Biological control using entomopathogenic fungi: an evaluation against the mites

*Psoroptes ovis* (Hering) and *Acarus siro* L.

by

Stephen Ralph Abolins

A dissertation submitted to the University of Bristol in accordance with the requirements of the degree of Doctor of Philosophy in the Faculty of Science.

April, 2008

Word count: 36,138
Biological control using entomopathogenic fungi: an evaluation against the mites

Psoroptes ovis (Hering) and Acarus siro L.

by

Stephen Ralph Abolins

A dissertation submitted to the University of Bristol in accordance with the requirements of the degree of Doctor of Philosophy in the Faculty of Science.

April, 2008

Word count: 36,138
ABSTRACT

The reduction in available insecticides for the treatment of sheep scab, has prompted interest in the development of non-insecticidal alternatives such as the use of entomopathogenic fungi for the control of *Psoroptes ovis*. The work presented here demonstrates that under controlled conditions it is possible to induce lethal infections in *P. ovis*, in vivo, using the fungal pathogens *Metarhizium anisopliae* and *Beauveria bassiana*.

Initial in vivo trials, in which conidia of *M. anisopliae* were applied directly to the lesions of sheep experimentally infested with scab, resulted in few or no infected mites being recovered. This may have been due to the inability of the conidia to penetrate the fleece. Under more controlled conditions, batches of 20 adult female *Psoroptes* mites were confined in 25 mm diameter arenas glued to the skin of 6 sheep, with 6 arenas per sheep. Some mites were exposed to the fungi for 48 h in vitro prior to being placed on the host while other mites had no prior exposure and were placed directly onto the skin of a treated host. For both species of fungi, the highest levels of infection were achieved when the mites were exposed for 48 h in vitro prior to 48 h exposure in vivo; infection rates of 100% for *B. bassiana* and 92.9% (±3.34) for *M. anisopliae* were observed. The lowest levels of infection were observed with the conidia in diatomaceous earth; even with prior exposure in vitro only 20.8% (±6.74) became infected with *M. anisopliae* and 88.6% (±4.58) with *B. bassiana*.

Further arena-based in vivo trials showed that formulation had a significant but highly variable effect on pathogenicity making it difficult to discern general patterns. It seems likely that formulation will be an important factor in the development for a treatment of sheep scab so that viable fungal conidia can be delivered to the skin surface, where the mites are present.

Due to the difficulty of studying *P. ovis* in vivo and in vitro, a model system using *Acarus siro* was designed. The population dynamics of the mite in the absence of a fungal pathogen were first studied over an 80 day period, under conditions of restricted food availability (0.3g; wholemeal flour:yeast, 3:1, w/w) at 20 °C and 70% r.h.. The populations initially consisted of 50 adult female and 50 adult male mites and repeated sampling showed that it grew to reach a peak of 10080 mites, 54 days post-establishment, after which it declined as a result of food shortage. A Leslie matrix model constructed from the life-history data gathered in the present study, proved to be too simple to accurately model the dynamics of a population that had a high degree of intrinsic variability.

It was also shown that *A. siro* is susceptible to infection by *M. anisopliae* and *B. bassiana*, with 92% (± 7) and 99% (± 2) of mites infected when exposed to unformulated conidia for 1 min. Infected mites had lower LT50 values of 3 - 5 days compared to the controls, 9.5 days (±2.8). Horizontal transmission was possible between cadavers infected with *M. anisopliae* and live, naïve mites; 30% (± 10) of live, naïve mites became infected. Transmission also occurred between live, exposed mites and live, naïve mites; 43% (± 12) of live, naïve mites became infected. Transmission was shown to be possible in small but dense populations of naïve mites when 20 infected cadavers were introduced to populations of 100 adults (1:1 sex ratio). However, there was no evidence of transmission or persistence of the fungal pathogen in larger but initially less dense populations. It may be that a density threshold exists, below which the fungal pathogen cannot persist and transmit within a population of mites.

This study shows that fungal pathogens have clear potential to be valuable agents contributing to the control of sheep scab mites but many problems must be overcome. Further work needs to be conducted to identify suitable formulations and application methods and to assess the transmission of the fungal pathogens through populations of *Psoroptes* mites in vivo.
ACKNOWLEDGEMENTS

First and foremost I would like to thank Professor Richard Wall for his help, patience, encouragement and guidance throughout my Ph.D.

I am grateful to staff at the CSL including Prof. Mike Taylor, Vicky Jackson, Bhushy Thind and Louise Ford for their help with the animal trials and supply of mites. I am also grateful to Dr Dave Moore, Dr Belinda Luke and Steve Edgington at CABI for the supply of fungal conidia and helpful comments.

I am also very thankful for the help of Patricia Wells for spending a summer in the laboratory, counting a ridiculously large number of mites.

Thanks also go to the staff in the biological sciences animal facility for the maintenance of the rabbits.

I am also very grateful for the support from the members of the Veterinary Parasitology and Ecology group: Jenny Broughan, Kat Pegler, Betty Bisdorff, Laura Briggs, Jan Van Dijk, Luke Dickson, Ryan Jefferies, Jacqueline Lusat but especially Hannah Rose for providing accommodation and lots of tea.

I would also like to thank Risha Patel and Jon Flanders for letting me a room and cooking some great curries (Risha, not Jon!), as well as my many other friends in Bristol who made my time here very enjoyable.

I am grateful for the funding received from DEFRA.

Special thanks go to my parents, Linda and Gunar Abolins for their constant help and support.
AUTHOR'S DECLARATION

I declare that the work in this dissertation was carried out in accordance with the Regulations of the University of Bristol. The work is original except where indicated by special reference in the text and no part of the dissertation has been submitted for any other degree.

Any views expressed in the dissertation are those of the author and in no way represent those of the University of Bristol.

The dissertation has not been presented to any other University for examination either in the United Kingdom or overseas.

Signed: ____________________________ Date: 15/7/08

IV
CONTENTS

CHAPTER 1 Introduction 1

1.1 Fungal control using *Metarhizium anisopliae* and *Beauveria bassiana* .................................................. 1

1.2 Fungal control of ectoparasites ................................................................. 3

1.3 Fungal control of mite species ................................................................... 4

1.4 Mechanisms of infection ......................................................................... 6

1.5 Aims ........................................................................................................ 8

CHAPTER 2 General Materials and Methods 10

2.1 Culture of *Psoroptes ovis* ....................................................................... 10

2.2 Collection and identification of *Psoroptes ovis* ........................................ 10

2.3 Culture of *Acarus siro* ........................................................................... 13

2.4 Collection and identification of *Acarus siro* mites .................................. 15

2.5 Incubation of mites .................................................................................. 15

2.6 Culture and collection of fungal conidia .................................................... 19

2.7 Formulating conidial suspensions ............................................................... 19

2.8 Inoculation of mites with conidia ............................................................... 20

2.9 Determination of the point of death of mites ............................................. 20

CHAPTER 3 Control of *Psoroptes ovis* in vitro and in vivo using entomopathogenic fungi 22

3.1 Introduction ............................................................................................. 22

3.1.1 Sheep scab ....................................................................................... 22
3.1.2 Life-history of *Psoroptes ovis* ................................................................. 24
3.1.3 The classification of *Psoroptes ovis* ......................................................... 25
3.1.4 Prevalence and distribution of scab .......................................................... 27
3.1.5 Control of scab ...................................................................................... 28
3.1.6 Entomopathogenic fungi to control scab .................................................... 31

3.2 Methods and Results .................................................................................... 34
3.2.1 Analysis ................................................................................................. 34
3.2.2 The Pathogenicity of *Metarhizium anisopliae* against *Psoroptes ovis*
*in vivo* (Trial 1) ............................................................................................. 34
3.2.3 The pathogenicity of two strains of *Metarhizium anisopliae* *in vitro* .......... 39
3.2.4 Treatment of sheep infected with sheep scab with *Metarhizium anisopliae* (Trial 2) ............................................................................................................. 42
3.2.5 The pathogenicity of *Metarhizium anisopliae* to *Psoroptes ovis*
*in vitro* in the presence of sheep skin ............................................................. 45
3.2.6 The pathogenicity of five high temperature tolerant strains of
*Metarhizium anisopliae* against *Psoroptes ovis* .............................................. 48
3.2.7 The pathogenicity of *Beauveria bassiana* against *Psoroptes ovis*
*in vitro* .......................................................................................................... 49
3.2.8 Control of *Psoroptes ovis* using entomopathogenic fungi *in vivo*
(Trial 3) .......................................................................................................... 55
3.2.9 The pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana*
against *Psoroptes ovis* in various formulations *in vitro* ................................ 62
3.2.10 The pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana*
in various formulations *in vivo* (Trial 4) ........................................................ 67

3.3 Discussion .................................................................................................... 73
CHAPTER 4 Life-history and population dynamics of Acarus siro

4.1 Introduction to Acarus siro ................................................................. 81

4.1.1 Life-cycle ...................................................................................... 81

4.1.2 Effects of infestation ...................................................................... 83

4.2 Materials and Methods .................................................................... 84

4.2.1 Longevity, development and fecundity of Acarus siro .................. 84

4.2.2 The population dynamics of Acarus siro .................................... 86

4.2.3 Modelling the population dynamics of Acarus siro .................... 89

4.2.4 Analysis ....................................................................................... 92

4.3 Results ............................................................................................ 92

4.3.1 Longevity, development and fecundity of Acarus siro ................. 92

4.3.2 The population dynamics of Acarus siro .................................... 98

4.3.3 Comparison of the modelled and observed population dynamics of Acarus siro ..... 98

4.4 Discussion ..................................................................................... 110

CHAPTER 5 Effects of entomopathogenic fungi on Acarus siro

5.1 Introduction .................................................................................... 117

5.2 Materials and Methods .................................................................. 118

5.2.1 Pathogenicity of Metarhizium anisopliae and Beauveria bassiana 118

5.2.2 Horizontal transmission of fungal infection ............................... 119

5.2.3 Effect of a fungal pathogen on the population dynamics of Acarus siro ..... 122

5.2.4 Analysis ....................................................................................... 122

5.3 Results ........................................................................................... 123

5.3.1 Pathogenicity of Metarhizium anisopliae and Beauveria bassiana 123
5.3.2 Horizontal transmission of fungal infection ................................................ 127
5.3.3 Effect of a fungal pathogen on the population dynamics of Acarus siro ............... 131
5.4 Discussion ................................................................................................ 139

CHAPTER 6 Discussion 143

REFERENCES 157

APPENDIX

Control of the sheep scab mite, Psoroptes ovis in vivo and in vitro using fungal pathogens.
Despite the ongoing health, welfare and economic need to control parasites of veterinary and medical importance there is increasing pressure to reduce the use of many of the insecticides that have formed the primary means of effecting control in the U.K. over the last 50 years (Kirkwood and Quick, 1981, 1982; O'Brien 1999). The reasons for this include the development of resistance, fears over safety of the chemicals to humans and non-target organisms and concerns over damage to the environment (Stephens et al., 1995; Synge et al., 1995; Clark et al., 1996; Cooke et al., 2004). This has lead to calls for increased research into the development of alternative treatment methods (Wall, 2007). One such alternative for the control of arthropod pests is the use of entomopathogenic fungi.

1.1  Fungal control using *Metarhizium anisopliae* and *Beauveria bassiana*

*Metarhizium anisopliae* (Metschnikoff) Sorokin (Deuteromycotina: Hyphomycetes) and *Beauveria bassiana* (Balsamo) Vuillemin (Ascomycota: Hypocreales) are two of the most commonly used and researched fungi (Samish and Rehacek, 1999; Inglis et al., 2001; Brooks, 2004; Scholte et al., 2005; Ansari et al., 2007). These species provide good candidates as control agents because of their wide geographical spread and host range as well as their ability to germinate at relatively low humidity levels (Zimmerman, 1993; Samish and Rehacek, 1999). Both of these fungal pathogens have been proved effective against many insect pests of crops and in certain cases have been made commercially available. One commercially available product containing *M. anisopliae* is BioGreen®, a product marketed as a control agent of the red-
headed cockchafer, *Adoryphorus couloni* (Burmeister), in Australia (Inglis et al., 2001). When BioGreen® was applied to the soil of pasture land there was a 58% reduction in the number of pupae, 27 weeks after application (Rath et al., 1995). The two subsequent generations after treatment had 68% and 65% fewer larvae than the controls indicating the long-term effects of the fungal pathogen (Rath et al., 1995).

Another commercially available product containing *M. anisopliae* is Green Muscle® (Lubilosa, 2004). This product is marketed for the control of locusts and grasshoppers in regions of Africa and Australia. A study, conducted between November 1998 and March 1999, treated areas of land infested with *Locusta migratoria* L. nymphs in Australia with an aerial spray of *M. anisopliae* (Hunter et al., 1999). At a dose of $3 \times 10^{12}$ conidia ha$^{-1}$ there was less than 10% survival of treated nymphs (Hunter et al., 1999). Another trial found that approximately 50 droplets per cm$^2$ of a $1 \times 10^{12}$ conidia per litre suspension of *Metarhizium flavoviride* Gams Rozsypal were required to achieve 90% mortality of the desert locust, *Schistocerca gregaria* (Forskål) within 10 days (Bateman et al., 1998). The same study also observed that locusts released into an area that had already been treated with the mycoinsecticide became infected. The time taken to achieve 50% mortality was quicker in the groups exposed directly to the mycoinsecticide but there was no significant difference in the average survival time of infected locusts between either of the groups (Bateman et al., 1998).

