.4.5 Red mite feeding challenge

There were no significant effects of treatment on red mite feeding and fecundity parameters. However, there was a trend for lower survival and both higher mortality and oviposition rates for red mite in the Antigen treatment, although this was not significant. Immunisation against other mite species (*Ornithonyssus sylviarum* and *Sarcoptes scabiei)*, as well as the mosquito (*Anopheles gambie*) have also failed to generate clear protective responses in previous studies, where parasites survived despite increased antibody levels (Minnifield *et al.*, 1993; Foy *et al.*, 2003; Tarigan and Huntley, 2005). Although birds in the Antigen treatment showed significant increases in the production of antibodies against red mite proteins, they might not have been presented in the optimum fashion to evoke a significant reduction in red mite numbers after feeding. This may have resulted from damage to the tertiary structure of proteins during the extraction process, a structure which may be important in eliciting protective responses (Lee *et al.*, 2002). Wikel *et al.* (1989) reported that birds generated a significant IgY response to northern fowl mite antigens, although this did not elicit a protective response by reducing mite numbers.

The same author suggested that exposure to mite antigens which contained immunodominant epitopes may stimulate antibody responses unrelated to protection, and that actually resistance to infestation could be induced by antigens which do not show immunodominance. It is also possible that potential protective epitopes become denatured during the extraction procedure, thus inhibiting antibody binding (Tarigan and Huntley, 2005).

Alternatively, the red mite antigens used in the current study may have induced the 'wrong type' of immune response. In some parasitic mite species there is evidence that there is an insignificant or limited role for IgG antibodies in protecting hosts from blood-feeding ectoparasites and they have actually been inversely related to protection (Dubois *et al.*, 1993; Tarigan and Huntley, 2005). In these instances, other antibody isotypes, such as IgE, or even Th1-type cellular responses have been demonstrated to be more important in host immunity (Nisbet and Huntley, 2006).

Despite there being no significant difference between treatments in the current study, sampling point did have a significant effect on red mite mortality, with the number of dead mites increasing significantly from 2 to 10 days post first infestation and decreasing thereafter. In contrast, Bruneau *et al.* (2001) saw high levels of mortality after initial infestations and suggested that this was due to natural selection, with red mite which are unable to feed in the experimental system dying, whilst red mite which adapt continue to feed and reproduce. However, it is likely that the higher mortality seen in the current study was simply due to the longer incubation period between counting points (i.e. 2 versus 10 days) allowing the greater accumulation of dead mites.

This study also revealed there were no significant differences in red mite survival and fecundity parameters between *in vivo* and *in vitro* feeding systems. This development of an *in vitro* feeding system is promising in the quest for a reliable, non-animal model to test protection against parasitism conferred to the host. It offers a controlled method for testing therapeutic compounds, whilst allowing for a reduction in the requirement of animals (Carroll *et al.*, 1992; Voigt *et al.*, 1993; McDevitt *et al.*, 2006a).

6.5 Conclusions

Immunisation with an antigen extracted from the poultry red mite resulted in a significantly greater antibody response compared to Control treated birds. However, birds in the Control treatment also showed an increase in the level of circulating IgY, which was thought to be due to the occurrence of non-specific antigen recognition.

Western blotting showed few differences in specific antibody binding between treatments and confirmed ELISA results suggesting non-specific antigen recognition. Several explanations were given for this effect, such as the presence of *Mycobacterium* or other red mite dwelling pathogens and/or cross-reactivity of natural endogenous antibodies as well as antibody recognition of poultry blood proteins present in the mite, although it is likely to be a combined effect of several of them.

Analysis of cytokine expression revealed that immunised red mite extracts appeared to have the capacity to manipulate production in the favour of the feeding mite. There was no change in the Th1-type cytokine expression as a result of immunisation, but increases in the levels of Th1-inhibitory and anti-inflammatory IL-10 cytokine were seen in the antigen treatment. In addition, there appeared to be some immunosuppression of anti-parasitic/helminthic Th2-type IL-5/IL-4 cytokines.

Red mite survival and fecundity appeared to be reduced following immunisation with mite extract, although these differences were not significantly different to the Control treatment. It was suggested that this protective failure may be as a result of insufficient antigen levels or improper presentation of mite antigens.

This study also resulted in the development of an *in vitro* feeding system using skin membranes, which was validated against an *in vivo* feeding system and will assist in future refinement of the immunisation strategy by providing a means for rapid and reproducible testing, without recourse to testing on live birds.

Chapter 7

The effect of immunisation with different poultry red mite extracts on humoral immunity and subsequent efficacy

7.1 Introduction

In the previous chapter immunisation of birds with an antigen prepared from whole red mite crushed in a PBS buffer resulted in a significant increase in IgY levels. However, the same antigen showed apparent cross-reactivity with *Mycobacterium* found in both the adjuvant used (CFA) and red mite extracts, which was confirmed by PCR analysis in Section 3.8. Despite increases in red mite specific IgY, immunisation failed to generate a significant protective response against red mite parasitism, which was revealed after the development of a robust *in vitro* feeding system for estimating the survival and fecundity of red mite. In addition, the previous chapter showed some apparent changes in the levels of cytokine expression, although overall effects on this type of the immune response were modest.

Since red mite antigens were observed to clearly facilitate humoral immunoglobulin responses, it would therefore be beneficial to quantify additional humoral antibody response in the form of immunoglobulin-M (IgM). IgM is a pentameric molecule binding less specifically than IgY which makes it ideal for defence against primary pathogen invasion (Abbas *et al.,* 2003). Despite having a short lifespan IgM is capable of producing protective immunity as it can recognise different epitopes to IgY and utilize alternative mechanisms for the elimination of parasites (Tizard, 2002; Ligas *et al.,* 2003).

Therefore this study had several aims, firstly was the eradication/limitation of the apparent Mycobacterial effect observed in the previous trial, allowing the establishment of a true negative. The second aim was to determine the effect of immunisation with red mite antigens on the level of IgM response. Finally, an *in vitro* feeding system was used to evaluate the survival and reproductive parameters of red mite after immunisation of pullets with different red mite antigen extracts.

7.2 Materials and methods

7.2.1 Experimental treatments

Birds were subject to one of 4 immunisation treatments; Treatment 1 was a true negative with birds receiving PBS only; Treatment 2 was a second control treatment, with birds receiving Incomplete Freund's adjuvant (IFA) only; Treatment 3 was an antigen treatment, with birds receiving the same PBS-extracted red mite antigen as used in the previous chapter (PBS Antigen); Treatment 4 was another antigen treatment, with birds receiving a red mite antigen extracted in a urea buffer (Urea Antigen). Treatments 3 and 4 both received antigen in IFA. Each treatment had 20 replicate birds, with 3 naive birds sacrificed at the start of the trial to generate baseline parameters giving a total of 83 birds (see Table 7.1).

Table 7.1 *Summary of experimental treatments*

Treatment No.	Immunisation constituents
1	1 ml PBS
2	Incomplete Freund's adjuvant + PBS
3	0.5 ml Incomplete Freund's adjuvant + 0.5 ml PBS Antigen (1 mg)
4	0.5 ml Incomplete Freund's adjuvant + 0.5 ml Urea Antigen (1 mg)

7.2.2 Birds and housing

Female domestic fowl of a commercial egg-laying strain were used in this experiment (Shaver-579), housed according to recommendations for floor pens (DEFRA, 2001). Birds were purchased at day-old and maintained in a floor pen, using a brooder lamp and wood-shavings for bedding. At 5 weeks of age the birds were allocated to 4 separate floor pens located in the same room. For the duration of the trial they were provided with *ad libitum* water and standard commercial feed for growing and subsequently laying hens. Ambient temperature was adjusted to maintain birds within their thermal comfort zone, which is approximately 32°C at day-old and falling to 18°C when they became fully feathered adults (Rose, 2001). The illumination program followed the standard for commercial production using a ratio of light to dark of 14L:10D designed to bring birds to the point of lay at 20 weeks of age. Leg rings were used to identify individual birds within a group. Birds were monitored daily for signs of ill health following immunisation, which included intolerance to exercise and at a more extreme level, an increased respiratory rate (Morton *et al.*, 1993). Individual bodyweight, mean weekly feed intake and room temperature were all recorded for the duration of the study.

7.2.3 Preparation of red mite antigen

Soluble, unfed whole red mite antigens were extracted using either a PBS or urea buffer, as described in Section 3.1 and were either immunised into birds or used in ELISA/western blotting. Antigens were defrosted for approximately 30 min at room temperature on the day in which they were required.

7.2.4 Immunisation and sampling schedule

Birds were immunised subcutaneously on the back, between the wings, on three separate occasions at 5, 8 and 11 weeks of age (Table 7.2). The volume of injectate in all four treatments was approximately 1 ml and so did not exceed the recommendations i.e. 2 to 5 ml/kg (Morton *et al.*, 2001).

Blood samples were taken at 5 weeks of age, prior to the first immunisation and then at 8, 11 and 14 weeks of age, one day prior to subsequent immunisations. A blood sample was taken from the brachial (wing) vein, removing a volume of approximately 1 ml. At the end of the experiment the remaining birds were dispatched humanely via cervical dislocation and bled directly from the heart, removing approximately 5 ml of blood. This blood was divided equally and either allowed to clot and serum removed for subsequent immunological analysis, or placed inside lithium heparinised vacutainers (Becton Dickinson vacutainer systems, Oxford, UK) to yield full blood for use in an *in vitro* red mite feeding system.

Table 7.2 *Schedule of immunisation and sampling*

Week	Age (weeks)	Treatment	Sampling
1	5	Immunise 1	#Serum (All)
4	8	Immunise 2	#Serum (All)
7	11	Immunise 3	#Serum (All)
10	14	*In vitro* mite feeding	#Serum and full blood (All)

#All samples were taken 1 day prior to that of treatment from all birds

7.2.5 ELISA

Both IgY and IgM specific ELISA's were conducted on serum from all birds at each sampling point to quantify the relative levels of antibody. The ELISA's were carried out using only PBS-extracted mite antigens to coat the plates, as a pilot validation assay performed comparing both plates coated with PBS/urea-extracted antigens and sera from birds immunised with both PBS/urea-extracted antigens showed no significant difference between the optical density for the two methods (see Tables 21-

22, Appendix II). The protocol for IgM ELISA is outlined below, however, for IgY ELISA protocol see Section 3.2.

7.2.5.1 IgM ELISA

An ELISA assay for the determination of red mite IgM levels in poultry serum was developed as follows. Firstly, 10 µg of PBS extracted red mite antigen (Section 3.1), diluted in 0.1 M $NaHC0_3$ (pH 9.5) was used to coat Nunc flat-bottomed 96-well ELISA plates (Nunc, Denmark) and left overnight in an orbital shaker at 4°C. The following day, the plates were washed 3 times in 200 µl PBS-Tween-20 (0.15 M NaCl, 0.02 M Na_2HPO_4, 0.01 % Tween-20, pH 7.2) and then blocked in 100 µl of 1 % milk powder and incubated at 37°C for 45 min. Plates were washed again using 200 µl PBS-Tween-20 and then 100 µl of serum, diluted 1:500 in PBS-T was added and incubated at 37°C for 1.5 hours. After another three washes with 200 µl PBS-Tween-20, each well received 100 µl of goat anti-chicken IgM antibody (Serotec, Oxford, UK), diluted 1:8,000 in PBS-T and 1 % milk powder and incubated for a final time at 37°C for 1 hour. Plates were once again washed in 200 µl PBS-Tween-20 and developed using TMB substrate (Sigma, St. Louis, USA). Plates were held at room temperature for 17.5 min, after which the reaction was stopped by adding 100 µl 1 N HCl to each well and plates read immediately at 450 nm using a microplate reader (Bio-tek Instruments, Winooski, USA) to determine optical density.