Entomopathogenic fungi have also been tested against pests of stored-products. When the beetles *Rhyzopertha dominica* (F.), *Sitophilus oryzae* (L.) and *Tribolium confusum* du Val were exposed to wheat treated with unformulated, dry conidia of *M. anisopliae*, mortality increased with increasing concentration (Kavallieratos et al., 2006). After 14 days of exposure at a concentration of $8 \times 10^{10}$ conidia kg$^{-1}$ of wheat, mortality levels of 74.6%, 82.3% and 74.4% were achieved for adults of *R. dominica*, *S. oryzae* and *T. confusum* respectively. Control mortality was 6%, 3% and 4.5% respectively (Kavallieratos et al., 2006). The larvae of *T. confusum* are also susceptible to *M. anisopliae* (Michalaki et al., 2006). When the larvae were
exposed to wheat treated with dry conidia of *M. anisopliae* at a concentration of $8 \times 10^{10}$ conidia kg$^{-1}$, mortality of over 50% was achieved (Michalaki *et al.*, 2006). Another study using *M. anisopliae* mixed with oven ash (burned paper sheets) showed that when applied as a curative treatment, under controlled conditions, mortality levels of 86.7% can be obtained against *S. oryzae* (Batta, 2004).

### 1.2 Fungal control of ectoparasites

As yet there are no commercially available fungal pathogens for the control of ectoparasites. However, previous studies on a range of parasites have shown that there is a strong potential for the use of entomopathogenic fungi for the control of ectoparasites.

*Metarhizium anisopliae* has been shown to infect and kill many different species of tick. *Rhipicephalus appendiculatus* Neumann and *Amblyomma variegatum* Fabricius kept on rabbit ears were shown to be susceptible to infection by *M. anisopliae* but with relatively low mortality levels of 34% and 37% respectively (Kaaya *et al.*, 1996). In the same study (Kaaya *et al.*, 1996), a $1 \times 10^8$ conidia ml$^{-1}$ suspension of *B. bassiana* was responsible for a 34% mortality of adult *R. appendiculatus*, although it failed to kill any *A. variegatum*. However, *R. appendiculatus* that were naturally present on zebu cattle in the field had a much higher level of mortality; 83% death when treated with *M. anisopliae* and 77% when treated with *B. bassiana* (Kaaya *et al.*, 1996). Another study recorded 90% mortality of adult female *Boophilus annulatus* Say, 7 days post-inoculation (Gindin *et al.*, 2001). Larval mortality was 100% after 6 days.

A study testing the pathogenicity of 12 different isolates of *M. anisopliae* against *Boophilus microplus* (Canestrini), recorded 100% mortality 14 days post-inoculation for 10 of the 12 isolates (Frazzon, 2000). However not all ticks are so susceptible to infection. In *Amblyomma maculatum* Koch, mortality of 60% of adult females exposed to *M. anisopliae* was observed but only 15% mortality in exposed *Amblyomma americanum* L. females (Kirkland,
A similar study found that while one strain of *M. anisopliae* was able to induce mortality levels of 100% in *B. annulatus* and *Rhipicephalus sanguineus* (Latreille), only 5% mortality was recorded for *Hyalomma excavatum* (Koch) (Gindin, 2002).

The cattle louse, *Bovicola bovis* (L.) has been shown to be susceptible to infection by *M. anisopliae*. When maintained in arenas on the skin surface of cattle, 73% of lice became infected when exposed to a concentration of \(1 \times 10^8\) conidia ml\(^{-1}\) of *M. anisopliae* in Tween 80 (Briggs et al., 2006).

*Metarhizium anisopliae* has also been shown to be pathogenic to the mosquito vectors of malaria (Scholte et al., 2005, 2007). Field trials using black, cloth targets impregnated with *M. anisopliae* and hung in traditional houses in Tanzania found that out of 580 female *Anopheles gambiae* Giles sensu stricto collected in the houses, 132 were infected with the pathogen (Scholte et al., 2005). The LT\(_{50}\) values were significantly lower for infected mosquitoes; 3.5 days for infected females compared to 9.3 days for uninfected females (Scholte et al., 2005). *In vitro* bioassays had similar results; 87% of *Aedes aegypti* (L.) and 89% of *Aedes albopictus* (Skuse) became infected with *M. anisopliae* when exposed to impregnated filter papers for 24 h (Scholte et al., 2007). This study concluded that this level of infection could significantly reduce the transmission of plasmodium and therefore with careful application, fungal pathogens could be used in the control of malaria.

### 1.3 Fungal control of mite species

The Acari is a large and diverse sub-class of the class Arachnida, including many species of parasite and pest of veterinary, agricultural and economic significance. The ability to control these arthropods can be of vital importance to the viability of agriculture, food and farming industries.
A study testing the pathogenicity of various strains of *B. bassiana*, *M. anisopliae* and *Paecilomyces fumosoroseus* (Wise) against *Oligonychus yothersi* (McGregor), a pest of tea crops, found that strains of *B. bassiana* were the most pathogenic with mortalities of over 70% at 3 to 4 days post-inoculation (Oliviera et al., 2004). A study testing the pathogenicity of *M. anisopliae* on the two-spotted spider mite, *Tetranychus urticae* Koch found that levels of infection varied from 3.8% to 29.4% depending on the isolate used (Chandler, 2005). Mortality levels were also variable ranging from 6.7% to 43.2% (Chandler, 2005). When *B. bassiana* was tested against the green mite, *Mononychellus tanajoa* (Bondar) mortality levels of 9% to 91% were found for *B. bassiana*, depending on the isolate used (Barreto et al., 2004). The mortality levels when *M. anisopliae* was used ranged from 8% to 45%. LT$_{50}$ values ranged from 4.2 to 17 days for *B. bassiana* and 8.6 to 19.8 days for *M. anisopliae* (Barreto et al., 2004). *Beauveria bassiana* has also been shown to be pathogenic to the citrus rust mite, *Phyllocoptruta oleivora* Ashmead (Alves et al., 2005). A mortality rate of 91% was recorded after 5 days exposure to 2 ml of a 1x10$^8$ conidia ml$^{-1}$ of *B. bassiana* in 0.2% Tween 80 (Alves et al., 2005). The mean LT$_{50}$ at this concentration was 2.74 days (Alves et al., 2005).

*Metarhizium anisopliae* has shown great potential as a control agent of *Varroa destructor* Anderson and Trueman in bee hives. In laboratory bioassays, three isolates of *M. anisopliae* were found to kill 100% of *V. destructor* within 7 days at a concentration of 1x10$^8$ conidia ml$^{-1}$ and one isolate killed 97% at just 1x10$^6$ conidia ml$^{-1}$ (Shaw et al., 2002). Field trials recorded infected mites up to 42 days post inoculation even though peak mortality occurred 3 to 4 days after inoculation (Kanga et al., 2003). It was concluded that *M. anisopliae* was just as effective as the tau-fluvalinate acaricide, Apistan.
1.4 Mechanisms of infection

The conidia of entomopathogenic fungi such as *B. bassiana* and *M. anisopliae*, stick to the surface of a host by random, non-specific, hydrophobic interactions (Charnley, 1989; Boucias *et al.*, 1991). The conidia are known as dry spores as they do not have a mucoid layer surrounding them. Instead they are covered by hydrophobic rodlets that form interwoven bundles across the surface of the conidium (Charnley, 1989; Boucias *et al.*, 1991). Once the conidia have attached to the surface of the host, the fungus germinates and penetrates the cuticle (Inglis *et al.*, 2001). This is achieved by the production of germ tubes or apressoria (Boucias *et al.*, 1991). The stimulation to produce germ tubes or apressoria varies with each species. *B. bassiana* and *M. anisopliae* both have wide host ranges and therefore respond to non-specific stimuli. The germination of these two fungi is thought to be stimulated by non-specific sources of carbon and/or nitrogen as would be found in amino acids, proteins, carbohydrates and some lipids and requires an exogenous nutrient source for germ tube formation (StIeger *et al.*, 1986; Charnley, 1989). From the apressorium a penetration peg grows (Clarkson and Charnley, 1996; Zacharuk, 1970a,b,c) that adheres to the cuticle by secreting mucus similar to that secreted by *Nomuraea rileyi* (Farlow) which has been identified as a mixture of β-1,3 glucans (St. Leger *et al.*, 1986; Charnley, 1989). The penetration of the host cuticle relies upon mechanical force and enzymatic degradation but the relative contribution of either method is not yet known. It is probably dependant on many variables of the host and pathogen interaction (Charnley, 1989).

The first barrier that must be penetrated is the epicuticle and wax layer. Penetrating the wax layer might be achieved through the activities of lipases and esterases that are secreted by *M. anisopliae* and *B. bassiana* (Charnley, 1989; Zacharuk, 1970). However the exact mechanism is still unclear as lipids and esters are not the only components of the wax layer (Charnley, 1989). Degradation of the cuticle may also rely upon the action of enzymes (St. Leger 1995). The fungal pathogens produce proteases, lipases and chitin deacetylase that can convert
chitin into chitosan, a glucosamine polymer (Barreto et al., 2004; Nahar et al., 2004). St. Leger (1986) purified endoprotease and chitinase from cultures of M. anisopliae. When these enzymes were applied to locust cuticle they were found not only to degrade the cuticle but also provided possible sources of nutrient to the growing fungus from the breakdown products in the form of amino sugars. It has been suggested that the outer epicuticle is probably breached through physical force whereas the inner epicuticle may be broken down by a mixture of endoproteases produced by the apressorium (Charnley, 1989; Arruda et al., 2005).

Once the epicuticle has been breached, the fungus has to penetrate the procuticle. The procuticle is the largest part of the cuticle and is constructed from chitin fibrils embedded in a protein matrix (Charnley, 1989). The degree of sclerotization is an important factor influencing the ability of the fungus to cross the procuticle. More heavily sclerotized procuticles are much more difficult to traverse and may be impassable, as sclerotized cuticle is comparatively resistant to the enzymes produced during penetration and also provides a stronger physical barrier to the pathogen (St.Leger et al., 1986; Charnley, 1989). Heavily sclerotized arthropods are usually penetrated at the joints of appendages. Hosts with a lower degree of sclerotization provide an easier route for penetration through the cuticle (Charnley, 1989).

Once the cuticle has been breached the morphology of the fungus changes as hyphal bodies are produced which then circulate in the haemolymph (Gillespie and Moorhouse, 1989) and proliferate just before the host dies (Ferron, 1981). The hyphae of M. anisopliae have been observed invading the fat body, muscles and gut walls of infected tsetse flies (Glossina morsitans morsitans Westwood) (Kaaya, 1991). The hyphal bodies of M. anisopliae have been found in the haemolymph of S. gregaria 2-3 days after infection (Gillespie et al., 2000). Death of the arthropod usually occurs 3-14 days after infection due to a combination of mechanical damage of tissue, nutrient depletion and toxicosis (Gillespie and Moorhouse, 1989).

Some entomopathogenic fungi are able to produce toxins. These toxins may be one of the main causes of host death (Roberts, 1981). Metarhizium anisopliae produces a family of
cyclic peptide toxins known as destruxins (Clarkson and Charnley 1996; Liu et al., 2004). The destruxins are cyclic hexadepsipeptides consisting of 5 amino acids and an α-hydroxyl acid (Liu et al., 2004). The exact mechanisms by which the destruxins kill the host are not yet known although there is evidence that the inhibition of the host's immune response within the haemolymph may be crucial (Huxham et al., 1989; Kershaw et al., 1999).

Once the host has died, the fungus colonises the body and the hyphae grow through the cuticle and become apparent on the surface of the host before sporulation takes place (Gillespie and Moorhouse, 1989). If the outside conditions are not conducive to sporulation the entomogenous fungi can remain inside the host for up to several months (Gillespie and Moorhouse, 1989).

1.5 Aims

The aims of the work described here were to examine the potential of two species of fungal pathogen to act as control agents for sheep scab mites (Psoroptes ovis (Hering)) (Acari: Psoroptidae) and also to investigate the effect that fungal pathogens could have on the population dynamics of the mite species Acarus siro L. (Acari: Acaridae). Firstly, following on from previous in vitro studies, the ability of M. anisopliae and B. bassiana to infect Psoroptes mites on a host was examined (Chapter 3). The effect of skin and fleece and various different formulations were also investigated both in vitro and then in vivo (Chapter 3). Subsequently, studies were undertaken to consider transmission of entomopathogenic fungi in populations of mites. However, because P. ovis is an obligate ectoparasite and therefore extremely difficult to study in vivo and in vitro, a model species, A. siro is used. To consider the effects of a fungal pathogen on population dynamics, populations of A. siro were established and maintained under constant conditions. Data on the life-history of A. siro was gathered and used to construct a simple model against which populations of A. siro could be compared (Chapter 4). The
pathogenicity of *M. anisopliae* against *A. siro* was tested with direct exposure between mites and conidia (Chapter 5). With the pathogenicity established, the ability of *M. anisopliae* infection to transmit from infected cadavers to naïve, live mites was examined using small-scale, high density populations and large-scale, low density populations (Chapter 5).
2.1 Culture of *Psoroptes ovis*

A low level infestation of *Psoroptes* mites was maintained in the ears of two New Zealand white rabbits maintained at the University of Bristol, UK under Home Office Licence number PPL 30/1636. The infestation of mites was maintained at low levels to keep the pathology and welfare problems to a minimum. Infestation was initiated by placing a piece of scab, containing mites, inside the ear of a rabbit and then sealing the ear with zinc oxide adhesive tape (Boots the Chemists Ltd. Nottingham, UK) for 24 h after inoculation. As many attempts as necessary at inoculation were made (approximately once a week) until signs of infestation could be seen. These signs were manifested in scabs that were visible on the inner surface of the ears which resulted from hardening of serous exudates.