7.2.6 SDS-PAGE and western blotting

In order to establish specific antibody binding, SDS-PAGE/western blotting for IgY was carried out on serum samples both pre- and post- immunisation on samples from the 5 most immunogenic birds (i.e. birds which displayed the highest antibody levels in ELISA) in each treatment. Blotting was done using both PBS and urea extracted antigens to allow visualisation of binding differences. The full protocols for both SDS-PAGE and western blotting were described previously in Section 3.4.

7.2.7 In vitro *red mite feeding challenge*

The survival and fecundity of red mite feeding on blood from the four treatments was assessed using an *in vitro* feeding system. The protocol for feeding was described in Section 3.6. Mites were fed using day-old chick skin membranes and lithium heparinised full blood taken from the experimental birds. After feeding, blood reservoirs were removed and the number of fed mites established, along with mortality, oviposition and subsequent hatching rates. In contrast to the previous experiment (Chapter 6) red mite were only subject to one round of feeding. It is also

important to note that data for mite survival and fecundity was expressed as percentages, due to differing mite recovery rates between replicates and treatments.

7.2.8 Statistical analysis

Statistical analysis of the effect of treatment on hen parameters, humoral immune response and mite infestation was performed using ANOVA in MINITAB (V14). Response variables including weekly hen bodyweight, IgY and IgM optical density and red mite survival and fecundity, were analysed against the effect of treatment. Also, ANOVA for hen bodyweight was performed using the initial starting weight as a covariate to determine whether this had an effect on subsequent bodyweight after immunisation.

7.3 Results

7.3.1 Bodyweight and feed intake

When comparing mean weekly weights of experimental birds per treatment it was obvious that Treatment 4 was consistently lower than other treatments. This difference was apparent before birds received any immunisation, although not significantly so at this point (Table 7.3) and maintained throughout the duration of the trial. However, this initial weight difference did not have a significant impact on subsequent bodyweight (see Appendix II).

Table 7.3 *Effect of treatment on mean weekly bodyweight (g) (20 birds/group)*

Age (Weeks)	Treatment 1	Treatment 2	Treatment 3	Treatment 4	S.E. Mean	Significance
5	338.5	333.9	375.9	345.6	6.25	NS
6	419.7^{a}	416.4^{ab}	425.4^{ab}	383.2^{b}	7.81	*
7	511.7^{a}	525.1^{ac}	566.5^{ac}	520.3^{c}	9.79	*
8	629.1^{ab}	632.5^{ab}	683.9^{a}	637.6^{b}	10.39	*
9	777.0^{ab}	785.9^{a}	823.1^{a}	781.2^{b}	14.44	*
10	909.0	896.7	957.1	892.1	14.99	NS
11	1015.4^{ab}	1008.9^{ab}	1102.2^{a}	1004.0^{b}	15.79	*
12	1107.9	1122.9	1220.6	1207.1	27.58	NS
13	1238.6	1264.8	1372.5	1338.7	30.55	NS
14	1355.0	1417.0	1491.0	1493.0	19.86	NS

*= $P<0.05$; NS= no significant difference;
Means within a row followed by a different superscript letter are significantly different at $P<0.05$

Statistical analysis was not possible on feed intake as it was recorded on a mean pen basis. However, there appeared to be no difference in mean weekly feed intake between treatments (Table 7.4), although a lull in intake was seen for all treatments

after the first immunisation (6 weeks of age). No decreases were observed after further immunisation, although there was a degree of fluctuation between weeks. Overall means suggest that Treatment 4 consumed the most feed, yet on average weighed less (Table 7.3).

Table 7.4 *Effect of treatment on mean weekly feed consumption (20 birds/group)*

Age (Weeks)	Treatment 1 (g/bird/week)	Treatment 2 (g/bird/week)	Treatment 3 (g/bird/week)	Treatment 4 (g/bird/week)
5	71.4	62.5	65.2	69.4
6	40.8	53.6	37.3	42.9
7	70.4	59.5	65.2	53.6
8	61.2	56.5	58.0	64.3
9	62.9	56.5	77.9	67.9
10	74.8	77.4	74.7	57.1
11	68.0	65.5	67.4	89.3
12	79.9	68.5	78.2	82.1
13	66.3	74.4	66.3	92.9
Mean:	**66.2**	**63.8**	**65.6**	**68.8**

7.3.2 Immunoglobulin-Y response

Immunoglobulin-Y levels are given in Table 7.5 and show that prior to immunisation, there was no significant difference between treatment optical densities. However, after the first immunisation, Treatments 3 and 4 had a significantly higher optical density than Treatment 2, which in turn had a significantly higher optical density than Treatment 1, these differences between treatments were maintained for the remaining duration of the trial.

Table 7.5 *Effect of treatment on mean IgY optical density (20 birds/group)*

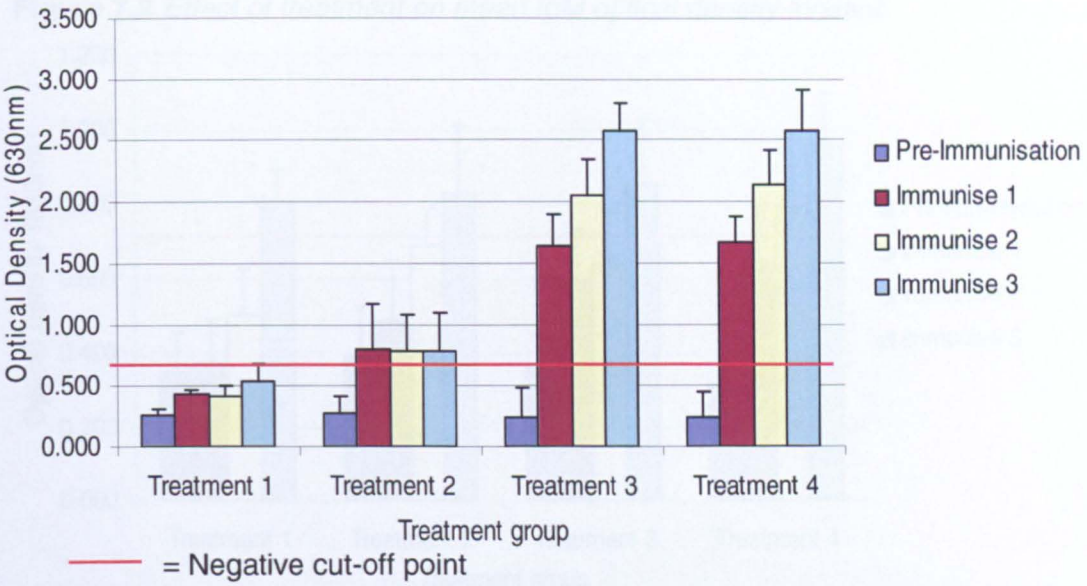
Sampling point	Age (Weeks)	Treat. 1	Treat. 2	Treat. 3	Treat. 4	S.E. Mean	Significance
Pre-immunisation	5	0.27	0.28	0.24	0.25	0.017	NS
Immunisation 1	8	0.43^{a}	0.81^{b}	1.64^{c}	1.67^{c}	0.064	***
Immunisation 2	11	0.43^{a}	0.79^{b}	2.06^{c}	2.14^{c}	0.055	***
Immunisation 3	14	0.54^{a}	0.79^{b}	2.57^{c}	2.58^{c}	0.057	***

***= P<0.001; NS= no significant difference;
Means within a row followed by a different superscript letter are significantly different at P<0.05

Figure 7.1 illustrates the kinetics of the IgY response over the sampling period. Both Treatments 1 and 2 show a low level of immune response, with only minor fluctuations in optical density after immunisation. However, both Treatments 3 and 4 show a large rise in IgY levels following the first immunisation and a steady increase thereafter. All treatments, excluding Treatment 1, were observed to exceed the

negative cut-off point following the first immunisation and remain above this level for the duration of the trial.

Figure 7.1 *Effect of treatment on mean IgY optical density kinetics*



7.3.3 Immunoglobulin-M response

Table 7.6 shows that IgM optical density values were not significantly different between treatments before immunisation. After the first immunisation, Treatments 3 and 4 had a significantly higher optical density than Treatment 1, with an intermediate value for Treatment 2. After the second immunisation, Treatment 4 was significantly higher than Treatments 3 and 1, but not 2. Treatment 3 was also not significantly different to Treatment 2, but was significantly higher than Treatment 1. Following the final immunisation Treatment 4 was significantly higher than Treatments 1 and 2, with an intermediate value for Treatment 3.

Table 7.6 *Effect of treatment on mean IgM optical density (20 birds/group)*

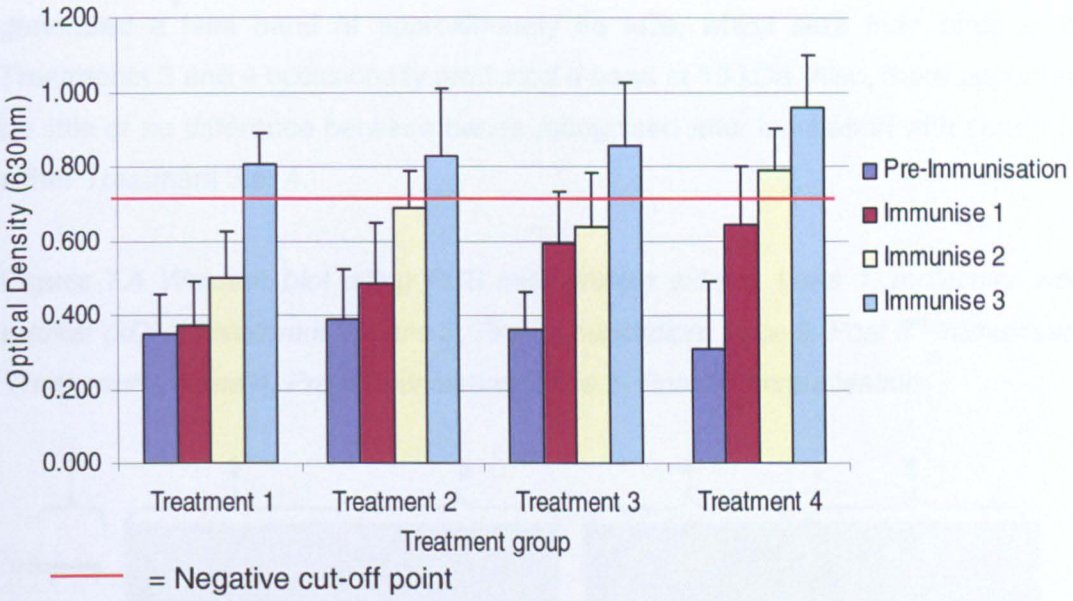
Sampling point	Age (Weeks)	Treat. 1	Treat. 2	Treat. 3	Treat. 4	SE Mean	Significance
Pre-immunisation	5	0.35	0.39	0.36	0.31	0.024	NS
Immunisation 1	8	0.37^{a}	0.49^{ac}	0.59^{bc}	0.64^{b}	0.032	***
Immunisation 2	11	0.50^{a}	0.69^{bc}	0.64^{b}	0.79^{c}	0.031	***
Immunisation 3	14	0.81^{a}	0.83^{a}	0.85^{ab}	0.96^{b}	0.033	*

*= P<0.05; ***= P<0.001; NS= not significantly different;
Means within a row followed by a different superscript letter are significantly different at P<0.05

The reaction kinetics of IgM in all treatments showed a steady increase after each immunisation (Figure 7.2). Prior to the third immunisation, IgM levels in all treatments

were lower than the cut-off point, with the exception of Treatment 4 which passed after the second immunisation, after which all treatments exceeded the negative cut-off value.

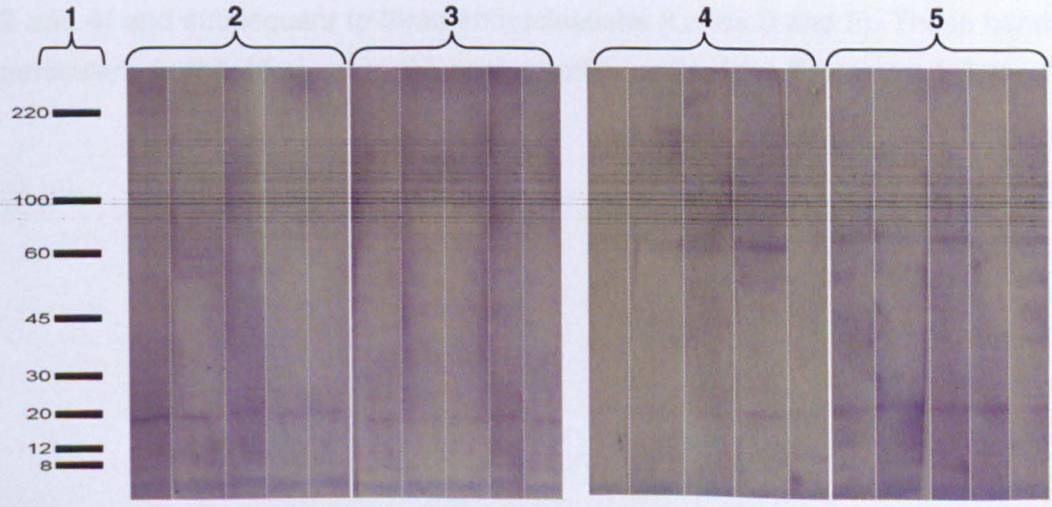
Figure 7.2 *Effect of treatment on mean IgM optical density kinetics*



7.3.4 Western blotting

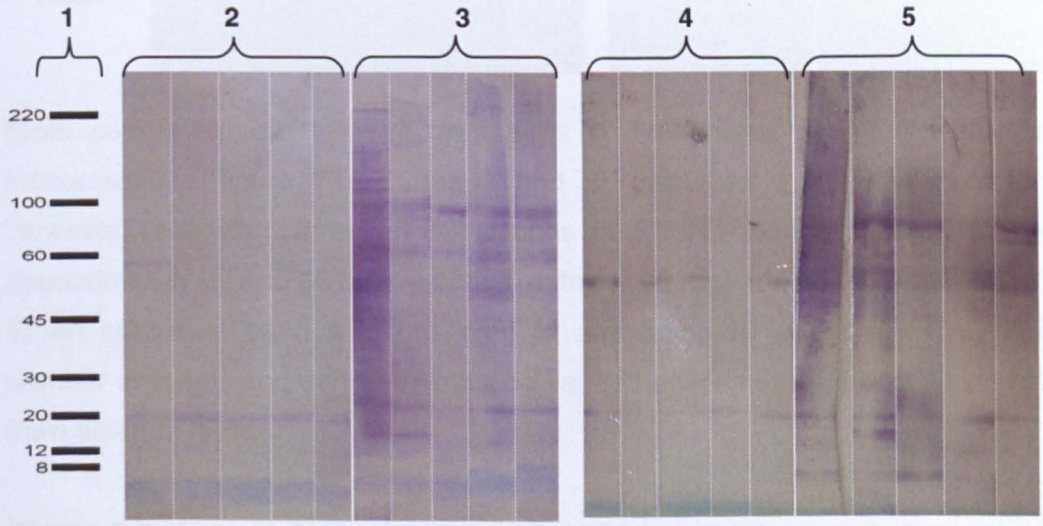
Figure 7.3 shows images of western blots on PBS-fractionated red mite antigens, for Treatments 1 and 2, using both pre-immunisation serum (Lanes 2 and 4) and serum after three inoculations (Lanes 3 and 5). In all lanes there is a degree of non-specific binding, with the presence of a band at around 20 kDa. There is also the appearance of a faint band at approximately 60 kDa for the birds in Treatment 2.

Figure 7.3 *Western blot using PBS mite protein extract. Lane 1, molecular weight marker (kDa); Treatment 1: Lane 2, Pre-immunisation; Lane 3, Post 3rd immunisation; Treatment 2: Lane 4, Pre-immunisation; Lane 5, Post 3rd immunisation*



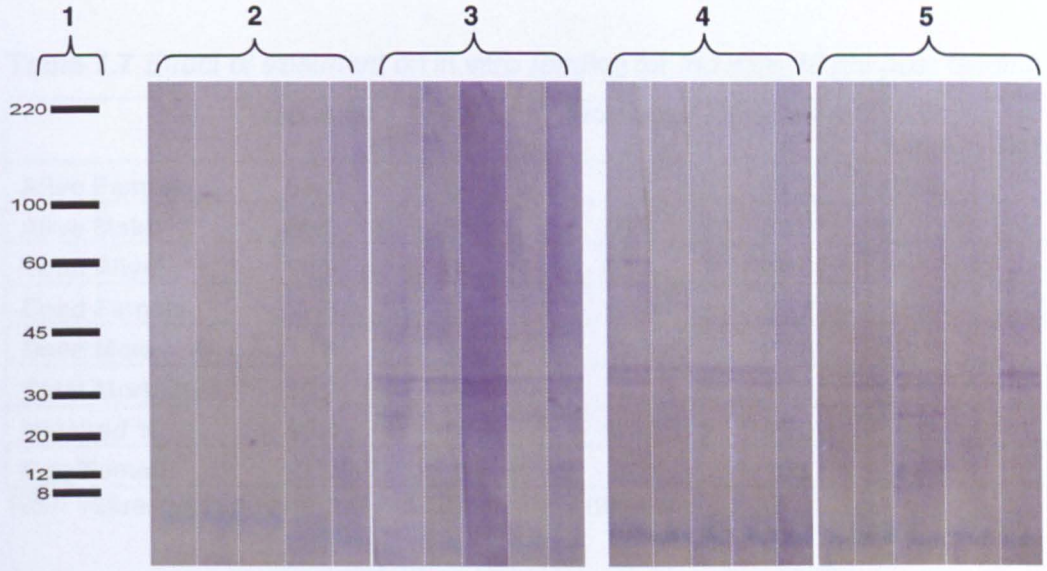
Pre-immunisation serum from Treatments, 3 and 4 generated only minor non-specific binding (Figure 7.4, Lanes 2 and 4) with faint bands at around 20 kDa and 60 kDa. Treatments 3 and 4 also recognised an additional dominant band at around 80 kDa after incubating blots with post immunisation sera. In addition, birds in Treatment 3 generated a faint band at approximately 55 kDa, whilst sera from birds in both Treatments 3 and 4 occasionally produced a band at 18 kDa. Also, there appeared to be little or no difference between bands recognised after incubation with serum from either Treatment 3 or 4.

Figure 7.4 *Western blot using PBS mite protein extract. Lane 1, molecular weight marker (kDa); Treatment 3: Lane 2, Pre-immunisation; Lane 3, Post 3rd immunisation; Treatment 4: Lane 4, Pre-immunisation; Lane 5, Post 3rd immunisation*



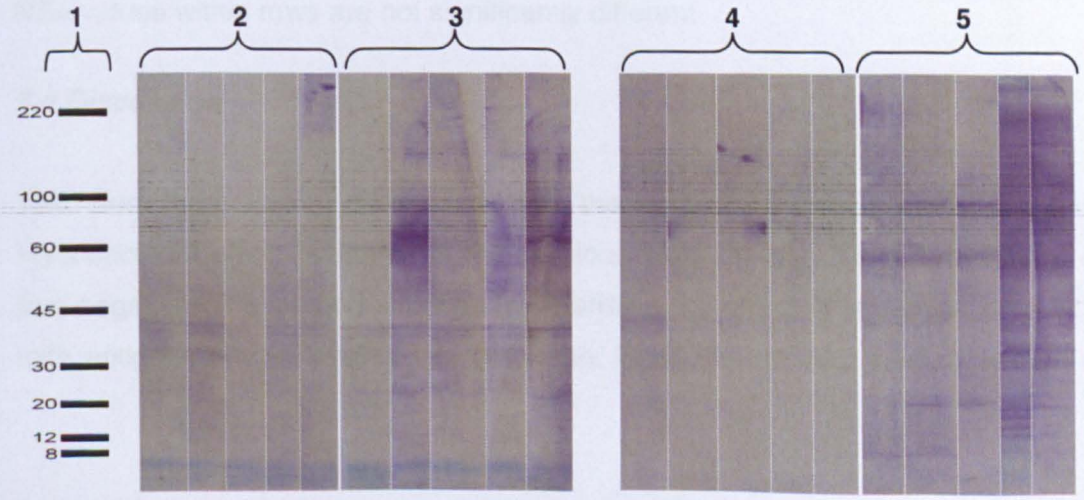
Western blotting was also performed using urea-fractionated red mite antigens as shown in Figure 7.5-7.6. Blots using serum from Treatments 1 and 2 consistently showed the presence of two bands at around 40 kDa, both pre-immunisation (Lanes 2 and 4) and subsequent to three immunisations (Lanes 3 and 5). These bands are particularly faint in blots using pre-immunisation serum from Treatment 1 (Lane 2).

Figure 7.5 *Western blot using urea mite protein extract. Lane 1, molecular weight marker (kDa); Treatment 1: Lane 2, Pre-immunisation; Lane 3, Post 3rd immunisation; Treatment 2: Lane 4, Pre-immunisation; Lane 5, Post 3rd immunisation*



Blots performed using serum from birds in Treatments 3 and 4 taken prior to immunisation (Figure 7.6, Lanes 2 and 4) displayed a 40 kDa dimeric band. However, following three immunisations additional bands were visualised at approximately 20 and 60 kDa in both Treatments 3 and 4. Also, in Treatment 4 (Lane 5) an additional band was observed at approximately 80 kDa. Once again the number of bands and degree of staining varied between individual blots using serum from different birds.

Figure 7.6 *Western blot using urea mite protein extract. Lane 1, molecular weight marker (kDa); Treatment 3: Lane 2, Pre-immunisation; Lane 3, Post 3rd immunisation; Treatment 4: Lane 4, Pre-immunisation; Lane 5, Post 3rd immunisation*



7.3.5 In vitro *red mite feeding challenge*

No significant differences were observed between treatments for red mite survival parameters 48 hours after infestation (Table 7.7). Similarly, the number of eggs laid and hatched was not significantly different between treatments.