2.2 Collection and identification of *Psoroptes ovis*

Scabs were removed from the ears of rabbits using blunt-nosed forceps. Scab was removed as often as required (usually twice per week) to keep the infestation in the ears of the rabbits at a low level. The scab and mites that had been removed were placed into clean, sealable, plastic containers, 40 mm in diameter and 60 mm high. Once the mites and scab had been removed from the host, the mites migrated from the scab and crawled up the sides of the container. A fine grade paintbrush was used to pick mites off the sides of the container. These mites were then placed onto a 50 mm diameter petri dish that was floated on 70% ethanol inside a larger 90 mm diameter petri dish. The ethanol collected and killed any mites that crawled out of the inner petri dish.
The life cycle stages of the mites were identified under a binocular microscope according to the descriptions given by Sanders et al. (2000). The adults are roughly oval in shape and white in colour, although they can become dark brown if eosinophils are consumed (Fig. 2.1 A, B) (Rafferty and Gray, 1987). The juvenile stages remain white. Adult females are morphologically the largest stage and are approximately 0.46 – 0.57 mm in length (Fig. 2.1 A) (Sanders et al., 2000). The adult males are smaller, approximately 0.36 – 0.44 mm, and not as round as the females (Fig. 2.1 B) (Sanders et al., 2000). Sexual dimorphism can be observed from the protonymph stage onwards (Sweatman, 1958; Sanders et al., 2000). Female and male nymphs can be distinguished from each other by the presence of dorsoposterior tubercles on the female in both nymphal stages (Fig. 2.1 C-F) (Sanders et al., 2000). Adults can be easily sexed by the difference in overall size but also by a number of other features. Males have a jointed pretarsi and pulvilli on the first three pairs of legs but not on the fourth pair, which are much shorter (Fig. 2.1 B) (Sanders et al., 2000). Males also possess a pair of adanal suckers and paired podosomal lobes. Females have two pairs of metapodosomal setae on the ventral surface and a U-shaped vulva on the propodosoma (Fig. 2.1 A) (Sanders et al., 2000; Wall, 2007).

Protonymphs can be distinguished from tritonymphs by the presence of three pairs of metapodosomal setae only, rather than the five pairs of setae in the tritonymph (Sanders et al., 2007). Tritonymphs are also larger than the protonymphs. Larvae show no sexual dimorphism but can be identified from the other stages due to the presence of only 3 pairs of legs (Fig. 2.1 G) (Sanders et al., 2000).
Fig. 2.1. The features used to identify the different lifecycle stages of *Psoroptes ovis*. A = ventral view of an adult female; B = dorsal view of an adult male; C and D = dorsal view of a female protonymph and tritonymph, respectively; E and F = dorsal view of a male protonymph and tritonymph respectively; G = ventral view of a larva (Sanders *et al.*, 2000). Bar = 100μm.
2.3 Culture of *Acarus siro*

*Acarus siro* mites (strain 9258/2) were obtained, in 1992, from a co-operative feedmill, Latham, U.K., and reared under laboratory conditions at the Central Science Laboratory (CSL), Sand Hutton, U.K.. A sub-colony was obtained in 2004 and reared at the University of Bristol. For this, the mites were kept in 50 ml conical flasks (Fig. 2.2) (Fisher Scientific UK Ltd., Loughborough, U.K.) and fed on a mixture of wholemeal flour (Sainsbury’s Supermarkets Ltd., London, U.K.) and yeast extract (Sigma-Aldrich, Dorset, U.K.) in a 3:1 (w/w) ratio. The flasks were sealed with non-absorbent cotton wool. All colonies were maintained at 20 °C and 70% relative humidity, in a controlled climate incubator.

When the density of mites within the flasks was deemed to be too high, i.e. more mites than food was visible when the colony was inspected under a binocular microscope, the colony was split into several new flasks, each containing fresh food. This was achieved by gently pouring a small amount of the colony from the flask with the high mite density into a new flask with fresh food. Each new flask received mites from several different flasks so that no genetic segregation would occur between colonies. Latex gloves (Semperit Technische Produkte GmbH & Co Vienna, Austria) were worn and sprayed with 70% ethanol before handling mites or food to prevent contamination of the food or mite colonies with fungal pathogens.
Fig. 2.2. Conical flasks containing *Acarus siro* mites feeding on wholemeal flour and yeast extract in a 3:1 ratio (w/w). Flasks were sealed with non absorbent cotton wool.
2.4 Collection and identification of Acarus siro mites

Mites were collected from colonies by gently brushing the sides of the conical flask with a fine grade paintbrush to pick up the mites. These mites on the paintbrush were then deposited onto a 50 mm diameter petri dish (Bibby Sterilin, Stone, UK) that was floated on 70% ethanol inside a larger 90 mm diameter petri dish. The ethanol collected and killed any mites that crawled out of the inner petri dish. The life cycle stages of the mites were identified under a binocular microscope and could be picked up with a paint brush. Adult females were identified by their relatively large size and round appearance compared to the other stages. Adult males were identified by their slender body in comparison to adult females and the presence of a spur on the underside of the trochanter of each fore leg. Tritonymphs and protonymphs were identified by their relative sizes, with tritonymphs being bigger than protonymphs and the larvae were identified by the presence of only 3 pairs of legs rather than the 4 pairs seen in all other stages (Solomon, 1969). Sex was not determined for juvenile stages.

2.5 Incubation of mites

All Psoroptes mites were incubated in their respective treatment groups, in clean incubation chambers constructed from 6 x 25 x 75 mm glass blocks (Fig. 2.3). Each block had a 20 mm diameter hole drilled through its centre. However, for Psoroptes mites the bottom of the block was sealed with a fine grade cotton cloth glued to the glass with epoxy resin to which 500 μl of sheep serum had been added. Mites were placed in the hole and the chamber was sealed using a glass microscope slide with a 5 mm diameter hole drilled through its centre, which was also closed with cotton cloth. Another glass microscope slide was placed on the underside of the chamber. The slides and glass block were held in place with bulldog clips at each end of the chamber. The hole in the upper glass slide was to allow for regulation of the humidity of the air within the chamber. The chambers and mites were
incubated at 30 °C and 90% relative humidity. A further 200 μl of sheep serum was added every 48 h until all mites had died.

The cloth-based chambers were used to incubate *Psoroptes* mites so that more space was available when conducting trials involving sheepskin and fleece. The filter-based chambers were also more liable to damage when the sheep serum was added.

All *A. siro* mites used in experiments were incubated in their respective treatment groups in clean incubation chambers constructed from 6 x 25 x 75 mm glass blocks. Each block had a 20 mm diameter hole drilled through its centre. A piece of filter paper was placed over the hole and an indentation was made. A glass microscope slide was placed over the filter paper and the block and slide were clipped together with a bulldog clip at each end (Fig. 2.4). Approximately 0.01 g of food was placed in the chamber before the addition of the mites. The chambers and mites were then incubated at 20 °C and 70% r.h. until all mites had died.
Fig. 2.3. Experimental chamber used to incubate *Psoroptes ovis* mites during bioassays. For details see text.
Fig. 2.4. Experimental chamber used to incubate *Acarus siro* mites during bioassays. For details see text.
2.6 Culture and collection of fungal conidia

Both of the fungal pathogens used in this work, *M. anisopliae* and *B. bassiana*, were cultured using the same method. The fungi were cultured on plates of Potato Dextrose Agar (Sigma-Aldrich, Poole, UK) and Yeast extract (Sigma-Aldrich, Poole, UK) (PDAY). The plates of PDAY were made by mixing 39 g of PDA with 2 g of yeast extract in 1 l of distilled water. The mixture was then autoclaved at 120 °C for a freesteam time of 15 min. The liquid agar was then poured into the base of a 90 mm petri dish and left to set. The plates of PDAY were inoculated by pipetting 150 µl of conidia suspended in silicone oil (dimethylpolysiloxane hydrolyzate; Sigma-Aldrich, Poole, UK) onto the surface and spreading the inoculum over the plates with a sterile glass rod. The PDAY and fungi were then incubated at 25 °C. Conidia were collected from 10 to 14 day-old cultures by gently scraping the conidia off the agar plate using a sterile loop, into a clean, glass universal pot. Small amounts of conidia of each isolate were stored in eppendorf tubes containing 2 ml of silicone oil and stored in an ultra low temperature freezer at -80 °C for future use.

2.7 Formulating conidial suspensions

Once the conidia had been collected, they were ready to be either formulated in an excipient or used as unformulated, dry conidia. To make a conidial suspension using an oil-based excipient (silicone oil, vegetable oil, sunflower oil or output®) conidia were added to 4 ml of the excipient. The concentration was calculated using an Improved Neubauer Haemocytometer (Weber Scientific International, Middlesex, UK). The suspension was then diluted to make a concentration of $1 \times 10^9$ conidia ml$^{-1}$. The suspensions were then further diluted when necessary by serial dilution.

Conidia suspended in water with Tween 80 (BDH Chemicals Ltd, Poole, UK) were initially suspended in 4 ml of 0.05% Tween 80 and then diluted to a concentration of $1 \times 10^9$ conidia ml$^{-1}$ using 0.03% Tween 80. Serial dilutions were carried out using the lower
concentration of Tween 80. This lower concentration was used for further dilutions to reduce any toxic effects that Tween 80 may have on the conidia (Butt and Goettel, 1997).

Conidia that were formulated in Codacide® (vegetable oils and polyethoxylated esters; Microcide Ltd, Stanton, UK) were first suspended in 0.4 ml of neat Codacide® before 3.6 ml of water was added to make 4 ml of 10% Codacide®. The conidial suspension was then further diluted to the required concentration using 10% Codacide®.

Formulations of conidia in diatomaceous earth were supplied by Dr Belinda Luke at CABI, Ascot, UK. Concentrations were calculated by mixing a known volume of conidia with a known volume of diatomaceous earth.

2.8 Inoculation of mites with conidia

When using conidia suspended in liquid, mites (both *A. siro* and *P. ovis*) were inoculated by completely immersing them in 2 ml of suspension for 1 min in a 50 mm diameter Petri-dish lined with a clean filter paper. After 1 min, the mites were removed and placed onto a clean piece of filter paper for 1 min to allow excess fluid to run off before being transferred to clean incubation chambers (Fig. 2.3, Fig. 2.4).

When dry conidia were used to inoculate mites, the appropriate volume of conidia was weighed out, such that mites would be exposed to a similar absolute number of conidia as if exposed to 2 ml of a $1 \times 10^8$ conidia ml$^{-1}$ suspension.

2.9 Determination of the point of death of mites

Death was determined when there was no movement from the mite when touched with a paintbrush. Where several treatments were compared, separate paint brushes were used for each of the treatments. The brushes were washed in 70% ethanol and wiped dry between each sample. Any dead mites were removed from the chambers and surface sterilised in 2% (w/v) sodium hypochlorite solution (Sigma-Aldrich, Dorset, UK) for 30 s and then washed in sterile water for 30 s to remove any conidia that had not penetrated the
mite cuticle before the point of death. Each individual dead mite was then transferred to a damp piece of sterile filter paper at the base of a well in a 96 well microtitre plate (Bibby Sterilin, Stone, UK). The filter papers were cut into discs with a diameter of 6 mm using a hole punch and autoclaved before being placed into the wells. The microtitre plates were then sealed with Parafilm® M (Pechiney Plastic Packaging, Chicago, USA) and incubated at 30 °C. The plates were inspected for mites with fungal infections every day for 2 weeks following the observation of the first infected corpse. Fungal infections were recorded as present when external hyphae could be observed protruding through the cuticle of a mite.

Proportions of infected mites, as presented in the results sections of Chapters 3 and 5, were calculated by dividing the number of mites showing signs of infection within each group by the number of mites treated in that group.