Table 7.7 *Effect of treatment on* in vitro *feeding for fed mite 48 hrs post feeding*

	Treatment 1	Treatment 2	Treatment 3	Treatment 4	S.E. Mean	Significance
Alive Female	68.1	69.3	72.7	69.2	3.02	NS
Alive Male	60.2	55.1	56.0	53.0	4.31	NS
Total Alive	66.0	65.1	67.6	64.8	1.53	NS
Dead Female	11.3	14.0	11.4	10.7	2.57	NS
Dead Male	18.8	13.7	19.5	22.8	2.43	NS
Total Mortality	13.2	14.8	14.6	14.8	1.27	NS
Hatched %	99.4	97.7	97.8	95.2	0.15	NS
Egg/Female	0.8	0.7	0.7	0.8	2.27	NS

NS= values within rows are not significantly different

There were also no significant differences between treatments for parameters effecting mite survival 10 days after feeing (see Table 7.8). Red mite in Treatment 4 had both lower total survival and higher mortality, although this was not significantly different from the other treatments.

Table 7.8 *Effect of treatment on* in vitro *feeding for fed mite 10 days post feeding*

	Treatment 1	Treatment 2	Treatment 3	Treatment 4	S.E. Mean	Significance
Alive Female	73.9	75.0	75.6	65.3	4.33	NS
Alive Male	66.5	69.0	69.3	67.7	5.96	NS
Total Alive	71.1	73.0	73.4	66.7	4.33	NS
Dead Female	27.5	25.9	25.7	35.3	5.61	NS
Dead Male	34.2	31.3	31.1	33.6	3.17	NS
Total Mortality	29.8	27.7	17.9	34.4	3.17	NS

NS= values within rows are not significantly different

7.4 Discussion

This study had several aims, firstly was the eradication/limitation of the apparent Mycobacterial effect observed in the previous trial, allowing the establishment of a true negative. The second aim was to determine the effect of immunisation with red mite antigens on the level of IgM response. Finally, an *in vitro* feeding system was

used to evaluate the survival and reproductive parameters of red mite after immunisation of poultry with different red mite antigen extracts.

7.4.1 Bodyweight and feed intake

As with the immunisation experiment in the previous chapter, birds used were of a commercial genotype, growing and consuming food within commercial recommendations (Nix, 2000; Rose, 2001). However, there was a significant difference in bodyweight with Treatment 4 being lighter than other treatments after the first immunisation. This appeared to be as a direct result of immunisation, since starting weight was eliminated as possible factor after statistical analysis and also birds in this group displayed some mild clinical signs of ill health. However, examination by a veterinary practitioner confirmed that these symptoms not severe and birds recovered soon after with no significant differences in bodyweight observed by week 12 of the experiment.

7.4.2 Immunoglobulin response

In order to satisfy the primary objective, *Mycobacterium* containing CFA was replaced with IFA. Also an additional saline only treatment was included to allow determination of the adjuvant effect. Since the generation of false positives from non-specific Mycobacteral binding should have been eliminated it would be reasonable to assume that there would be no difference in antibody levels between control Treatments 1 and 2. However, inoculation with IFA plus saline in Treatment 2 did elicit a significantly higher IgY response when compared to saline alone (Treatment 1). The reasons for this are unclear and may simply be as a result of a greater proportion of non-specific endogenous antibodies produced following stimulation of the birds' immune system due to the use of IFA rather than just saline. Similar effects can be seen with age (Meulemans and Halen, 1982), as birds are continually challenged by numerous environmental pathogens birds increase the number and diversity of circulating endogenous antibodies, which results in a greater non-specific cross-reactivity (Tizard, 2002). In the current study, even in the saline immunised control treatment there is a small, progressive increase in optical density as time elapsed. Previously, Nobrega *et al.* (1996) also reported an age related increase in the production of non-specific mouse serum immunoglobulins reactive to several autoantigens.

IgM ELISA showed a much higher degree of non-specific binding than for IgY in both control Treatments 1 and 2. This was not an unexpected result, since IgM molecules

are pentameric with ten potential antigen binding sites and therefore the capacity for non-specific binding compared to IgY in much greater (Tizard, 2002). Previous research has demonstrated similar problems with IgM ELISA generating false positive results as a consequence of non-specific antigen binding (Liesenfeld *et al.*, 1997; Miyakawa *et al.*, 2001). In addition, some of this background activity may be accounted for by the unusually high affinity of avian immunoglobulins to plastic and polystyrene surfaces, and also the nature of the antigen coated in the plate wells (Bauer *et al.*, 1999). In current study, since antigens were extracted from whole crushed mites, it is likely that non-specific cross reactivity would result from impurities such as the presence of carbohydrates/glycoproteins or blood protein residues from feeding. Also, as suggested in Chapter 5, mites could potentially contain any number of pathogens which control birds may have been inadvertently exposed too, including Salmonella, *Mycobacterium*, smallpox, cholera spirochetes, amongst others (Zeman *et al.*, 1982; Durden and Turell, 1993; Chirico *et al.*, 2003). If pathogenic residues remain inside the mites and are subsequently immunised into hosts, then these may be potential sources of false positive results.

Several methods for reducing non-specific antibody binding have been suggested, including replacement of non-ionic detergents (Tween-20), with non-reactive protein blocking buffer (Bovine Serum Albumen) and also by increasing both primary and secondary antibody dilutions (Kenna *et al.*, 1985; Miyakawa *et al.*, 2001). However, preliminary ELISA validation tests described in Section 3.2 showed that changes to these parameters did not induce significant differences in optical density or reaction kinetics. The replacement of *Mycobacterium* containing CFA with IFA in the current study did, however, appear to reduce non-specific binding.

Irrespective of the degree of non-specific binding a significant increase in IgY levels ($P<0.001$) was seen in both PBS and urea treatments, compared to the controls. These same differences were present but not at the same magnitude with IgM ELISA, possibly due to the timing of the sampling in this study which may have missed the optimal IgM response, which is produced as early as 72 hours after immunisation, peaks between 4 and 8 days and declines thereafter (Tizard, 2002).

ELISA did not however show any quantitative differences between the level of IgY in the two antigen treatments and no clear-cut differences in IgM response. Therefore, it appeared that the differences in protein profiles seen when conducting preliminary SDS-PAGE analysis in Section 3.3 were not reflected in the humoral immune

response. Failure to generate significant antibody responses after immunisation with different arthropod fractions has been documented before (e.g. Lee *et al.*, 2002; Patarroyo *et al.*, 2002; Smith and Pettit, 2004). Previously studies which have been successful in displaying significant differences between the protective capacities of antigen fragments were performed using salivary glands and midgut sections, against whole extracts of ticks, mosquitoes and sandflies (Ingonga *et al.*, 1996; Foy *et al.*, 2003; Jittapalapong *et al.*, 2004). The inherent problem with red mite is their size, which is approximately 0.75 mm in length (Wood, 1917), compared to ticks which can range from 2-13 mm depending on their fed status (Stafford, 2004). Consequently this precluded using dissection to determine whether this approach to immunisation was a feasible proposition for red mite. Similar problems with size have been encountered with obtaining gut antigens from the sheep scab mite (Smith *et al.*, 2004). Therefore less direct methods were adopted for extracting antigens, bearing in mind the difficulty and expense in obtaining large enough quantities of clean mites as starting material. Thus the present study set about comparing two whole mite protein extractions. However, it is important to highlight that not using dissection was not necessarily a problem since previous reports have observed that, in fact, preparations of soluble mite proteins induced a greater level of protection than concealed antigens (Jayawardena *et al.*, 2000; Smith *et al.*, 2002).

PBS-extracted red mite proteins were therefore used since it was seen to significantly increase IgY levels and showed a trend towards the reduction of red mite survival in the previous trial (Chapter 6). Also, PBS-antigen extracts have previously been observed to show protective immunity against the sheep scab mite causing significant reductions ($P<0.01$) in populations (Nisbet and Huntley, 2006). However, PBS isolates only soluble antigens and lacks the ability to penetrate membrane proteins, such as the extracellular peritrophic matrix (Tellman *et al.*, 1999), which lines the gut lumen and has evoked protection in the blowfly (*Lucillia cuprina*) causing 50 % reductions in larval growth (Casu *et al.*, 1997). Such proteins can be solubilized using strong denaturants such as urea (Smith *et al.*, 2002). This was therefore the second extraction method used in the current study. However, since no significant increase in immune response was observed when using urea-extracted red mite antigens compared to PBS-extracted antigens, it appears that solubilization of epithelial membranes had no additive effect. Smith *et al.* (2002) made similar observations where whole *Psoroptes ovis* antigens extracted using urea showed no difference in IgG response and in fact showed poorer protection in comparison to saline antigen extracts. The reason for this is unclear, but it is possible that during the

extraction process the more aggressive disruption using urea alters the tertiary structure of the antigen, thus damaging or blocking potentially protective epitopes (Lee *et al.*, 2002).

7.4.3 Western blotting

Western blotting analysis substantiated findings from ELISA results, as there was a reduction in the degree of non-specific binding of control sera reported in the previous experiment (Chapter 6). In addition an increase in the number of bands observed between control and antigen treatments and also between serum pre- and post- immunisation was seen in this experiment. Pre-immunisation and control treatment serum was observed to generate some non-specific bands, which can be attributed to secondary anti-chicken antibodies binding directly to chicken blood antigens (Devaney and Augustine, 1987), as was described earlier (Chapter 6).

Serum recognised bands as a result of immunisation at 80, 55 and 18 kDa on blots using PBS-fractionated proteins and at 80, 60 and 20 kDa when using urea-fractionated antigens. The fact that control treatment birds did not generate bands reactive to protein fractions at these weights would indicate that antibody responses were targeted directly at poultry red mite antigens. The next step in this research would be to purify these proteins from the red mite in sufficient quantities and immunise birds with a higher concentration of these particular antigens. If this subsequently lowered survivability of mites, this would indicate that these antigens would be suitable as vaccine candidates (Minnifield *et al.*, 1993).

Western blotting also demonstrated the lack of IgY differentiation between fractionated proteins following immunisation with either PBS or Urea-extracted mite protein. As with the ELISA results it is perhaps not surprising that no obvious differences were seen between blots comparing antigen treatments, since previous research has observed the same effect where extraction using urea offered no additional immune response (Smith *et al.*, 2002).

7.4.4 In vitro *red mite feeding challenge*

Efficacy assessment of immunisation using an *in vitro* feeding device revealed no significant differences between treatments. It is possible that this is simply due to the absence of antigens capable of eliciting protective responses as previously discussed (see Chapter 6). Alternatively, it could be due to the failure of red mite to ingest a sufficient quantity of immunoglobulins to induce protection. It has been

documented that red mite feed relatively quickly and infrequently, with engorgement taking place for approximately 0.5 to 2 hours at intervals of 1 to 4 days, allowing the consumption of as little as 0.2 µl of blood (Sikes and Chamberlain, 1954; Chauve, 1998; DEFRA, 2001; Kilpinen, 2001). Previously a successful ectoparasite vaccine was developed against the African cattle tick (*Boophilus microplus*, Willadsen *et al.*, 1996) which have been estimated to consume as much as 15 ml blood whilst continuously attached to their host for 10 days or more (Nuttall, 1998). Red mite have been observed to consume IgY molecules during feeding, although the quantity and consequential effect on the mite is unknown (Nisbet *et al.*, 2006b).

Western blotting analysis identified several red mite antigens which elicited specific antibody responses. It may be that if these were to be isolated and re-injected into birds in a recombinant form, as suggested by Minnifield *et al.* (1993), then they might display protective properties. Employing similar methods, Trimnell *et al.* (2002) observed nearly 50 % reductions ($P<0.001$) in tick survival after immunisation with a specific tick cement protein. Also, Hartmann *et al.* (1997) induced immune responses in rodents causing more than 60 % reduction in nematode burdens when administering an allergenic actin-binding protein. Many other examples of protective immune response exist as a result of immunising with specific antigens. However, much of the current/future research aims at characterising antigens which share genetic homology to previously determined protective antigens in other parasitic species. One example of this is the protein tropomyosin, which has previously been shown to be present in house dust mite (Saarne *et al.*, 2003), sheep scab (Huntley *et al.*, 2004) and several species of shellfish (Shanti *et al.*, 1993) and has been observed to produce protective immunoglobulins in host species. More recently, a red mite tropomyosin homologue was identified and assessed for its immunogenic characteristics in poultry by Nisbet *et al.* (2006a). These authors found that natural red mite exposure was not sufficient to generate tropomyosin specific antibodies, indicating that hens are not directly exposed to this antigen. However, both western blotting and immunolocalization using dissected red mite antigens and serum raised specifically for tropomyosin in mice recognised tropomyosin antigens. This kind of approach is worthy of further exploration as molecules such as tropomyosin may represent candidates for the 'concealed antigen' approach to vaccination (Nisbet *et al.*, 2006a).

7.5 Conclusions

As with Chapter 6, a significant increase in IgY levels was observed after immunising birds with red mite extracts when compared to control treatments. Similarly, but to a lesser magnitude, the levels of circulating IgM were observed to be significantly higher in birds in the antigen treatment. However, ELISA did not demonstrate differences in IgY responses between birds immunised with different red mite antigen extracts.

It appeared that by replacing *Mycobacterium* containing CFA with IFA, that there was a reduction in the level of non-specific binding in both IgY specific ELISA and western blotting, allowing significant differences between treatments after just one immunisation.

Immunoblotting revealed several bands in the antigen treatments which were not observed in control animals. These antigens are worthy of further investigation since they appear to elicit immunoglobulin responses directly as a result of immunisation with red mite extracts. However, as for ELISA, immunising with different red mite antigen extracts did not result in differences in IgY responses to these fractionated antigens.

There was no difference between survival and fecundity of red mite observed between treatments after the *in vitro* feeding challenge, which posed several questions regarding the structural and protective qualities of the antigen, the volume of the blood ingested and the sensitivity of the assays used.

Chapter 8

General Discussion

8.1 Introduction

The aim of this thesis was to develop a vaccine against the poultry red mite (*Dermanyssus gallinae*). In order for this to be accomplished it was necessary to achieve a number of underlying milestones. This involved firstly undertaking a review of the literature to consider the biology of the red mite, assess current control practices and investigate the immune mechanisms of host species which have previously been exploited for development of other haematophagous ectoparasite vaccines. This was then followed by experiments to evaluate the relationship between red mite population, poultry production parameters and acaricide application, as well as the concurrent development of a series of immunological, proteomic and genetic laboratory techniques. These techniques included the optimisation of extraction of IgY from egg yolk and red mite antigens, followed by the validation of IgY-specific ELISA, SDS-PAGE and western blotting protocols. In addition *in vivo* and *in vitro* red mite feeding systems were designed as a means of testing the impact of natural red mite exposure on both immunological and productive parameters. Finally, these techniques were used in a pair of experiments to determine the effect of immunisation with red mite antigens on avian immune response and subsequent impact on survival and fecundity of red mite populations.

8.2 Poultry red mite population dynamics in relation to acaricide application

An experiment was designed to investigate the impact of several environmental parameters on red mite population dynamics, amongst which was the application of the commercially available acaricide Bendiocarb (Ficam® W, AgrEvo, Berlin) on a commercial free-range system. After repeated application of Bendiocarb, significant reductions in mite numbers were observed, to a point where the red mite population were almost completely eradicated. However, this chemical has only a limited sustainability since resistance to carbamate compounds has been repeatedly observed (Fiddes *et al.*, 2006). It has also been frequently seen that following acaricide application, the population of red mite soon returns to the level of parasitism observed prior to spraying, which has been attributed to insufficient

exposure of mites to the acaricide during application (Nordenfors and Höglund, 2000). In addition, as with many previously available and now withdrawn compounds, Bendiocarb has been linked to potential human/animal health issues (Zheng *et al.*, 2001). For these reasons, it can be argued that the application of acaricides is therefore only a temporary measure (Nordenfors and Höglund, 2000).

8.3 Estimation of the level of poultry red mite infestation using trap sampling

Research undertaken in experiment 1 to develop a means of monitoring red mite populations in laying hen housing systems illustrated the importance of trap placement. Previously, it has been suggested that red mite are sensitive to both genotype of the hen and physical positioning of traps within the house (Nordenfors and Höglund, 2000). Results from Section 4.2.3 support this and displayed unequal red mite distribution resulting from the design of the building, which has in turn been shown to have a significant effect on bird distribution within the house (Abrahamsson and Tauson, 1995). Red mite were found in greater abundance in the part of the house which permitted access to pasture, where birds were more likely to dwell upon re-entry into the house. This extensive variation in red mite distribution between trap positioning leads to problems associated with reproducibility of the data when estimating the red mite population in different houses/systems. In the current research, this often meant that significant differences or correlations between red mite and other parameters could not be established. It was therefore recommend that careful consideration is given to trap placement in order to obtain a more reliable estimate of red mite numbers.

8.4 Effect of the poultry red mite on egg production parameters

Part of this research programme focused on quantifying the effect of red mite on productive parameters, since it has previously been suggested that infestation with this parasite may cause a reduction in egg production and quality and an increase in mortality of the hens (Wojcik *et al.*, 2000; Cosoroaba, 2001). Therefore several separate laying units were sampled, representing free-range, barn and cage systems. Records were made at regular intervals of the population of red mite and hen production parameters, including mean egg output per bird, hen mortality, feed and water consumption and building temperature.

Relationships between red mite population and each one of these production variables were found at some point on one of the individual laying units, displaying both positive and negative correlations. However, few of these observations were consistent when all laying units were analysed as one dataset. By far the most predominant observation was the positive relationship between poultry red mite population and mortality of hens. As the red mite burden increased, there was a significant rise in bird mortality. Increases in hen mortality as a result of red mite-induced anaemia have previously been observed to be as high as 50 % (Wojcik *et al.*, 2000). An additional observation which showed some consistency across laying units was a positive relationship between red mite population and building temperature. Here an increase or decrease in building temperature had an equivalent effect on red mite population. These findings are consistent with previous investigations into the effect of temperature fluctuations on mite populations (Maurer and Baumgartner, 1994; Kilpinen, 2001; Kilpinen, 2004). However, it has been previously suggested that it is in fact the rate of change in temperature which has a greater effect on red mite, than temperature alone (Kilpinen, 2004).

Failure to demonstrate consistent relationships between production parameters and red mite population levels across all farms was again attributed to the high variability observed in the data collected, particularly with red mite numbers in traps. It was therefore suggested that in order to limit this variability more controlled experiments were necessary to eliminate the influence of external variables, as was previously achieved by Kilpinen (2005). This may, however, limit the application of results from such studies to the commercial situation of egg production, where multiple integrated variables exist.

In an attempt to establish the greatest determinant of variability between red mite population and production parameters in the current research, values which generated significant correlations were subject to regression analysis. It was revealed that actually many of the apparent significant associations between red mite populations and production data were affected by other external variables. In reality it was observed that parasitism by red mite was not generally the predominant predisposing factor effecting production, but this was in fact other production variables including. However, red mite parameters were often the second largest explanatory variable, particularly for hen mortality and building temperature. Therefore, it was concluded that whilst the population of red mite may not be the

exclusive driving force behind variation in the levels of egg production, they may serve to amplify existing trends and problems.

8.5 Effect of the poultry red mite on immunoglobulin production

Previous vaccines which have displayed protective immunity against ectoparasites often did so as a result of increased immunoglobulin production, particularly IgG (Minnifield *et al.*, 1993; Tarigan and Huntley, 2005; Nisbet and Huntley 2006). Therefore, considerable effort was directed at developing protocols to quantify levels of IgY (the avian equivalent to mammalian IgG) raised against the poultry red mite for both naturally and artificially exposed birds. This was achieved by the development of red mite specific ELISA and western blotting assays.

8.5.1 Natural red mite exposure

An ELISA assay was used to quantify the presence of naturally circulating red mite antibodies from birds exposed to varying degrees of red mite parasitism across a range of different commercial egg production systems. Prior to statistical analysis, examination of the data on a mean site level suggested a numerical association between the estimated population of red mite and levels of IgY, a trend previously documented by Lee *et al.* (2002). However, when performing correlation analysis between red mite populations and IgY levels using sampling data from all laying systems, a significant relationship was only found on one site. The same problem was described by Maurer (1993), who found that antibody response did not appear to be linked to red mite infestation levels. In the current study the reasons for this lack of relationship were once again attributed to the large variation observed, not only in the population of red mite in traps, but also in IgY levels. In general, relationships between red mite population and IgY levels which have been reported in the literature followed repeated sampling of the same animals, thus reducing sampling variation (Lee *et al.*, 2002; Pruett *et al.*, 2006). However, in the present study, IgY was collected from commercial laying systems which meant that successive samples could not be collect from the same individual bird, thus resulting in further variability.

Sampling commercial laying systems also gave the opportunity to obtain both serum and yolk IgY to evaluate the relationship between these two sources. It has previously been documented that yolk IgY can be used as a tool to determine serum immunoglobulin levels in response to exposure to particular antigens (Woolley and Landen, 1995). However, in the current research program this was not found to be

true since correlations between yolk and serum were absent. In fact significantly higher IgY levels were observed in yolk samples. One possible explanation for this was the deposition of yolk IgY over a 21 day period, whereas serum antibody levels are representative of the level of circulating antibodies at any given time point (Mohammed *et al.*, 1986). When correcting data for this delay in the current research an apparent relationship was seen between the two IgY sources, although this was not significant. However, as before, samples were collected on a mean flock basis, not from repeat samples on the same individual bird, therefore the variability between samples was high. Irrespective of this variation, it does raise the question whether indeed yolk-derived IgY is a reliable predictor of serum IgY levels, even though it reduces the need for procedures to be undertaken on birds.

8.5.2 Immunisation with red mite antigens

Despite the lack of direct correlation between red mite population numbers and IgY response, monitoring of commercial flocks did demonstrate the ability of birds to elicit varying degrees of immune response to infestation by the poultry red mite. This provided justification to progress with the immunisation of birds with red mite antigen extracts. In the first immunisation experiment, birds were initially immunised with a phosphate buffered saline (PBS) red mite protein extract using Complete Freund's adjuvant (CFA), subsequently replaced with Incomplete Freund's adjuvant (IFA) in the second and third immunisations. This was compared to a control treatment which was immunised with PBS and CFA and subsequently IFA.