$LT_{50}$ values were determined by the point at which half of the mites within a group had died.
CHAPTER 3

CONTROL OF PSOROPTES OVIS IN VITRO AND IN VIVO USING ENTOMOPATHOGENIC FUNGI

3.1 Introduction

3.1.1 Sheep scab

Sheep scab or psoroptic mange, is a disease of global importance, caused by the astigmatic mite Psoroptes ovis. The clinical signs of the disease include wool loss, weight loss, decreased feeding, intense pruritus, biting and scratching, epileptiform fitting and can lead to the death of the sheep (Downing, 1936a; Tarry 1974; Kirkwood, 1980). Not only are there serious welfare implications associated with the disease, there is also a large economic impact of sheep scab as a result of the reduction in weight gain, damage caused to the skin and the cost of treatment. In a study in 1986, it was estimated that the disease could cost the U.K. £600 million over a 30 year period (Kirkwood, 1986). The same study calculated that a 30% loss in weight gain would lead to a cost of £1000 for a flock of 100 sheep (Kirkwood, 1986). A more recent study calculated that sheep scab would cost Britain £8.3 million per annum (Nieuwhoff and Bishop, 2005). It was reported that the majority of the expenditure was on prevention (Nieuwhoff and Bishop, 2005). The loss of wool and weight and the damage caused to the skin, combined with the cost of treatment, means that sheep scab is a disease of great economic importance, not only in Britain but also in other parts of the world such as Asia, southern Africa and South America (Kirkwood, 1986).

Although the exact feeding mechanisms are unknown, it was originally assumed that mites punctured the skin surface and fed on dermal cells and serous fluid (Shilston, 1915; Downing, 1936a; Sweatman, 1958). However, it has since been shown that Psoroptes mites
feed on the lipids and serous exudates present on the surface of the skin and do not bite or burrow (Blake et al., 1978; Sinclair and Kirkwood, 1983; Sinclair and Filan, 1989, 1991). The mouthparts of P. ovis appear more suited for abrading the skin and imbibing the surface fluids than puncturing the dermis (Blake et al., 1978; Rafferty and Gray, 1987; Mapstone et al., 2002). A fluid pumping action has been observed around the pharyngeal region when Psoroptes mites are immersed in serum (Rafferty and Gray, 1987).

The presence of actively feeding mites as well as antigenic material in the faeces is thought to trigger a hypersensitivity response in the host, leading to inflammation and serous exudate at the skin surface, providing a more suitable microclimate and more available food for the mite (Sinclair and Kirkwood, 1983; Mathieson and Lehane, 1996). Suppression of the immune response in sheep using Cyclosporin A dramatically reduces lesion growth and mite numbers, compared to control sheep, demonstrating the importance of the immune response in the development of scab (Huntley et al., 2005). It is these skin secretions that dry and harden to form the characteristic yellow scabs from which the disease derives its name.

As the serum on the skin surface dries to form scabs and wool is lost from the areas of active infestation, the conditions become less suitable for the mites, which move outwards to the edge of the lesions where wool is still present and the skin secretions have yet to dry (Downing, 1936b). As the population of mites grows, the lesion size increases and the disease spreads across the body of the sheep. The areas of the sheep that are usually affected are the shoulders and sides of the body but, as the lesion grows, up to three quarters of the body could be covered (Tarry, 1974).

After the initial infestation there may be a pre-clinical phase before the disease is detected. This period can be variable in length depending on factors such as sheep breed, health of the host, initial number of mites and previous exposure (Bates, 1997a, b, 2000). The population will then undergo a period of rapid growth, usually lasting 40 – 50 days until a peak is reached, after which the numbers of mites plateau (Bates, 1997a, b, 2000; Berriatua, 1999, 2001). Transmission of mites between hosts is most likely to occur during the plateau
phase when mite numbers are at their highest (Berriatua, 1999, 2001). At this stage the host will either die or the immune response will cause the number of mites to decline, leading to the apparent recovery of the sheep. However, some of the sheep that appear to have recovered may be harbouring sub-clinical levels of mites which can easily go undetected and remain as a reservoir for re-infestation within the flock (Babcock and Black, 1933; Downing 1936 a,b).

The survival of the mite off-host is highly dependant upon environmental conditions. One study which kept mites on scab and wool at room temperature, found that adult females could survive for 16 days (Shilston et al., 1915). Another found that mites could survive for a mean of 12 days (range of 1 – 38 days) at 21 – 27 °C (Babcock and Black, 1933). More recent studies have shown that mites may remain alive and infective for 18 – 20 days off-host (O’ Brien, 1994; Smith et al., 1999).

3.1.2 Life history of Psoroptes ovis

_Psoroptes ovis_ has a 5 stage life-cycle: egg, larva, protonymph, tritonymph and adult (Sanders et al., 2000). Unlike many other astigmatic mites, _P. ovis_ does not possess a deutonymph stage (Sanders et al., 2000).

Several studies have investigated the time spent at the various stages of development and show that, although there is some variation, on the host each stage in the life-cycle generally requires approximately 2 days; the total time taken to complete the egg to adult life-cycle has been recorded as taking between 10.7 – 16 days (Stockman, 1910; Shilston, 1915; Downing, 1936a,b).

The number of eggs a female can produce in its lifetime has been recorded as ranging from 35 (Downing, 1936 b) to 86 (Shilston et al., 1915) and the number of eggs laid per day per female estimated to be from 0.5 to 5.6 (Shilston, 1915; Downing, 1936b).

A more recent study created a Leslie matrix model, simulating the population dynamics of _P. ovis_, used values of 2 days per life-cycle stage, with egg production of 2.9 female eggs per female per day and a female longevity of 16 days (Wall et al., 1999). The
model gave a good fit with data collected from scab infested sheep and therefore appears to provide a good estimation for the rate of population growth of *P. ovis*.

### 3.1.3 The classification of *Psoroptes ovis*

The first description of *Psoroptes* mites on sheep was given by Hering in 1838 when he classified them as *Sarcoptes ovis*. It was Gervais however in 1841 that showed that the sheep scab mites were different to those belonging in the *Sarcoptes* genus and subsequently reclassified them as *Psoroptes ovis*. Then in 1861 the classification changed again as Fürstenburg labelled them as *Dermatokoptes communis* before Megnin reassigned them as *Psoroptes* in his 1877 review.

Meginin (1877) used the species name *Psoroptes longirostris* to describe mites that were found on the bodies of sheep, cattle, horses and the ears of rabbits. The mites found on each host were labelled as different variants of *P. longirostris*. Since Meginin’s original classification there has been various additions and changes to the taxonomy of the *Psoroptes* genus although they have generally been referred to as *P. communis* with different varietal names appended depending on the host upon which the mite is found (Stockman and Berry, 1913; Shilston, 1915; Hirst, 1922; Downing, 1936; Bates, 1999). However, a reclassification by Sweatman (1958) organised the genus into 5 different species split between 2 groups; body mites and ear mites. *Psoroptes ovis*, *P. equi* and *P. natalensis* were grouped as body mites while *P. cuniculi* and *P. cervinus* were grouped as ear mites.

The criteria Sweatman (1958) used to distinguish between the species were the location on the host’s body where the mites were found and the length of the outer opisthosomal seta of the adult males. However, these criteria are not definitive, as ear mites have been observed to move from the ear to the body of the host and the lengths of the outer opisthosomal setae can overlap between body and ear forms (Wright et al., 1984; Bates 1999). A later study showed that there were no morphological differences between mites of different hosts in sympatric populations, only in allopatric populations, and there were no morphological differences between ear and body forms (Boyce et al., 1990).
Antigenic studies also support the hypothesis that sympatric populations of mites from different hosts may not be ecologically or reproductively isolated (Boyce and Brown 1991). One study has claimed to have shown that it is possible for *P. ovis* and *P. cuniculi* to mate and produce viable offspring, however, this paper is unclear on how this was achieved and this result has never been validated or repeated (Wright et al., 1983).

*Psoroptes* mites have been experimentally transferred from one host to another host of a different species. For example, Kirkwood (1985) transferred *P. ovis* from the bodies of sheep to the ears of rabbits, causing a severe infestation, and was then able to transfer the mites back and forth from rabbit to sheep for three generations. The same study also managed to transfer mites from rabbits to cattle. Sweatman (1958) was successful in transferring mites from the ears of rabbits to the ears of sheep.

However, some studies have reported a failure of transferred *Psoroptes* mites to establish (Ochs, 2002). For example, a recent study was unable to establish *P. cuniculi* from the ears of rabbits onto sheep or even onto the backs of rabbits (Ochs, 2002). Shilston (1915) was only able to establish ear mites from rabbits onto sheep for 17 days before the population died out at the second generation. Another study was unable to produce a skin infestation on the backs of rabbits with *P. ovis* from sheep but could induce an infestation within the rabbit’s auditory canal; however, an infestation on the backs of sheep or in the auditory canal could not be established using *P. cuniculi* (Siegfried et al., 2004).

Clearly, establishment of a new population is probabilistic and experimental studies often suffer from low replicate number, so it is difficult to draw conclusions from many of the small scale individual studies.

Recent studies using ELISA tests show cross-reactivity between various isolates of *Psoroptes* mites taken from different hosts (Boyce and Brown, 1991). Genetic analysis has provided further evidence for the conspecificity of *Psoroptes* mites (Zahler et al., 1998). Characterisation of the second internal transcribed spacer of the rRNA in 15 different isolates of *Psoroptes* mites collected from various hosts from 4 continents, found that the isolates were highly homogenous with no discernable segregation (Zahler et al., 1998).
Another study using 9 polymorphic microsatellite markers showed that although there was some genetic segregation between mites taken from different species there was not enough difference to suggest separate species status (Pegler et al., 2005). Morphological characterisation of mites from different hosts showed that there was no clear relationship between morphology of the mites and the different host species or geographic regions from where they were taken (Pegler et al., 2005).

In the light of the recent genetic and morphological evidence supporting the grouping of *Psoroptes* mites into one species with phenotypically adapted host-derived populations, it has been suggested that the genus should be synonymized and that taxonomic priority should be given to the name *Psoroptes ovis* (Hering) (Wall and Kolbe, 2006).

### 3.1.4 Prevalence and distribution of scab

Sheep scab is prevalent in many countries across Europe, Asia, southern Africa and South America (Kirkwood, 1986). It was introduced but then eradicated in Australia and New Zealand and it has also largely been eliminated from Norway, Denmark, Sweden, Canada and the U.S.A. (Seddon, 1964; Kirkwood, 1986).

Scab has been a problem in Britain for many hundreds of years with the first systematic data on prevalence dating back to 1807, when 2573 outbreaks were recorded nationally (Kirkwood, 1986). In 1905, the British government made dipping with approved dips compulsory, leading to a reduction in the number of recorded outbreaks. By 1914 there were just 226 outbreaks (Kirkwood, 1986). After a slight rise in the prevalence of scab during the first world war, the number of cases decreased until, in 1952, the disease was thought to have been eradicated (Kirkwood, 1986). However, in 1971, the disease was inadvertently reintroduced into Britain, probably as a result of importing a scab infested sheep from Ireland and was initially misdiagnosed (Loxam, 1974; Tarry, 1974). In 1973, 27 new outbreaks were recorded.

As the incidence of scab continued to rise, dipping was once again made compulsory in 1976. This led, initially to a decrease in the number of outbreaks. However, a switch
from autumn to summer dipping in 1983 resulted in an increase in scab outbreaks (French et al., 1999). Twice-yearly dipping was then made compulsory in 1984, causing outbreak numbers to decline substantially. However, in 1989 the summer dip was removed (French et al., 1999). In that year, approximately 40 outbreaks were recorded (French et al., 1999). In 1991 the final compulsory dip was abolished; in that year there were approximately 120 outbreaks (French et al., 1999). With the deregulation of scab and the abolition of the compulsory dip the incidence of scab then increased and in 1997 up to 3000 outbreaks were estimated to have occurred nationally (Lewis, 1997).

By 1999, a postal questionnaire survey estimated that 5000 farms were likely to have been infested with scab in a one year period from October 1998 to September 1999 (Corke and Broom, 2000). More recently, another postal questionnaire survey estimated that approximately 9% of flocks in Britain had experienced at least one case of scab between March 2003 and February 2004 (Bisdorff et al., 2005). However in Scotland the prevalence was as high as 17% (Bisdorff et al., 2005). The authors estimated that the number of outbreaks in Britain was close to 7000 (Bisdorff et al., 2005). Clearly the prevalence of scab in the U.K. is currently growing rapidly.

3.1.5 Control of scab

The first dip to become commercially available was a wettable powder containing arsenic and sulphur produced by William Cooper in 1843 (Kirkwood, 1986). Previous treatment methods included ointments made from tar, butter and lard and washes containing lime, mercury, nicotine, turpentine or arsenic (Kirkwood, 1986). Following the development of the first commercial dip, formulations containing coal tar, creosote and other arsenic preparations were also used but none of these dips provided long term protection and only persisted in the fleece for a few days requiring a second dipping 10 – 14 days later (Kirkwood, 1985). The chemicals were also harmful to the sheep and would damage the fleece. In 1947, Downing tested organochlorines including gamma-hexachlorocyclohexane (lindane) and DDT and found that residual activity could last for up to 3 months. Lindane was approved for use in
dips in 1948 and was not withdrawn from the market until 1984 (Kirkwood, 1985). The use of lindane with the combined effort and cooperation of farmers and vets is believed to have been a major factor in the eradication of the disease in sheep in 1952 (Kirkwood, 1986).

The organophosphates diazinon and propetamphos, were approved for use in dips against scab in the early 1980s. The organochlorines were withdrawn from the market in 1985 after concerns about residues in meat and environmental damage (O'Brien, 1999). This meant that the organophosphates were the only available formulations for use in dips (Kirkwood and Quick, 1981, 1982; O'Brien 1999). The pyrethroid flumethrin was introduced onto the market in 1987 and later high cis-cypermethrin was also registered for use (Bates, 2004). Along with the organophosphates, diazinon and propetamphos, these were the only compounds available for use in dips. In 1999 however, all the organophosphates and pyrethroids were withdrawn (O'Brien, 1999). In the case of pyrethroids this was due to fears over environmental damage, as they are much more stable and able to persist in the environment for longer than the organophosphates (Cooke et al., 2004). Propetamphos was withdrawn for economic reasons. Diazinon was temporarily withdrawn to allow for the development of safer packaging and then placed back onto the market in 2001.

An alternative to dips for the control of scab are the macrocyclic lactones. These were developed in the 1970s as agricultural pesticides and first used in veterinary medicine in the 1980s as anthelmintic treatments against gastrointestinal parasites. Ivermectin is effective against scab following two subcutaneous injections of 200 μg/kg, given seven to ten days apart (Soll, 1992; O'Brien et al., 1993). Two other injectable avermectins have been found to be effective with just one subcutaneous injection: moxidectin at 200 μg/kg (O'Brien et al., 1994, 1996) and doramectin at 300 μg/kg (Bates et al., 1995). However the injectable avermectins, particularly ivermectin, have a relatively short period of residual activity meaning that great care must be taken to treat every sheep correctly and prevent reinfestation of the flock from contaminated housing (O'Brien, 1999).
Recently a bolus of ivermectin was developed to overcome the problems of the short residual activity seen with the injectables. A polypropylene cylinder is inserted into the rumen of the sheep where flexible wings expand from the side to prevent the cylinder from leaving the rumen. The bolus releases ivermectin at a rate of 20 - 40 µg kg\(^{-1}\) day\(^{-1}\) for 100 days and is able to provide 100% therapeutic efficacy (Forbes et al., 1999; O’Brein et al., 1999). However, while used in Australia, it is not registered for use in the U.K.

Insecticide resistance has become a growing concern in recent years. Resistant mites have been found in Scotland and South West England, in 1994, on sheep that had been dipped three times in flumethrin at the recommended dose (Synge et al., 1995). Organophosphate resistance was identified in 1996 with sheep dipped in propetamphos harbouring live mites up to 70 days (the end of the trial) after exposure (Clark et al., 1996). Resistance to flumethrin, cypermethrin and diazinon has also been demonstrated in vitro with a large proportion of mites surviving after a 1 minute immersion in a variety of concentrations (Coles and Stafford, 1999).

There is growing pressure to ban the use of the remaining diazinon organophosphate dip. The pressure has come from groups and lobbies concerned about the effects of dips on the environment as well as the effects they may have on human health. A study of 146 sheep farmers who had experienced long-term exposure to organophosphate dips performed significantly worse than a control group of 143 unexposed quarry workers in neuropsychological tests (Stephens et al., 1995). A study of 612 sheep farmers, suggested that acute poisoning from organophosphate dips is more likely to be responsible for the neurological problems seen in exposed farmers rather than long-term low level exposure (Pilkington et al., 2001). A telephone survey of 367 people who believed that they had become ill due to organophosphate exposure also concluded that it was those individuals that had high levels of exposure that reported more illness than those with long-term, low-level exposure (Fletcher and MacLehose, 2005).

Currently the only available organophosphate dip is diazinon and the only available alternatives are the injectable avermectins. Resistance, environmental concerns and potential health problems associated with the organophosphate dips mean that diazinon might not be
available for use much longer. The avermectins have short residual activity, are expensive and require long withdrawal periods for meat and milk. When these problems are combined with the increasing prevalence and incidence of the disease there is clearly a pressing need for alternative treatments.

One alternative being investigated is the use of natural plant oils. In vitro bioassays have shown that extracts of Cinnamomum zeylanicum Blume, can induce 100% mortality in mites collected from rabbits ears after 24 h exposure to 2.5 ml paraffin oil containing 0.3% C. zeylanicum oil. (Fichi et al., 2007). The same study also demonstrated a reduction in mite numbers and clinical signs of otoacarasis in infected rabbits treated with the oil. Previous studies have shown, in vitro, that the compounds containing free alcoholic or phenolic groups have the most potent effect (Perrucci et al., 1995).

Attempts have also been made at producing a vaccine against P. ovis. However, only limited success has been achieved. Sheep immunized with soluble extracts of P. ovis showed a two-fold decrease in lesion size and a seven-fold decrease in mite number compared to the control sheep (Smith et al., 2002). A later experiment that fractionated the extracts to potentially enrich the inoculum with target antigens found a three-fold decrease in lesion size with a thirteen-fold decrease in mite numbers (Smith and Pettit, 2004). However, suitable antigens have yet to be confirmed and tested as targets for use in a vaccine, although there are several candidates such as homologues of tropomyosin (Der p 10), paramyosin (Der p 11) and an apolipoprotein-like Ig E reactive protein (Der p 14) (Huntley et al., 2004). Although progress has been made in the identification of suitable target antigens, a potential vaccine still seems to be long way in the distance.

3.1.6 Entomopathogenic fungi to control scab

A potential alternative to conventional insecticides for the control of scab is the use of entomopathogenic fungi. In vitro trials showed that M. anisopliae infected 71% of the adult female mites which were exposed to 3 ml of a 1x10⁷ conidia ml⁻¹ suspension for 10 s (Smith et al., 2000). Hirsutella thompsonii however showed no pathogenicity and did not
infect any mites (Smith et al., 2000). Another study had similar success infecting 77% of P. ovis which were immersed in a 1x10^6 conidia ml^-1 suspension (Brooks and Wall, 2002). It was also shown in the Brooks and Wall study that cadavers of infected mites could be infectious to live mites that came into contact with the sporulating corpse. Chambers that had various ratios of live naïve mites and infected cadavers showed that when 10 infected cadavers were housed with 10 live naïve mites, over 65% of naïve mites became infected (Brooks and Wall, 2002). However, it only took 1 infected cadaver introduced to 19 live naïve mites to infect up to 40% of naïve mites (Brooks and Wall, 2002).

Psoroptes ovis completes its life-cycle on the host and as such it inhabits a relatively stable microclimate. This could be advantageous to the use of entomopathogens, provided that the conditions on the host animal are suitable for the germination of the fungus. The conditions at the skin surface range between 31 °C to 37 °C and in dry conditions the humidity at the skin surface is usually 10% below atmospheric (Wall et al. 1992). Conidia of M. anisopliae have been found to germinate at temperatures between 15 °C and 35 °C with relative humidities above 92% needed (Walstad et al., 1970). This means that temperatures are generally appropriate to allow M. anisopliae to infect P. ovis on the host animal, although the humidity is often relatively low. Nevertheless, the peak germination temperature for M. anisopliae is 25 °C (Walstad et al., 1970), hence strain selection is important to find an isolate with a higher peak germination temperature. Various isolates of M. anisopliae have been examined for their infectivity against Psoroptes taken from the ears of rabbits, at a range of different temperatures between 28 °C and 40 °C (Brooks et al., 2004). Comparisons were made between isolates from Brazil, Denmark, France and the USA suspended in Tween 80. The number of fatal infections was reduced significantly at temperatures above 30 °C in all except a French isolate, where there was a slight, although insignificant, increase at 32.5 °C (Brooks et al., 2004). No infections were seen at 40 °C. Significantly higher levels of infection were seen when the conidia were formulated in silicone oil rather than Tween 80 (Brooks et al., 2004).
Another fungal pathogen that could potentially be used for the treatment of scab is *Beauveria bassiana*. When adult female mites were exposed, *in vitro*, to suspensions of $10^7$ conidia ml$^{-1}$ in Tween 20, 100% of the mites showed signs of infection and increased mortality, compared to the controls (Lekimme et al., 2006). Fungal infection with *B. bassiana* is also transmissible between infected cadavers and live naïve mites (Lekimme et al., 2006). When 10 infected cadavers were housed in chambers with 10 live naïve mites, all mites showed signs of infection at the end of the trial (Lekimme et al., 2006). Although several studies have demonstrated the lethal effects and potential for transmission of infection between mites, no previous studies have been able to induce fatal infections in *Psoroptes* mites *in vivo*.

The aims of the work described in this chapter were to investigate the effects of formulation on the pathogenicity of fungal pathogens and pathogenicity of fungal pathogens to *P. ovis* in the presence of sheepskin and fleece. The potential for *M. anisopliae* and *B. bassiana* to be used as a preventative or curative treatments for sheep scab were also examined *in vivo*. Because of the complexity of the series of experiments described in this chapter, they will be presented as methods followed by results for each individual experiment.

Due to the nature of the collaboration between Central Science Laboratory (CSL), Sand Hutton, CAB International (CABI), Ascot and the University of Bristol, there were several logistical constraints imposed upon the work that could be carried out during the course of this project. This impacted heavily upon the planning and conduct of the various trials presented in this chapter. Previous work (Brooks, 2004) had already screened many isolates of *M. anisopliae* and identified an appropriate isolate for further testing. Therefore the project started with *in vivo* trials rather than with more *in vitro* work. However, due to a miscommunication between collaborators, the first *in vivo* trial used the less virulent Danish strain of *M. anisopliae* rather than the French strain that was previously identified as the most promising strain. This led to the *in vitro* bioassay (section 3.2.2) to demonstrate the
greater virulence and pathogenicity of the French strain used throughout the rest of this study. Another in vivo trial was then conducted using the more pathogenic strain.

In vivo and in vitro trials were carried out alternately due to time constraints and a necessity to demonstrate the effects of fungal pathogens to *P. ovis* in vivo. The methods and results are presented in a chronological order to demonstrate the problems and how they were addressed so that a better understanding of the overall project can be obtained.

### 3.2 Methods and Results

#### 3.2.1 Analysis

All data were checked for normality using a Kolomogorov-Smirnov test. Proportional data were arcsine transformed prior to statistical analysis, however, figures show the untransformed data for clarity.

#### 3.2.2 The pathogenicity of *Metarhizium anisopliae* against *Psoroptes ovis* in vivo (Trial 1)

**Materials and methods**

An *in vivo* trial was used to test whether *M. anisopliae* could be used as a pour on treatment for sheep scab. Eighteen pathogen-free, Scottish blackface cross, female sheep, aged between 6 and 9 months were used in this trial. The sheep were maintained indoors in approved containment facilities at the Central Science Laboratory (CSL), Sand Hutton.

To infest the sheep with *P. ovis*, a small tuft of fleece (5 mm in diameter) was removed from the withers approximately 30 cm from the base of the head and 25 adult female mites were placed onto the exposed skin. Fleece from the surrounding area was then pulled over the bare skin and mites and held in place with a rubber band. Each sheep received a single inoculum of mites. The sheep were checked for the presence of mites 14 days after the initial seeding. The infestations were allowed to develop for 28 days with no intervention. On day 28 the size and position of the lesions were recorded and the sheep were divided into 3 groups of 6 animals. One group of sheep received no treatment, one
Chapter 3 – Effects of fungal pathogens on *Psoroptes* mites

group of sheep had 100 ml of silicone oil applied per sheep and one group had 100 ml of *M. anisopliae* IM1386698 (Danish strain isolated from *Agrotis segetum* (Denis and Schiffermuller), Lepidoptera: Noctuidae) conidia in silicone oil at a concentration of $5 \times 10^8$ conidia ml$^{-1}$ applied per sheep. The fungal suspension and silicone oil were supplied by CABI Bioscience, Ascot.

The silicone oil and the conidial suspension were applied to the dorsal surface of the sheep using a dial-a-dose drench gun with a spray nozzle (Forlong and Masey, Ripon) in 4 rows of 25 ml from the base of the head to the base of the tail. The rows were evenly spaced across the back of the sheep to ensure an even application of treatment over the dorsal surface of the sheep. The drench gun was primed with the relevant treatment before application to the sheep. The sheep that received a dose of silicone oil were treated first to prevent any contamination from the conidial suspension. During application, the sheep were restrained by holding the head of the sheep between the legs of the handler. After the sheep had received their treatment they were housed separately in their respective groups ensuring that no contact could occur between the groups.

Wash samples from the lesion were taken from each sheep 42 days after the initial seeding with mites (14 days after the groups had been treated with silicone oil or the conidial suspension). The wash samples were taken along a transect of the lesion, taking two samples from the edge of the lesion and one sample from the middle of the lesion (Fig. 3.1). This was achieved by flushing a $1 \text{ cm}^2$ area of the lesion with phosphate buffered saline (PBS) using a syringe with a dome attachment on the nozzle. A secure fit was achieved by pressing the dome of the collection nozzle against the surface of the skin before flushing the area with the PBS. A second wash sample was also taken from each sheep 49 days after the initial seeding (21 days after treatment). The mites were removed from the wash samples by straining the PBS through a filter paper. The dampened filter paper and mites were placed into a petri dish which was sealed with parafilm, refrigerated over night and transported to the University of Bristol usually a maximum of 24 h after collection from the sheep. The following day, up to 20 adult female mites were removed from each wash sample using a
fine grade paint brush that had been washed in 70% ethanol, and incubated in the standard incubation chamber (Fig. 2.4) with 500 μl of sheep serum at 30 °C and 90% r.h. If there were not enough adult females, female tritonymphs, adult males and male tritonymphs were used. Chambers were checked for the presence of dead mites every 24 h. Dead mites were removed and checked for signs of infection as described in Chapter 2.