Analysis by ELISA revealed that antigen immunised birds produced significantly higher IgY levels when compared to the controls. However, there was also an increase in IgY in control birds over the course of the study, which after western blotting analysis was seen to give the same degree of specific red mite protein binding as the antigen birds. A number of potential explanations could be given for this lack of difference in specific binding, including the increased level of endogenous antibodies with age and exposure to environmental pathogens. An alternative reason was that there had been maternal transfer of immunoglobulins or the presence of serum anti-chicken IgY antibodies equivalent to RF and HAMA, found in humans. However, after analysis by PCR and RT-PCR using gene specific primers, it was suggested that this non-specific binding could be largely be attributed to the presence of *Mycobacterium* which was found to be present not only in CFA but also in red mite. A similar situation was described by Hou *et al.* (2006), who reported the presence of antigen specific IgG in both control and house dust mite

(Dermatophagoides farinae) infested dogs. Hou *et al.* (2006) suggested that the reason for this apparent non-specific antigen recognition in control animals was due to IgG responses being directed towards environmental proteins or allergens similar to, but not those of *D. farinae*, which both control and mite infested animals had been exposed too.

After highlighting this homology between CFA and red mite extracts, a second immunisation experiment was carried out. This time the *Mycobacterium*-containing CFA was replaced with IFA, which had a positive effect in reducing non-specific binding, since even after the first immunisation with red mite antigens, a significant increase in IgY levels were observed compared to the control group. A degree of non-specific binding was still apparent, but this may have been due either to homology of red mite with other environmental antigens (Hou *et al.*, 2006) or circulating host endogenous antibodies which are capable of mimicking other antigens/antibodies (Wingren *et al.*, 1995). Western blotting analysis of sera from antigen immunised birds also confirmed a reduction in the degree of non-specific binding. In addition, blotting revealed several fractionated proteins which were recognised only in groups receiving red mite antigen and not controls. These proteins require characterisation in order to determine their potential as vaccine candidates and justify their use in further research.

8.5.3 Immunisation with different poultry red mite extracts

An additional aim of the second immunisation experiment was to determine the effect of immunisation on immune response using different poultry red mite extracts. Two extraction buffers were used, firstly PBS which targets only soluble proteins (Huntley *et al.*, 2004), and secondly urea, which has the capacity to penetrate and release membrane bound proteins (Tellman *et al.*, 1999). However, no significant differences in the level of IgY were found between the two extraction methods and only minor, though significant differences in the level of IgM. Therefore, it was suggested that solubilization with urea offered little additive effect, as observed previously by Smith *et al.* (2002). Western blot analysis confirmed these observations, with no difference in the array of fractionated proteins being recognised by serum from either PBS- or urea-antigen immunised birds.

8.5.4 Immunoglobulin-M response

In addition to promoting an IgY response, several studies have reported increases in circulation of other antibody serotypes, such as IgM, after exposure to arthropod

antigens (Nisbet and Huntley, 2006). IgM has a lifespan of only several days (Tizard, 2002) but despite this is capable of producing protective immunity recognizing different epitopes to IgY and also utilizing alternative mechanisms for the elimination of parasites (Ligas *et al.*, 2003). Therefore, an ELISA was used to determine the specific IgM response to immunisation with red mite extracts. Significantly higher levels of IgM were found in the two antigen treatments when compared to controls, although these were not of the same magnitude as for IgY. This was perhaps due to the timing of the post-immunisation sampling in this study which may have missed the optimal IgM response, said to occur around 4-8 days after immunisation and declining thereafter (Tizard, 2002). Also, the pentameric structure of IgM molecules, providing 10 potential binding sites, leads to a higher degree of non-specific binding (Tizard, 2002) which may also determine why clear differences between treatments were not apparent.

8.6 Effect of the poultry red mite on cytokine expression

In the assessment of the effect of exposure to red mite antigens, either naturally or through immunisation, on the host's immune system, this study also evaluated the expression of certain classical Th1- and Th2-type cytokines. Th1-type responses were determined by screening RNA from spleen samples for the cytokines IL12α and IFNγ, which are typically associated with the cellular immune response. Th2-type cytokine production on the other hand was assessed by levels of expressed IL-4, IL-13, IL-10 and IL-5, which are typically associated with humoral immune responses.

8.6.1 Effect of natural red mite exposure on cytokine expression

Using samples collected from commercial poultry farms, it was found that natural exposure to red mite did not produce any significant correlations with the levels of cytokines expressed. Despite this, there were both numeric and statistically significant associations between IgY and cytokine levels. However, even these results lacked consistency. For example, IL-4 was found to be up-regulated on some farms and down-regulated on others. Although not significant, there was also some suggestion of a relationship between IgY levels and IL-5 and IL-12α expression, which would be rational since IL-5 is involved in increasing B-cell and immunoglobulin secretion (Harriman *et al.*, 1988), whilst IL-12α is important in resistance against pathogens (Trinchieri, 2003). However, these correlations were not significant, a result of either failure to stimulate the immune system to elicit a

cytokine response to red mite antigens (Cross *et al.*, 1994) or alternatively due to the large variability observed within parameters.

8.6.2 Effect of immunisation with red mite extracts on cytokine expression

Immunisation with red mite extracts gave rise to significant differences in cytokine expression between treatments. These differences were seen between cytokines associated with the Th2-type response, but not for cytokines associated with the Th1-type. The possible explanation for this was attributed to the significantly higher levels of IL-10 found in the antigen treatment. IL-10 is known as the cytokine synthesis inhibitory factor (CSIF) and is capable of suppressing expression of other cytokines, in particular those of the Th1-type (Waal Malefyt *et al.*, 1991). Therefore, it would appear that an increase in IL-10 production was induced by a fragment of the immunised red mite antigen, which in turn reduced Th1-type immunity as indicated by levels of IFNγ.

Significant differences were also observed for IL-4 and IL-5, although surprisingly antigen immunised birds had lower levels. It was suggested that perhaps the immunised red mite antigen extracts contained an element which had an immunosuppressive ability, such as molecules found in the saliva (Schoeler and Wikel, 2001). However, it has been frequently documented that arthropod components typically act by up-regulating cytokines associated with Th2-type response, whilst driving down Th1-type response (Schoeler and Wikel, 2001). This was not completely true in the present research as Th2-type cytokine expression was lower in the antigen treatment, although IgY production, which is a facet of the Th2-type response, showed the opposite effect. It is difficult to draw further conclusions about cytokine expression without conducting more refined experiments which solely target changes to cytokine response. It was perhaps true of the current study that only limited differences in cytokine expression were observed due to the locality and timing of the response (Zeidner *et al.*, 1999).

8.7 Efficacy of poultry red mite immunisations

In order to determine the effects of immunisation with red mite antigen on the survival and fecundity of live red mite, an *in vitro* feeding system was developed. In the first immunisation experiment the *in vitro* system was compared against an *in vivo* feeding system and since few significant differences were observed for red mite

survival and fecundity parameters between the two systems, the *in vitro* system alone was used for the second immunisation experiment.

Immunisation with poultry red mite extracts did not have any significant effect on the survival or fecundity of red mite in either *in vivo* or *in vitro* feeding system. Although in the first immunisation experiment mite mortality was greater in the antigen treatment, this difference was not significant from the control treatment. Similarly, there was no evidence of protection against parasitism by the red mite following immunisation in the second experiment. There are several explanations for this. Firstly, it has been suggested that red mite are able to consume IgY (Nisbet *et al.*, 2006a), but the question remains whether IgY can cross the gut epithelium without being destroyed. However, even if the IgY is fragmented in the gut, protection can still be induced provided that the relative *F(ab')* and *Fc* antibody fragments remain intact (Silver *et al.*, 2002). Perhaps an important question is whether red mite are able to ingest sufficient quantities of IgY to provide protection to the bird (Sikes and Chamberlain 1954).

Another possible explanation for the failure to generate protection could be that the antigen treatment elicits the wrong type of immune response, towards the Th2-type rather than the Th1-type response (Nisbet and Huntley, 2006). However, the most likely explanation for the inability to induce protection that has been suggested concerns the antigenic structure. It is possible that protective antigen epitopes are denatured during the extraction procedure (Tarigan and Huntley, 2005) causing damage to their tertiary structure (Lee *et al.*, 2002). Tellman *et al.* (2001) emphasised the importance of antigenic structure when inoculating sheep with the glycoprotein, peritrophin-95, isolated from blowfly *(Lucilia cuprina)* larval peritrophic matrix. They discovered that immunisation with the native protein induced a strong, protective IgG response causing a 50 % reduction in larval growth, whereas immunisation with recombinant forms of the proteins raised in bacteria and baculovirus-infected insect cells generated no significant reductions in larval growth. Tellman *et al.* (2001) later showed that these recombinant proteins were not glycosylated and were incorrectly folded. Thus Tellman *et al.* (2001) concluded that the oligosaccharides attached to native peritrophin and its unique polypeptide structure were essential for the induction of larval growth inhibitory activity in the sera of sheep vaccinated with this antigen. Therefore, it is essential for future isolation and characterisation of potential protective red mite antigens that all of these suggestions are taken into consideration.

8.8 Future poultry red mite research

As with other ectoparasite vaccines, the development of a vaccine against the poultry red mite is in its infancy. According to Nisbet and Huntley (2006), the approaches used thus far in developing anti-parasitic vaccines across most species have been met with little or no success. These authors maintain that despite more than 20 years of research into the development of ectoparasitic vaccines, using ever increasingly sophisticated biochemical and molecular tools, TickGARD™ (Intervet, Australia) and Gavacv™ (Heber Biotec, Cuba) vaccines against the tropical cattle tick *Boophilus microplus,* remain the only commercially available ectoparasitic vaccines. Following this success, strategies adopted for the development of vaccines against ectoparasites have largely employed methods based on these two vaccines (Willadsen *et al.,* 1996). The approach used against *Boophilus microplus* is based around immunising hosts with gut protein fragments which induce immunoglobulin production. As the parasite subsequently engorges, it ingests host antibodies as part of a large blood-meal which target antigenic sites in the gut and result in death. Research on development of vaccines in alternative ectoparasitic species has had to make the assumption that they display physiological similarity with the tropical cattle tick and also share similar protective traits. However, these assumptions may not be valid, which may then partly explain why progress with alternative haematophagous arthropod vaccines has been slow. Not only this, but direct assumptions have been made regarding the avian immune pathways, with parallels being drawn from mammalian species (Sam-Sun *et al.,* 2002; McDevitt *et al.,* 2006b). Care must be taken when making such assumptions, since the avian immune system displays evolutionary differences to the mammalian equivalent, such as the presence of IgY, rather than IgG or IgE (Tizzard, 2002; Karlsson *et al.,* 2005). Success in developing alternative ectoparasitic vaccines will depend upon a better understanding of their digestive immunology and its critical protective antigens (Raynard *et al.,* 2002).

The current research, like much of that previously published, has focused on identification of the mechanisms of protection operating in naturally-acquired immunity and comparing these to the mechanisms functioning after vaccination with native proteins. This may subsequently offer direction to the selection of isolated protective antigens for use in vaccine development against ectoparasites (Nisbet and Huntley, 2006). An alternative and more recent approach is to use genetic technologies, more specifically the informed identification of antigens by investigating

genetic data using immunoscreening and/or expressed sequence tags (EST's) (Schoeler and Wikel, 2001).