At the end of the trial, day 55 (27 days after treatment) the sheep were killed by a schedule 1 method and the lesions and surrounding skin were removed. Four samples of skin were excised from each sheep, placed into re-sealable plastic bags and refrigerated over night at CSL and then posted to the University of Bristol by special delivery (sent on day 56 of the trial), arriving the next morning (day 57). The skin samples were removed along a transect of the lesion taking two samples from the edge of the lesion, one sample from the middle and one sample from outside of the lesion (Fig. 3.1). The samples were approximately 15 x 15 cm squares, stored in sealable plastic bags. Once the samples had been received at Bristol up to 30 adult female mites were removed from each sample. Mites were removed using a fine grade paintbrush and forceps that had been washed in 70% ethanol. Different forceps and brushes were used for each treatment group. If there were not enough adult females, female tritonymphs, adult males and male tritonymphs were used. Mites were incubated in standard incubation chambers and checked for signs of infection as previously described.

For both of the wash samples and the skin samples, mites were removed from 3 randomly selected sheep from each group and the same sheep were used for both wash samples and the skin samples.

Application of the mites, treatment and euthanasia of the sheep were carried out by suitably registered staff at the CSL.
Fig. 3.1. Diagram depicting the 3 sites within a scab lesion and 1 site outside of the lesion that were excised from the sheep skin at the end of the first *in vivo* trial and sent to the University of Bristol for examination. A 1 cm$^2$ area within each site was sampled using PBS washes at days 42 and 49 of the trial. The diagram is not drawn to scale.
Results

For the wash samples taken at day 14, a total of 39 mites were removed from the control group, 47 mites were removed from the group treated with silicone oil and 50 were removed from the group treated with conidia suspended in silicone oil. For the wash samples taken at day 21, a total of 56 mites were removed from the control group, 51 mites were removed from the group treated with silicone oil and 39 were removed from the group treated with conidia suspended in silicone oil. For the skin samples taken at the end of the trial (day 27), a total of 187 mites were removed from the control group, 248 mites were removed from the group treated with silicone oil and 202 mites were removed from the group treated with conidia suspended in silicone oil. Hence, for the whole trial, a total of 282 mites removed from the control sheep, 346 mites removed from sheep treated with silicone oil and 291 mites removed from sheep treated with conidia suspended in silicone oil were examined for infection. However, only 2 mites showed any infection with *M. anisopliae*. Both mites were removed in the wash samples taken at day 14 from two sheep in the group treated with conidia suspended in silicone oil.
3.2.3 *The pathogenicity of two strains of* Metarhizium anisopliae *in vitro*

To try to account for the failure of the fungal inoculation to infect mites in the *in vivo* trial, an *in vitro* investigation was undertaken to compare the Danish *M. anisopliae* strain used, against a French strain.

*Materials and methods*

Adult female mites were obtained from the ears of artificially infested rabbits. The mites were then inoculated with one of three treatments: silicone oil, *M. anisopliae* IM1386697 (French strain isolated from *Sitonia discoides* Gyllenhal, Coleoptera: Curculionidae) suspended in silicone oil and *M. anisopliae* IM1386698 (Danish strain) suspended in silicone oil. The conidial suspensions were made to a concentration of $1 \times 10^8$ conidia ml$^{-1}$ for both fungal isolates. Once the mites had been inoculated, as described in Chapter 2, they were transferred in their respective treatment groups to glass incubation chambers (Fig. 2.4) and incubated at 30 °C and 90 % relative humidity. Each chamber housed 20 adult female mites. The chambers were checked every 24 h for dead mites which were then removed and checked for signs of infection every 24 h as previously described. There were 4 replicates for each treatment.

*Results*

The proportion of infected mites was highest for the groups of mites treated with the French strain of *M. anisopliae* (IM1386697) with a mean proportion of 0.91 (± 0.18) (Fig. 3.2.). For the Danish strain, a mean proportion of 0.38 (± 0.32) of mites became infected. There were no infections in the groups treated with silicone oil only. This difference was highly significant (ANOVA; $F_{2,12} = 19.99, P < 0.001$; Fig. 3.2.). There was a significantly greater proportion of infected mites in the groups exposed to the French strain than the groups of mites exposed to the Danish strain (Tukey HSD; $P = 0.013$).
It seems likely therefore that the failure of the first *in vivo* trial may have been caused by the lack of pathogenicity of the Danish strain used.
Fig. 3.2. The proportion of adult female mites that became infected with *M. anisopliae* after exposure to silicone oil only, conidia of a French strain (IMI386697) suspended in silicone oil and conidia of a Danish strain (IMI386698) suspended in silicone oil at 30 °C and 90% r.h.. Conidia of both strains were suspended at a concentration of $1 \times 10^9$ conidia ml$^{-1}$. Each data point represents 1 replicate of 20 adult female mites. There were 4 replicates for each group.
### 3.2.4 Treatment of sheep infected with sheep scab with *Metarhizium anisopliae* (Trial 2)

A second *in vivo* trial was used to investigate the potential use of *M. anisopliae* against sheep scab using a more carefully controlled application procedure.

**Materials and methods**

Nine pathogen-free, poll Dorset female sheep, aged between 6 and 12 months were used in this trial. As for the previous sheep trial the animals were maintained indoors in approved containment facilities at the Central Science Laboratory (CSL), Sand Hutton.

On day 1 of the trial, each sheep had an area of fleece sheared down to the skin to create an area of unsheared fleece 10 x 10 cm square, surrounded by a margin of sheared fleece 2 cm wide. This margin of sheared fleece was designed to act as a deterrent to migration for the mites that were placed within the enclosed 10 x 10 cm square area. The sheep were divided into groups of 3 and housed in separate units depending upon the treatment each group would receive. The first group, to act as a control, was inoculated with mites on day 1 of the trial and received no treatment. The second group was inoculated with mites on day 1 of the trial and then exposed to conidia of *M. anisopliae* IMI386697 on day 21 to act as a curative treatment. The third group was exposed to conidia of *M. anisopliae* IMI386697 on day 20 of the trial and then inoculated with mites on day 21. The same method of inoculation with 25 adult female mites was used in this trial as for the previous sheep trial, except that the mites were placed into the centre of the area of skin surrounded by the sheared margin. For the groups treated with *M. anisopliae*, sheep were exposed to 0.4g of the unformulated conidia (1 x 10^{11} conidia g^{-1}). The conidia were applied by parting the fleece in 3 evenly spaced lines horizontally and then 3 lines vertically and finally one line diagonally across the 10 x 10 cm square with the conidia sprinkled evenly into the parted fleece.

By the end of the trial the control sheep had been exposed to mites for 42 days with no treatment. The curative group had been exposed to mites for 42 days but were treated with *M. anisopliae* conidia on day 21. The preventative group had been treated with *M.
Anisopliae conidia on day 20, before inoculation with mites on day 21, and therefore had only been exposed to mites for 21 days.

At the end of the trial, day 42, the sheep were killed by a schedule 1 method and the skin containing and surrounding the lesions was excised and placed into re-sealable plastic bags and refrigerated over night. The following day (day 44), 3 groups of 30 adult female mites were removed from each skin sample in the same way as described for the previous sheep trial. These mites were then incubated with 30 mites per chamber (Fig. 2.4) at 30 °C and 90% r.h. with 500 μl of sheep serum. Each chamber was inspected every 24 h for dead mites which were removed and checked for signs of infection as described in Chapter 2.

The skin samples and any remaining mites were then frozen at -13 °C (± 2 °C). After 28 days in deep freeze (day 72), the samples were thawed at room temperature and then washed with distilled water in an attempt to remove as many mites as possible. The mites removed by the washing were placed into 90 mm petri dishes with damp filter paper and incubated at 30 °C and 90 % R.H. and inspected every 24 h for 14 days to check for signs of infection.

Results

Inspections made during the trial showed that not all of the sheep inoculated with P. ovis developed lesions. The sheep that showed no obvious signs of infestation during the trial were not found to have any mites present on the skin samples inspected at the end of the trial. There were 4 sheep in total where no mites could be found, including 1 sheep from the control group, 2 sheep from the curative group and 1 sheep from the preventative group. This meant that on day 44 of the trial, 180 mites were examined from the control group, 180 mites were examined from the preventative group and 90 mites were examined from the curative group. However, none of these mites showed any signs of infection. On day 72 of the trial, 203 mites were removed from the control group, 63 mites were removed from the
preventative group and 104 mites were removed from the curative group. Surprisingly, only 4 mites, removed from the control sheep B909, showed signs of infection (Table 3.1).

Table 3.1. The number of mites removed from the skin samples of each sheep after euthanasia (day 44) and from the wash of the skin on day 72 and the number of infected mites found from each sheep.

<table>
<thead>
<tr>
<th>Sheep</th>
<th>Treatment</th>
<th>Mites removed day 44</th>
<th>Mites removed day 72 (wash)</th>
<th>Number of infected mites</th>
</tr>
</thead>
<tbody>
<tr>
<td>B909</td>
<td>Control</td>
<td>90</td>
<td>106</td>
<td>4</td>
</tr>
<tr>
<td>B919</td>
<td>Control</td>
<td>90</td>
<td>97</td>
<td>0</td>
</tr>
<tr>
<td>B900</td>
<td>Preventative</td>
<td>90</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>B904</td>
<td>Preventative</td>
<td>90</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td>B911</td>
<td>Curative</td>
<td>90</td>
<td>104</td>
<td>0</td>
</tr>
</tbody>
</table>
3.2.5 The pathogenicity of Metarhizium anisopliae to Psoroptes ovis in vitro in the presence of sheep skin

To try to account for the second failure to infect mites in vivo, it was considered that the skin or fleece itself may have some inhibitory compounds. An in vitro bioassay was conducted to test this.

Materials and methods

The skin of a freshly-killed lamb with no history of treatment for P. ovis was obtained from an abattoir. A series of 20 mm diameter circles were cut from the skin and the fleece attached to these circles was cut to a length of 20 mm. Once a disc of skin and fleece had received its appropriate treatment, it was placed into an incubation chamber (Fig. 2.4).

Four treatment groups were used: untreated fleece only, fleece to which conidia of M. anisopliae suspended in silicone oil had been added, fleece to which only silicone oil had been added and, as a positive control, cotton cloth to which conidia suspended in silicone oil had been added.

Treatments containing conidia used a suspension of $1 \times 10^8$ conidia ml$^{-1}$ of M. anisopliae IMI386697 (French strain) in silicone oil (dimethylpolysiloxane hydrolyzate, Sigma-Aldrich, Dorset, UK). The silicone oil, or silicone oil with its suspended conidia, were applied using a household plant sprayer (Hozelock Group Ltd. Aylesbury, UK) fixed 20 cm directly above the skin or cloth using a clamp stand. This distance between the nozzle of the sprayer and the discs of skin or cloth was chosen to give an even application of the treatment across the skin or cloth and to simulate a possible application method for use on sheep. The sprayer delivered a standard 0.08 ml of conidia suspended in silicone oil to each surface resulting in the application of $8 \times 10^6$ conidia per disc.

After application, the skin and cloth were placed into the chambers and left for 1 h before the addition of 20 adult, female Psoroptes mites, collected from the ears of experimentally infested rabbits on the same day that they were used in the experiment.
In the cloth treatment, 500 µl of ovine serum was also added (Sigma-Aldrich, Poole, UK) before the addition of the mites. Five replicates of each treatment were set up. The mites were then left in their treatment chambers for 24 h at 30 °C and 90% relative humidity. After 24 h, all mites were removed from the chambers and transferred, in their respective treatment groups, to clean incubation chambers where they were incubated at 30 °C and 90% r.h. with 500 µl of sheep serum. The chambers were inspected every 24 h for the presence of dead mites which were removed with a fine grade paint brush and checked for signs of infection as previously described. Different paint brushes were used for each of the treatments. The brushes were washed in 70% ethanol and wiped dry between each sample.

Results

In the groups that received no treatment or silicone oil only, there were no infected mites (Fig. 3.3). In the group that was exposed to silicone oil plus conidia on the skin, a mean of 0.52 (± 0.34) mites became infected and for the group exposed to silicone oil plus conidia on the cloth, a mean of 0.34 (± 0.31) mites became infected. This difference was highly significant (ANOVA; F3, 19 = 8.4, P = 0.001; Fig. 3.3). There was no significant difference in the proportion of infected mites in the two groups exposed to silicone oil and conidia whether on fleece or cloth (Tukey HSD post-hoc test, P > 0.05; Fig. 3.3). Clearly the sheep fleece itself did not inhibit germination of conidia and infection of mites.
Fig. 3.3. Mean proportion of mites (± S.D.) showing signs of fungal infection after 24 h exposure to untreated fleece, fleece treated with silicone oil, fleece treated with silicone oil plus *Metarhizium anisopliae* (IMI386697) conidia ($1 \times 10^8$ conidia ml$^{-1}$) and cloth treated with silicone oil plus *M. anisopliae* (IMI386697) conidia ($1 \times 10^8$ conidia ml$^{-1}$).
3.2.6 The pathogenicity of five high temperature tolerant strains of Metarhizium anisopliae against Psoroptes ovis in vitro

One possible reason for the failure of the first two in vivo trials was that the temperature at the sheep skin was too high to allow germination of the conidia. An in vitro trial was conducted to test 5 new strains of M. anisopliae that had exhibited tolerance to high temperatures for their pathogenicity against P. ovis.

Materials and methods

The standard inoculation method as described in Chapter 2, was used to inoculate adult female mites with the 5 new strains of M. anisopliae at 37 °C and 90% r.h. Each strain was suspended in silicone oil to give a concentration of 1 x 10⁸ conidia ml⁻¹. Each treatment was tested against 4 replicates of 20 mites. Another 4 replicates of 20 adult female mites was set up that were treated with silicone oil only. Table 3.2 gives information on the strains used.

Table 3.2. The strains of Metarhizium anisopliae, reference numbers and the original host from which the fungi were isolated.

<table>
<thead>
<tr>
<th>Species</th>
<th>Reference number (ARSEF)</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. anisopliae</em></td>
<td>1545</td>
<td>Scotinophara coarctata</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Hemiptera: Pentatomidae)</td>
</tr>
<tr>
<td><em>M. anisopliae var. acridum</em></td>
<td>2575</td>
<td>Curculio caryae</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Coleoptera: Curculionidae)</td>
</tr>
<tr>
<td><em>M. anisopliae var. acridum</em></td>
<td>3609</td>
<td>Patanga succincta</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Orthoptera: Acrididae)</td>
</tr>
<tr>
<td><em>M. anisopliae var. acridum</em></td>
<td>3619</td>
<td>Oxya multidentata</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Orthoptera: Acrididae)</td>
</tr>
<tr>
<td><em>M. anisopliae var. acridum</em></td>
<td>324</td>
<td>Austracris gutulosa</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Orthoptera: Acrididae)</td>
</tr>
</tbody>
</table>
Results

There were no infected mites found in any of the treatment groups. None of the strains tested could infect mites at 37 °C.

3.2.7 The pathogenicity of Beauveria bassiana against Psoroptes ovis in vitro

It was decided also to consider the pathogenicity of \textit{B. bassiana} as an alternative to \textit{M. anisopliae}. Using the standard inoculation method described in Chapter 2, the pathogenicity of \textit{B. bassiana} was tested against adult female \textit{P. ovis}. The strain of \textit{B. bassiana} was obtained from a naturally infected mite found on a sheep at the CSL and will be described as CSL strain 1.

Materials and methods

The conidia of \textit{B. bassiana} were suspended in silicone oil at a concentration of $1 \times 10^8$ conidia ml$^{-1}$. There were 5 replicates of 20 mites used. A further 5 replicates of 20 mites were set up and treated with silicone oil only as controls.

In a further experiment, the pathogenicity of \textit{B. bassiana} formulated in 0.05% Tween 80 at various concentrations was tested against \textit{P. ovis}. For each concentration, 3 replicates of 20 adult female mites were inoculated using the standard method. The concentrations tested were: Tween 80 only, $1 \times 10^5$, $1 \times 10^6$, $1 \times 10^7$, $1 \times 10^8$, $1 \times 10^9$ conidia ml$^{-1}$. A further 3 replicates of 20 mites were left untreated to act as a control.

For both experiments, mites were collected from the ears of experimentally infested rabbits kept at CSL the day before the experimental tests and sent to the University of Bristol overnight by special delivery.

Results

A mean proportion of 0.58 (± 0.08) of mites inoculated with \textit{B. bassiana} suspended in silicone oil at $1 \times 10^8$ conidia ml$^{-1}$ became infected, whereas none of the mites exposed to silicone oil became infected (Fig. 3.4). The mites exposed to \textit{B. bassiana} had a mean LT$_{50}$
of 6 (± 0.55) days and the mites exposed to silicone oil had an LT$_{50}$ of 6.4 (± 0.71) days (Fig. 3.5). This was not a significant difference (ANOVA; $F_{1.8} = 1.0, P > 0.05$).

When *B. bassiana* was formulated in Tween 80, a high proportion of infected mites were found at all the concentrations tested. No infected mites were found in the groups exposed to Tween 80 only or groups that received no treatment (Fig. 3.6). This was a significant difference (ANOVA; $F_{6.14} = 974.06, P < 0.001$; Fig. 3.6). However, there was no significant difference in the proportion infected between the groups treated with *B. bassiana* suspended in Tween 80, regardless of concentration (Tukey HSD; $P > 0.05$). All of the groups treated with *B. bassiana* had significantly more infected mites than the control or Tween 80 only groups (Tukey HSD; $P < 0.001$).

There was no significant difference in LT$_{50}$ between any of the different concentration groups exposed to *B. bassiana*. The mites in the control groups had significantly longer LT$_{50}$ values than any of the groups of mites exposed to conidia (ANOVA; $F_{6.14} = 20.9, P < 0.001$; Fig. 3.7).

The results show that this strain of *B. bassiana* is highly pathogenic to *P. ovis* even at relatively low concentrations.
Fig. 3.4. Proportion of adult female mites infected with *Beauveria bassiana* when exposed to silicone oil only or *B. bassiana* suspended in silicone oil at a concentration of $1 \times 10^8$ conidia ml$^{-1}$ at 30 °C and 90% r.h. Each data point represents 1 replicate of 20 adult females.
Fig. 3.5. LT_{50} (± S.D.) of mites surviving after exposure to silicone oil or *Beauveria bassiana* conidia suspended in silicone oil at a concentration of $1 \times 10^8$ conidia ml^{-1} at 30 °C and 90% r.h.. There were 4 replicates used for each group.
Fig. 3.6. Mean proportion of mites infected with *Beauveria bassiana* when exposed to no treatment, Tween 80 only or *B. bassiana* conidia suspended in Tween 80 at various concentrations at 30 °C and 90% r.h.. There were 3 replicates used for each group.
Fig. 3.7. LT50 of adult female mites after exposure to no treatment (control), Tween 80 only or Beauveria bassiana conidia suspended in Tween 80 at various concentrations; 1 x 10^5, 1 x 10^6, 1 x 10^7, 1 x 10^8 and 1 x 10^9 conidia ml⁻¹ at 30 °C and 90% r.h.. There were 3 replicates of 20 mites used for each treatment. Letters show groups between which there was no significant difference (P > 0.05).
3.2.8 Control of Psoroptes ovis using entomopathogenic fungi in vivo (Trial 3)

A yet more controlled approach was used for the third in vivo trial to investigate whether or not *P. ovis* can be infected by fungal pathogens on a host animal.

**Materials and methods**

Six, pathogen-free, poll Dorset sheep aged between 6 to 12 months were used in this trial. The sheep were maintained indoors as a single group, on straw in approved containment facilities, at the CSL.

Each sheep had six circular rubber arenas glued to the skin on its back (three either side of the midline) giving a total of 36 arenas (Fig. 3.8). Prior to attachment, the fleece was shorn close to the skin and the arenas were attached with surgical glue. Each arena had an internal diameter of 25 mm and was 10 mm high. Each arena was sealed with fine mesh cotton held in place with a tight fitting rubber washer placed on the inside of the arena (Fig. 3.8).

Treatments were allocated to specific sheep/arenas at random, with the constraint that no one sheep had more than one treatment of any particular type and that each sheep had one arena to which no treatment was applied. Thus, the skin in a total of six arenas acted as controls and had no treatment applied. The skin of six of the arenas received 0.01 g of dry spores of *M. anisopliae* (isolate IMI386697) at a concentration of $1.8 \times 10^{10}$ conidia g$^{-1}$, three arenas were treated with 2 ml of the same isolate of *M. anisopliae* conidia suspended at $1.5 \times 10^9$ conidia ml$^{-1}$ in sheep dip excipient (70% Octyl oil plus stabilizers, diluted to 15% w/v in water) and three arenas were treated with 0.01g of the same isolate of *M. anisopliae* conidia mixed in 0.001g of diatomaceous earth giving a concentration of $1.6 \times 10^{10}$ conidia g$^{-1}$. A further six arenas were treated with 0.01 g of a mixture of two different isolates of *M. anisopliae* (ARSEF3297 isolated from *Boophilus* spp., Acari: Ixodidae, Mexico) and (ARSEF4556 isolated from *Boophilus* spp., Acari: Ixodidae, U.S.A.) at a concentration of $8 \times 10^9$ conidia g$^{-1}$. 
Beauveria bassiana (CSL strain 1) was also examined. For this, six arenas were treated with 0.01 g of dry spores of B. bassiana (1.2 x 10^{11} conidia g^{-1}), three arenas were treated with 2 ml of B. bassiana conidia suspended at 1 x 10^{10} conidia ml^{-1} in sheep dip excipient and three arenas were treated with 0.01 g of B. bassiana conidia mixed in 0.001 g of diatomaceous earth (1.1 x 10^{11} conidia g^{-1}). The skin in each of the arenas was treated 1 h before mites were applied.

For the trial, 25-30 adult female Psoroptes mites were placed inside each arena. The mites had been removed from infested sheep 72 h prior to being placed on the experimental sheep. Prior to application to the experimental sheep, half of the groups of mites were maintained in clean chambers at 90% r.h. and 30 °C and offered 500 μl of sheep serum.

The other half of the mites were exposed to formulations of either M. anisopliae or B. bassiana. Among these groups, the mites to be exposed to dry conidia on the host were first dipped in suspensions of 1 x 10^8 conidia ml^{-1} of either M. anisopliae or B. bassiana in Tween 80 for 1 min. The mites were then transferred to a dry piece of filter paper for 1 min to allow any excess fluid to run off before being placed into a clean incubation chamber. The mites to be exposed on the sheep to conidia in sheep dip excipient or conidia mixed in diatomaceous earth were placed onto filter paper in a 45 mm diameter Petri-dish which had been treated with the same volume and concentration of the treatment to which they were to be exposed on the sheep for 48 h.

Hence, on the experimental sheep, half the arenas containing dry spores, contained mites that had been pre-exposed, while half contained mites that had not. All the arenas treated with conidia in dip excipient and all the arenas treated with conidia in diatomaceous earth contained mites that had been pre-exposed. Table 3.3 summarizes the treatment regimes used.

At the end of a 48 h period on host, the sheep were euthanized by a schedule 1 method and the skin within and surrounding the chambers was excised. The skin and arenas were then packed into individual sealable plastic bags and refrigerated at 5 °C over night. The following day the mites were removed from the arenas, surface sterilised with 2%
sodium hypochlorite solution, placed into clean incubation chambers (Fig. 2.4) with 500 μl of sheep serum and incubated at 30 °C and 90% r.h. The chambers were inspected every 24 h for the presence of dead mites. A further 200 μl of sheep serum was added every 48 h until all the mites had died. Dead mites were treated as described previously and fungal infections were recorded when external hyphae could be observed protruding through the cuticle.

Fig. 3.8 Rubber arena attached to the skin on the dorsum of a sheep. The arenas had an internal diameter of 25 mm and a height of 10 mm.
Table 3.3. Summary of the fungal pathogens, excipients, concentrations and amounts applied to the arenas on the experimental sheep and the exposure method used to inoculate adult, female mites for the third *in vivo* trial.

<table>
<thead>
<tr>
<th>Fungal species</th>
<th>Excipient</th>
<th>Concentration</th>
<th>Amount applied</th>
<th>Exposure method</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. anisopliae</em> (IMI386697)</td>
<td>Dry conidia</td>
<td>$1.8 \times 10^{10}$ conidia g$^{-1}$</td>
<td>0.01g</td>
<td>48 h on host</td>
</tr>
<tr>
<td><em>M. anisopliae</em> (IMI386697)</td>
<td>Dry conidia</td>
<td>$1.8 \times 10^{10}$ conidia g$^{-1}$</td>
<td>0.01g</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td><em>M. anisopliae</em> (IMI386697)</td>
<td>Sheep dip excipient</td>
<td>$1.5 \times 10^9$ conidia ml$^{-1}$</td>
<td>2ml</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td><em>M. anisopliae</em> (IMI386697)</td>
<td>Diatomaceous earth</td>
<td>$1.6 \times 10^{10}$ conidia g$^{-1}$</td>
<td>0.01g + 0.001g</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td><em>B. bassiana</em> (CSL1)</td>
<td>Dry conidia</td>
<td>$1.2 \times 10^{11}$ conidia g$^{-1}$</td>
<td>0.01g</td>
<td>48 h on host</td>
</tr>
<tr>
<td><em>B. bassiana</em> (CSL1)</td>
<td>Dry conidia</td>
<td>$1.2 \times 10^{11}$ conidia g$^{-1}$</td>
<td>0.01g</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td><em>B. bassiana</em> (CSL1)</td>
<td>Sheep dip excipient</td>
<td>$1 \times 10^{10}$ conidia ml$^{-1}$</td>
<td>2ml</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td><em>B. bassiana</em> (CSL1)</td>
<td>Diatomaceous earth</td>
<td>$1.1 \times 10^{11}$ conidia g$^{-1}$</td>
<td>0.01g + 0.001g</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td><em>M. anisopliae</em> (ARSEF3297 + ARSEF4556)</td>
<td>Dry conidia</td>
<td>$8 \times 10^{9}$ conidia g$^{-1}$</td>
<td>0.01g</td>
<td>48 h on host</td>
</tr>
<tr>
<td><em>M. anisopliae</em> (ARSEF3297 + ARSEF4556)</td>
<td>Dry conidia</td>
<td>$8 \times 10^{9}$ conidia g$^{-1}$</td>
<td>0.01g</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td>Control (no fungal pathogen)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Results

There were no infected mites in any of the control groups. Infected mites were found in the treatment groups but there was no significant difference in the proportion infected between the group of mites that were exposed to the fungi for 48 h \textit{in vitro} followed by 48 h exposure \textit{in vivo} and the group of mites that only had the 48 h exposure \textit{in vivo} (ANOVA; $F_{2,12} = 11.15, P > 0.05$; Fig. 3.9). There was however, a significant interaction, as those mites exposed to the mixed strains of \textit{M. anisopliae} for 48 h \textit{in vitro} prior to exposure for 48 h \textit{in vivo} showed fewer infections than those mites exposed to the fungus for only 48 h \textit{in vivo} ($F_{2,12} = 11.15, P = 0.002$; Fig. 3.9).