8.8.1 Complementary DNA libraries and immunoscreening

Both immunoscreening and EST's rely on information from complementary DNA (cDNA) libraries, which represent the data encoded in the mRNA of a specific organism. However, since RNA molecules in their natural form are very unstable, the encoded information is converted in to stable double-stranded cDNA and subsequently used for screening applications (Ljunggren, 2005). Immunoscreening itself is a technique where antibodies are used to screen a cDNA library for identification of polypeptides with antigenic properties (Ljunggren, 2005). This method can be used to identify molecules at the host-parasite interface, with the potential of discovering candidate antigens for use as vaccine components. Screening of cDNA in this fashion has led to the identification and characterisation of antigenic proteins from a range of parasites, including *Sarcoptes scabiei* antigens -1 and -2 (Ssag1 and Ssag2, Harumal *et al.,* 2003), *Psoroptes ovis* (Nisbet and Huntley, 2006) and *Dermatophagoides farinae* (Fujikawa *et al.,* 1996). However, the function of immunoscreening is restricted, since it can only recognize naturally exposed and not 'concealed' antigens.

8.8.2 Expressed sequence tags

Unlike immunoscreening, EST technology can be used to screen cDNA for both natural and/or concealed antigens. First reported by Adams *et al.* (1991), EST's are partial nucleic-acid sequences (300-500 bp) derived from cDNA generated from the mRNA present in the tissue of interest (Ljunggren, 2005). Since EST's are generated from cDNA, they represent expressed and thus frequently utilized genes. EST's can be generated on a relatively large scale. A recent estimate suggested that up to 2,000 EST's could be produced by a single researcher per month (Whitton *et al.,* 2004). The use of EST's is dependant upon information entered into public databases, which is subsequently used to compare gene sequences and is the fastest growing division of this type of research (Schoeler and Wikel, 2001). However, since this is a relatively novel approach in arthropod vaccination strategies, the number of EST's available in this area is limited. Nonetheless, as interest in this technology grows so does the database and to date includes sequence data for several blood feeding arthropods including mites such as *P. ovis* and *S. scabiei* (Kenyon *et al.,* 2003; Ljunggren *et al.,* 2003), ticks such as *B. microplus, R. appendiculatus* and *D. variabilis* (Nene *et al.,* 2004; Simser *et al.,* 2004; Guerrero *et*

al., 2005), mosquitoes, *Aedes aegypti* and *Anopheles gambiae* (Dimopoulos *et al.,* 2000; Sanders *et al.,* 2003), as well as several other parasites, worms and protazoans (Ljunggren, 2005). As a consequence of the limited repertoire of heamatophagous ectoparasite EST's, unique genes might not be encountered in public databases. However, there are currently several protective antigens which have shown genetic homology across a number of parasitic species as a result of EST technology, examples of these include tropomyosin, peritrophic matrix (PM) proteins and glutathione S-transferase (GST) (Hartman *et al.,* 1997; Wang and Granados, 1997; Pettersson *et al.,* 2005).

8.8.3 Exploiting recombinant genetic technology in poultry red mite research

It would be logical that the next step in developing a vaccine against the poultry red mite would be firstly to construct a comprehensive cDNA library similar to that created for other mite species (Ljunggren, 2005; Nisbet and Huntley, 2006). This could subsequently be screened in the EST database (dbEST, GenBank) in order to confer sequence homology to other EST's. Alternatively, cDNA could be probed using gene specific primers to previously submitted protective parasitic antigens, such as tropomyosin, PM proteins or GST's to determine homology. The next step in this research programme would depend on the outcome of this genetic screening. If homology was observed then a decision might be made to immunise birds with specific recombinant DNA vaccines. In the absence of EST homology, an alternative approach would be required such as micro-dissection and subsequent immunisation of known mite fractions.

8.9 Summary and conclusions

- In conclusion, this research programme has demonstrated that the application of acaricides was an effective short-term control for the poultry red mite. However, multiple reports of red mite resistance to acaricides, as well as the rapid recovery of red mite after application and concerns over acaricide toxicity to hens' means that this method is not sustainable and alternative methods must be sought.

- A number of significant correlations were observed between red mite populations and production parameters on individual laying sites, including egg output, feed intake and water consumption. However, red mite populations were observed to have the strongest relationships with hen

mortality and building temperature. These experiments also highlighted the large variation in red mite distribution throughout laying systems, which was attributed to building design and hen genotype.

- Natural exposure of laying hens to red mite populations was rarely significantly correlated with IgY levels, the high degree of variation seen for both of these parameters was thought to be the cause of this. Similarly, natural red mite infestation showed no significant relationship with cytokine expression, although the cytokine IL-4 was negatively correlated to serum IgY indicating immunosupression

- Immunisation experiments evaluated the IgY and cytokine responses of hens immunised with red mite antigen extracts and also assessed the effect that this had on the survival and fecundity of red mite using *in vivo* and *in vitro* feeding systems. In the first immunisation experiment a significant increase in IgY levels was seen in antigen immunised birds. However, a high degree of non-specific antibody recognition of red mite antigens was found in controls and later attributed to the presence of *Mycobacterium* in CFA which shared direct homology with that of red mite extracts. Significantly higher levels of the cytokine IL-10 were found in the antigen treatment which was thought to be responsible for the significant inhibition observed in IL-4 and IL-5. Both, *in vivo* and *in vitro* feeding systems showed no significant difference in survival or fecundity of red mite between treatments or between feeding systems suggesting that no protection was induced in hens following immunisation.

- The second immunisation trial eliminated the confounding effect of *Mycobacterium* in the adjuvant and demonstrated significant increases in the levels of IgY and IgM. However, no significant difference in antibody response was observed between the different red mite antigen extracts. However, as with the first immunisation experiment there were no significant effects of treatment on survival or fecundity of red mite, once again confirming that immunisation with red mite antigens did not elicit a protective response. Further research therefore is needed to identify a suitable antigen which does elicit protection to laying hens from predation by the poultry red mite.

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Appendix I

Table 1 *Stepwise regression of factors affecting production parameters on site 1.*

Response	Predictor	Significance	r^2
Mortality cumulative (%)	Age(Weeks)	***	98.44
Total:			98.44
Feed intake (g/b/d)	Water consumption	**	37.21
Total:			37.21
Water consumption	% Lay	**	42.68
	Fed Adult and Nymph	NS	10.10
Total:			52.78
Temperature (°C)	Mortality cumulative (%)	*	30.75
Total:			30.75
Yolk O.D.	Mortality cumulative (%)	*	29.99
Total:			29.99

In this and subsequent tables values with different superscripts are significantly different at *(P<0.05) **(P<0.01) ***(P<0.001) NS: no significant difference

Table 2 *Stepwise regression of factors affecting mite populations on site 1.*

Response	Predictor	Significance	r^2
Total Mite Popln	Fed Adult and Nymph	***	91.22
Total:			91.22
Fed Adult and Nymph	Total Mite Popln	***	91.22
Total:			91.22
Unfed Adult and Nymph	% Lay	*	21.17
Total:			21.17
Larvae No.	Mite egg No.	**	50.99
Total:			50.99
Mite egg No.	Larvae No.	**	50.99
Total:			50.99

Table 3 *Stepwise regression of factors affecting production parameters on site 2.*

Response	Predictor	Significance	r^2
Mortality cumulative (%)	Age(Weeks)	***	99.31
Total:			99.31

Table 4 *Stepwise regression of factors affecting mite populations on site 2.*

Response	Predictor	Significance	r^2
Total Mite Popln	Mite egg No.	***	96.7
	Larvae No.	***	2.21
	Fed Adult and Nymph	***	0.96
	Unfed Adult and Nymph	***	0.13
Total:			100.00
Fed Adult and Nymph	Mite egg No.	***	84.87
Total:			84.87
Unfed Adult and Nymph	Larvae No.	***	73.41
Total:			73.41
Larvae No.	Total Mite Popln	***	76.71
	Fed Adult and Nymph	**	14.14
	Mite egg No.	***	7.80
	Unfed Adult and Nymph	***	1.35
Total:			100.00
Mite egg No.	Total Mite Popln	***	96.7
	Larvae No.	**	1.58
	Fed Adult and Nymph	***	1.21
	Unfed Adult and Nymph	***	0.51
Total:			100.00

Table 5 *Stepwise regression of factors affecting production parameters on site 3.*

Response	Predictor	Significance	r^2
Mortality cumulative (%)	Age(Weeks)	***	96.22
Total:			96.22
% Lay	Water consumption	**	45.76
Total:			45.76
Feed intake (g/b/d)	Larvae No.	*	41.53
Total:			41.53
Water consumption	% Lay	**	45.76
	Unfed Adult and Nymph	*	13.90
Total:			59.66

Table 6 *Stepwise regression of factors affecting mite populations on site 3.*

Response	Predictor	Significance	r^2
Total Mite Popln	Fed Adult and Nymph	***	84.94
	Larvae No.	**	8.67
	Mite egg No.	***	5.60
Total:			99.21
Fed Adult and Nymph	Total Mite Popln	***	84.94
	Larvae No.	NS	3.82
	Mite egg No.	**	5.90
Total:			94.66
Unfed Adult and Nymph	Water consumption	*	27.92
Total:			27.92
Larvae No.	Total Mite Popln	***	62.04
	Fed Adult and Nymph	NS	9.64
Total:			71.68
Mite egg No.	Total Mite Popln	***	69.29
Total:			69.29

Table 7 *Stepwise regression of factors affecting production parameters on site 4*

Response	Predictor	Significance	r^2
Water consumption	Age(Weeks)	***	99.97
Total:			99.97
Yolk O.D.	Total Mite Popln	*	48.46
Total:			48.46

Table 8 *Stepwise regression of factors affecting mite populations on site 4.*

Response	Predictor	Significance	r^2
Total Mite Popln	Larvae No.	***	98.4
	Unfed Adult and Nymph	NS	0.53
	Fed Adult and Nymph	**	0.88
	Mite egg No.	***	0.19
Total:			100.00
Fed Adult and Nymph	Larvae No.	***	86.02
Total:			86.02
Unfed Adult and Nymph	Total Mite Popln	**	79.71
	Water consumption	***	19.43
	Fed Adult and Nymph	*	0.60
	Mite egg No.	**	0.23
	Larvae No.	***	0.03
Total:			100.00
Larvae No.	Total Mite Popln	***	98.40
	Mite egg No.	*	0.66
Total:			99.06
Mite egg No.	Age(Weeks)	***	90.75
	Yolk O.D.	NS	4.29
	Fed Adult and Nymph	NS	1.78
	Total Mite Popln	NS	1.72
	Unfed Adult and Nymph	NS	0.97
	Larvae No.	NS	0.15
Total:			99.66

Table 9 *Stepwise regression of factors affecting production parameters on site 5.*

Response	Predictor	Significance	r^2
Mortality cumulative (%)	Unfed Adult and Nymph	***	85.39
	Age(Weeks)	***	13.58
	Total Mite Popln	***	0.76
	Serum O.D.	NS	0.10
	Mite egg No.	*	0.12
Total:			99.95
Yolk O.D.	Age(Weeks)	**	58.37
Total:			58.37
Serum O.D.	Age(Weeks)	**	69.64
Total:			69.64

Table 10 *Stepwise regression of factors affecting mite populations on site 5.*

Response	Predictor	Significance	r^2
Total Mite Popln	Mite egg No.	***	96.25
	Larvae No.	***	3.18
	Unfed Adult and Nymph	**	0.38
	Fed Adult and Nymph	***	0.19
Total:			100.00
Fed Adult and Nymph	Total Mite Popln	***	92.58
	Mortality cumulative (%)	NS	1.83
	Mite egg No.	**	4.36
	Yolk O.D.	NS	0.06
Total:			98.83
Unfed Adult and Nymph	Mortality cumulative (%)	***	85.39
	Age(Weeks)	***	12.11
	Total Mite Popln	**	1.95
Total:			99.45
Larvae No.	Fed Adult and Nymph	**	67.06
Total:			67.06
Mite egg No.	Total Mite Popln	***	96.25
	Larvae No.	**	2.86
	Age(Weeks)	*	0.53
Total:			99.64

Table 11 *Stepwise regression of factors affecting production parameters on site 6.*

Response	Predictor	Significance	r^2
Mortality cumulative (%)	Age(Weeks)	***	88.99
	Larvae No.	**	6.77
	% Lay	*	1.57
	Mite egg No.	NS	0.76
Total:			98.09
% Lay	Temperature (°C)	**	64.94
Total:			64.94
Temperature (°C)	% Lay	**	64.94
	Mortality cumulative (%)	NS	8.50
Total:			73.44
Serum O.D.	Temperature (°C)	*	34.57
Total:			34.57

Table 12 *Stepwise regression of factors affecting mite populations on site 6.*

Response	Predictor	Significance	r^2
Total Mite Popln	Fed Adult and Nymph	***	89.1
	Larvae No.	***	9.40
	Mite egg No.	***	1.48
	Unfed Adult and Nymph	***	0.02
Total:			100.00
Fed Adult and Nymph	Total Mite Popln	***	89.10
	Larvae No.	**	6.85
	Mite egg No.	***	3.99
	Unfed Adult and Nymph	***	0.06
Total:			100.00
Unfed Adult and Nymph	Larvae No.	**	50.18
Total:			50.18
Larvae No.	Total Mite Popln	***	83.87
	Fed Adult and Nymph	**	10.14
	Mite egg No.	***	5.78
	Unfed Adult and Nymph	***	0.21
Total:			100.00
Mite egg No.	Total Mite Popln	***	86.55
	Fed Adult and Nymph	**	7.54
	Larvae No.	***	5.70
Total:			99.79

Table 13 *Stepwise regression of factors affecting production parameters on site 7.*

Response	Predictor	Significance	r^2
Mortality cumulative (%)	Age(Weeks)	***	97.57
Total:			97.57
Temperature (°C)	Age(Weeks)	*	53.43
Total:			53.43

Table 14 *Stepwise regression of factors affecting mite populations on site 7.*

Response	Predictor	Significance	r^2
Total Mite Popln	Fed Adult and Nymph	***	95.78
	Mite egg No.	***	3.16
	Larvae No.	***	1.02
Total:			99.96
Fed Adult and Nymph	Total Mite Popln	***	95.78
	Mite egg No.	**	2.44
	Larvae No.	***	1.67
Total:			99.89
Larvae No.	Total Mite Popln	***	80.38
Total:			80.38
Mite egg No.	Total Mite Popln	***	75.92
	Fed Adult and Nymph	**	13.91
	Larvae No.	***	9.59
Total:			99.42

Table 15 *Stepwise regression of factors affecting production parameters on all sites for yolk IgY.*

Response	Predictor	Significance	r^2
Mortality cumulative (%)	Age(Weeks)	***	43.28
	Unfed Adult and Nymph	***	13.28
Total:			56.56

Table 16 *Stepwise regression of factors affecting mite populations on all sites for yolk IgY.*

Response	Predictor	Significance	r^2
Total Mite Popln	Mite egg No.	***	94.67
	Larvae No.	***	3.60
	Fed Adult and Nymph	***	0.93
	Unfed Adult and Nymph	***	0.80
Total:			100.00
Fed Adult and Nymph	Total Mite Popln	***	90.80
	Unfed Adult and Nymph	***	5.12
	Mite egg No.	***	2.22
	Larvae No.	***	1.86
Total:			100.00
Unfed Adult and Nymph	Total Mite Popln	***	56.97
	Fed Adult and Nymph	***	19.57
	Larvae No.	***	10.99
	Mite egg No.	***	12.47
Total:			100.00
Larvae No.	Total Mite Popln	***	85.06
	Mite egg No.	***	5.48
	Fed Adult and Nymph	***	1.86
	Unfed Adult and Nymph	***	7.60
Total:			100.00
Mite egg No.	Total Mite Popln	***	94.67
	Larvae No.	***	1.95
	Fed Adult and Nymph	**	0.58
	Unfed Adult and Nymph	***	2.80
Total:			100.00

Table 17 *Stepwise regression of factors affecting production parameters on all sites for serum IgY.*

Response	Predictor	Significance	r^2
Mortality cumulative (%)	Age(Weeks)	***	75.36
	Temperature (°C)	***	20.17
Total:			95.53
Temperature (°C)	Mortality cumulative (%)	***	55.69
	Serum O.D.	NS	5.08
Total:			60.77
Serum O.D.	Mortality cumulative (%)	**	33.14
Total:			33.14

Table 18 *Stepwise regression of factors affecting mite populations on all sites for serum IgY.*

Response	Predictor	Significance	r^2
Total Mite Popln	Fed Adult and Nymph	***	92.08
	Mite egg No.	***	5.74
	Larvae No.	***	1.58
Total:			99.40
Fed Adult and Nymph	Total Mite Popln	***	92.08
	Mite egg No.	***	3.13
	Larvae No.	***	3.00
Total:			98.21
Unfed Adult and Nymph	Mortality cumulative (%)	***	29.47
Total:			29.47
Larvae No.	Total Mite Popln	***	72.99
	Fed Adult and Nymph	***	8.55
	Mite egg No.	***	8.02
	Unfed Adult and Nymph	***	10.44
Total:			100.00
Mite egg No.	Total Mite Popln	***	84.11
	Fed Adult and Nymph	***	6.28
	Larvae No.	***	4.17
	Unfed Adult and Nymph	***	5.44
Total:			100.00

Table 19 *Stepwise regression of factors affecting production parameters on all sites.*

Response	Predictor	Significance	r^2
Mortality cumulative (%)	Age(Weeks)	***	46.86
	Unfed Adult and Nymph	***	8.21
Total:			55.07
Feed intake (g/b/d)	Temperature (°C)	*	19.74
Total:			19.74
Temperature (°C)	Unfed Adult and Nymph	***	40.36
Total:			40.36

Table 20 *Stepwise regression of factors affecting mite populations on all sites.*

Response	Predictor	Significance	r^2
Total Mite Popln	Fed Adult and Nymph	***	93.71
	Mite egg No.	***	4.83
	Larvae No.	***	1.15
	Unfed Adult and Nymph	***	0.31
Total:			100.00
Fed Adult and Nymph	Total Mite Popln	***	85.64
	Unfed Adult and Nymph	***	10.24
	Mite egg No.	***	2.61
	Larvae No.	***	1.51
Total:			100.00
Unfed Adult and Nymph	Larvae No.	***	23.42
	Mortality cumulative (%)	**	8.72
Total:			32.14
Larvae No.	Total Mite Popln	***	82.14
	Fed Adult and Nymph	***	7.13
	Mite egg No.	***	5.50
	Unfed Adult and Nymph	***	5.23
Total:			100.00
Mite egg No.	Total Mite Popln	***	89.21
	Fed Adult and Nymph	***	4.62
	Larvae No.	***	3.12
	Unfed Adult and Nymph	***	3.05
Total:			100.00

Appendix II

Table 21 *Validation of capture antigen between antigen treatments.*

Bird No.	PBS antigen coated		Urea antigen coated	
	PBS antigen immunised	Urea antigen immunised	PBS antigen immunised	Urea antigen immunised
1	0.93	0.65	0.77	0.89
2	0.71	0.64	0.68	0.71
3	0.92	0.64	0.66	0.68
4	0.79	0.82	0.84	0.86
5	0.41	0.60	0.49	0.38
6	0.50	0.61	0.61	0.62
7	0.87	0.67	0.66	0.66
8	0.37	0.34	0.31	0.37
9	0.31	0.36	0.31	0.36
10	0.47	0.73	0.69	0.64
11	0.43	0.52	0.62	0.71
12	0.39	0.59	0.59	0.59
13	0.71	0.77	0.82	0.88
14	0.54	0.59	0.65	0.70
15	0.47	0.45	0.44	0.42
16	0.40	0.45	0.50	0.55
17	0.44	0.51	0.57	0.63
18	0.56	0.52	0.49	0.46
19	0.72	0.76	0.79	0.82
20	0.52	0.71	0.60	0.49
Mean:	**0.57**	**0.60**	**0.60**	**0.62**
STDEV:	**0.193**	**0.131**	**1.131**	**2.131**

NB: Validation was performed using serum from birds immunised three times with either PBS or Urea-extracted antigens, as described in Chapter 7.

Table 22 *Validation of capture antigen between antigen treatments.*

Bird No.	PBS coated		Urea coated		Significance
	PBS immunised	Urea immunised	PBS immunised	Urea immunised	
Mean:	**0.57**	**0.60**	**0.60**	**0.62**	NS
STDEV:	**0.193**	**0.131**	**1.131**	**2.131**	-

Table 23 *Effect of treatment on mean weekly bodyweight of birds, corrected for differences in initial starting weights*

Age (Weeks)	Treat. 1	Treat. 2	Treat. 3	Treat. 4	S.E. Mean	Significance
5	338.5	333.9	375.9	345.6	6.25	NS
6	419.7^{a}	416.4^{ab}	425.4^{ab}	383.2^{b}	7.81	*
adjusted	*361.1^{a}*	*357.5^{ab}*	*330.4^{ab}*	*307.6^{b}*	*4.49*	*
7	511.7^{a}	525.1^{ac}	566.5^{ac}	520.3^{c}	9.79	*
adjusted	*453.2^{a}*	*456.4^{ac}*	*451.0^{ac}*	*430.4^{c}*	*6.99*	*
8	629.1^{ab}	632.5^{ab}	683.9^{a}	637.6^{b}	10.39	*
adjusted	*547.3^{ab}*	*556.1^{ab}*	*557.8^{a}*	*527.0^{b}*	*8.65*	*
9	777.0^{ab}	785.9^{a}	823.1^{a}	781.2^{b}	14.44	*
adjusted	*667.4^{ab}*	*683.8^{a}*	*674.1^{a}*	*636.2^{b}*	*11.65*	*
10	909.0	896.7	957.1	892.1	14.99	NS
adjusted	*776.2*	*781.7*	*786.9*	*760.6*	*46.85*	*NS*
11	1015.4^{ab}	1008.9^{ab}	1102.2^{a}	1004.0^{b}	15.79	*
adjusted	*887.6^{ab}*	*897.0^{ab}*	*910.6^{a}*	*866.4^{b}*	*13.4*	*NS*
12	1107.9	1122.9	1220.6	1207.1	27.58	NS
adjusted	*1009.7*	*978.1*	*1037.0*	*972.8*	*27.32*	*NS*
13	1238.6	1264.8	1372.5	1338.7	30.55	NS
adjusted	*1076.0*	*1138.0*	*1154.0*	*1111.0*	*27.65*	*NS*
14	1355.0	1417.0	1491.0	1493.0	19.86	NS
adjusted	*1222.0*	*1242.0*	*1279.0*	*1239.0*	*16.89*	*NS*

Values with different superscripts are significantly different at *(P<0.05) **(P<0.01) ***(P<0.001) NS: no significant difference