Overall, \textit{B. bassiana} infected a significantly greater number of mites than \textit{M. anisopliae} (IMI386697) when compared in different formulations ($F_{2,12} = 9.93, P = 0.003$; Fig. 3.10). But formulation also had a significant effect on the level of infection ($F_{1,12} = 24.42, P < 0.001$; Fig. 3.10). Unformulated, dry conidia were significantly more infectious than conidia formulated in sheep dip excipient or diatomaceous earth for both \textit{M. anisopliae} and \textit{B. bassiana} (Tukey HSD post hoc test, $P < 0.05$). However, for \textit{B. bassiana}, the level of infectiousness remained high when in both sheep dip excipient and in diatomaceous earth, whereas for \textit{M. anisopliae} the level of infection observed with these two formulations was significantly lower (Fig. 3.10).

In this trial, the ability of \textit{M. anisopliae} and \textit{B. bassiana} to infect mites on a sheep was demonstrated but under very tightly constrained conditions.
Fig. 3.9. Mean proportion of adult female mites (± S.D.) showing fungal infection when treated with *Metarhizium anisopliae* (IMI386697), a mixture of *M. anisopliae* isolates (ARSEF3297 and ARSEF4556) or an isolate of *Beauveria bassiana* (CSL1) either for 48 h *in vivo* only (open squares) or 48 h *in vitro* followed by 48 h exposure *in vivo* (solid circles).
Fig. 3.10. Mean proportion (± S.D.) of adult female mites showing fungal infection when treated with either *Metarhizium anisopliae* (solid circles) or *Beauveria bassiana* (open squares) *in vivo* when formulated as dry spores, in sheep dip excipient or with diatomaceous earth.
3.2.9 The pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana* against *Psoroptes ovis* in various formulations in vitro

It was considered that one of the problems in *in vivo* trials 1 and 2 may have been associated with the formulation of the conidia, which might have prevented penetration to the skin surface. Therefore an *in vitro* bioassay was designed to examine the effects of formulation on the pathogenicity of fungal pathogens.

**Materials and methods**

Conidia of *Metarhizium anisopliae* IMI386697 (French strain) were suspended in various excipients at a concentration of $1 \times 10^9$ conidia ml$^{-1}$. The excipients used were: vegetable oil (rapeseed oil, Sainsburys, UK), Codacideo®, Output® and sunflower oil (Sainsburys, UK). Codacideo® is a commercially available oil-based adjuvant, containing rapeseed and emulsifiers (Microcide Ltd. U.K.; Jenkins and Thomas, 1996). It is designed to encapsulate the particles of the active ingredient, improving the solubility of hydrophobic compounds in water and also improving the spread of the active ingredient. Output® is also a commercially available oil-based adjuvant consisting of 60% mineral oils and 40% non-ionic surfactants (Syngenta Crop Protection Ltd. Switzerland). Each excipient, with and without fungal conidia, as well as a control group of no treatment, was tested for its pathogenic effects by inoculating 4 replicates of 20 adult female mites using the standard method as described previously. The mites were then incubated in the standard incubation chambers (Fig. 2.4) at 30°C and 90% r.h. with 500 µl of sheep serum. The chambers were inspected every 24 h for the presence of dead mites which were removed with a fine grade paint brush and checked for signs of infection as previously described.

The experiment was repeated replacing *M. anisopliae* with *B. bassiana* (CSL strain 1). The concentration used for the conidial suspensions was the same, $1 \times 10^9$ conidia ml$^{-1}$. Again, 20 adult female mites were inoculated using the same method as before but this time there were 5 replicates for each group.
Results

The highest proportion of infected mites was found in the group treated with *M. anisopliae* conidia suspended in vegetable oil where all of the mites became infected (Fig. 3.10). A high proportion of infection (0.99 ± 0.03) was also achieved with *M. anisopliae* suspended in Codacide® (Fig. 3.11). The groups treated with *M. anisopliae* suspended in Output® or sunflower oil had a lower proportion of infected mites at only 0.29 (± 0.125) and 0.25 (± 0.21) respectively (Fig. 3.11). There were no infected mites in the control group that received no treatment or any of the groups that were exposed to excipient only. The effect of treatment on the number of mites infected was highly significant overall (ANOVA; $F_{8,28} = 109.03, P < 0.001$) but there was no significant difference in the number of infected mites when exposed to *M. anisopliae* in vegetable oil and those mites exposed to *M. anisopliae* in Codacide® (Tukey HSD; $P > 0.05$; Fig. 3.11). There was also no significant difference between mites exposed to *M. anisopliae* suspended in Output® and those mites exposed to *M. anisopliae* suspended in sunflower oil ($P > 0.05$; Fig. 3.11). Each group of mites that were exposed to *M. anisopliae* showed significantly higher levels of infection than the groups of mites exposed to excipient only or no treatment ($P < 0.05$).

When the experiment was repeated with *B. bassiana* suspended in the various formulations, surprisingly a much lower number of infections was observed compared to when *M. anisopliae* was used (Fig. 3.12). Again, there were no infected mites for those exposed to excipient only. The highest proportion of infected mites (0.06 ± 0.065) was achieved with *B. bassiana* suspended in sunflower oil. All of the other treatments containing *B. bassiana* conidia had a mean proportion of 0.01 (± 0.02), infected mites. There was a significant difference in the number of infected mites between the different treatments when the controls were included (ANOVA; $F_{8,36} = 2.94, P = 0.012$). However, it was only the mites exposed to *B. bassiana* suspended in sunflower oil that had significantly greater numbers of infected mites than the control group and the excipient control group (Tukey HSD; $P = 0.016$; Fig. 3.12).
There was no significant difference in the number of infected mites between the groups that were exposed to *B. bassiana* in any of the formulations (*P* = 0.07; Fig. 3.12).
Fig. 3.11. Proportion of adult female mites showing infection after exposure to *Metarhizium anisopliae* conidia suspended in various formulations at a concentration of $1 \times 10^9$ conidia ml$^{-1}$ at 30°C and 90% r.h.. Each data point represents 1 replicate of 20 adult females. There were 4 replicates used for each treatment. Untreated and excipient only controls are not shown.
Fig. 3.12. Proportion of adult female mites showing infection after exposure to *Beauveria bassiana* conidia suspended in various formulations at a concentration of $1 \times 10^9$ conidia ml$^{-1}$ at 30 °C and 90% r.h. Each data point represents 1 replicate of 20 adult females. There were 5 replicates used for each treatment. Untreated and excipient only controls are not shown.
3.2.10 The pathogenicity of Metarhizium anisopliae and Beauveria bassiana in various formulations in vivo (Trial 4)

The fourth in vivo trial was designed to examine the effects of formulation and fleece length on the pathogenicity of fungal pathogens on the host under the same conditions as used in in vivo trial 3.

Materials and methods

Ten pathogen and insecticide free, poll Dorset sheep were used. The sheep were maintained indoors as a single group, on straw in approved containment facilities, at the CSL. Six circular rubber arenas, as used in the previous in vivo trial, with an internal diameter of 25 mm and height of 10 mm were attached to the dorsal surface of the sheep using surgical glue. Twenty four hours after the arenas were attached, approximately 25 adult, female *P. ovis* mites were placed into 58 of the arenas. The remaining two arenas had no mites placed in them. There was no pre-exposure in this trial.

The *Psoroptes* mites were obtained from experimentally infested sheep the day before the trial and maintained in standard incubation chambers with 500 µl of sheep serum and incubated at 30 °C and 90% rh. Seventy two hours after the mites had been placed into the arenas, the sheep skin enclosed within each arena received a dose of one of the following treatments. Six arenas were treated with 0.1 ml of *M. anisopliae* in Codacide at a concentration of $1 \times 10^8$ conidia ml$^{-1}$, another six were treated with 0.1 ml of *M. anisopliae* in sunflower oil at a concentration of $1 \times 10^9$ conidia ml$^{-1}$, another six were treated with 0.06g of *M. anisopliae* with 0.001g of diatomaceous earth and another six were treated with 0.06g of unformulated *M. anisopliae* (Table 3.4). These treatment groups were repeated for *B. bassiana*, so that, six arenas were treated with 0.1 ml of *B. bassiana* in Codacide at a concentration of $1 \times 10^8$ conidia ml$^{-1}$, another six were treated with 0.1 ml of *B. bassiana* in sunflower oil at a concentration of $1 \times 10^9$ conidia ml$^{-1}$, another six were treated with 0.009g of *B. bassiana* with 0.001g of diatomaceous earth and another six were treated with 0.009g of unformulated *B. bassiana* (Table 3.4). In 10 of the arenas, no treatment was applied to
provide controls. For each treatment, as well as the controls, half of the arenas had fleece that had been sheared down to the skin before the addition of the mites and half of the arenas had fleece that had been sheared to approximately 10 mm in length. This was done to test for any effects that fleece length may have on the pathogenicity of the fungi. Therefore, there were 3 replicates of each treatment at each fleece length. All treatments are summarised in Table 3.4.

Once the treatments had been applied, the arenas were sealed with a fine grade cotton mesh held in place with a rubber washer. The day that the treatments were applied to the sheep was recorded as day 0. The arenas were left untouched for 4 days after which the sheep were euthanized and the skin containing the arenas was excised. The skin, with the arenas attached, were placed into sealable plastic bags and refrigerated over night. The next day (day 5), all visible mites were removed from the arena with a paintbrush and forceps before removing the arena itself washing the skin with distilled water to try and remove any more mites that were not visible upon first inspection. The wash was filtered through a fine grade nylon cloth to catch all stages of mites. The fleece was inspected one more time after being washed. Different paint brushes and forceps were used for the controls and the also for the different species of fungi. The brushes and forceps were washed in 70% ethanol between treatments.

All mites were surface sterilised and live mites were placed into the standard incubation chamber (Fig. 2.4) while dead mites were treated as previously described in Chapter 2. Chambers were given 500 μl of sheep serum and incubated at 30 °C and 90% r.h. and were checked every 24 h for the presence of dead mites which were removed and checked for signs of infection as described previously.

To confirm the pathogenicity of the fungal formulations, each of the treatments applied to the arenas on the sheep were also used to inoculate mites in vitro. The standard method (as previously described) was used to inoculate 20 adult female mites with the same concentrations and amounts of each of the treatments applied to the sheep. The mites were then moved to clean chambers with 500 μl of sheep serum and incubated at 30 °C and 90%
The chambers were checked 5 days after inoculation and every day afterwards to check for the presence of dead mites which were removed and checked for infection as previously described. There were three replicates of each treatment.

Table 3.4. Summary of the fungal pathogens, excipient, concentration, amounts and the length of fleece within the arena used in the second arena based in vivo trial.

<table>
<thead>
<tr>
<th>Fungal Species</th>
<th>Fleece length</th>
<th>Excipient</th>
<th>Amount applied</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>M. anisopliae</td>
<td>Short</td>
<td>Codacide&lt;sup&gt;®&lt;/sup&gt;</td>
<td>0.1 ml</td>
<td>1 x 10&lt;sup&gt;8&lt;/sup&gt; conidia ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>Short</td>
<td>Diatomaceous</td>
<td>0.06g +</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt; conidia g&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>Short</td>
<td>Sunflower oil</td>
<td>0.1 ml</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt; conidia ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>Short</td>
<td>Dry conidia</td>
<td>0.06g</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt; conidia g&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>Long</td>
<td>Codacide&lt;sup&gt;®&lt;/sup&gt;</td>
<td>0.1 ml</td>
<td>1 x 10&lt;sup&gt;8&lt;/sup&gt; conidia ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>Long</td>
<td>Diatomaceous</td>
<td>0.06g +</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt; conidia g&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>Long</td>
<td>Sunflower oil</td>
<td>0.1 ml</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt; conidia ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>M. anisopliae</td>
<td>Long</td>
<td>Dry conidia</td>
<td>0.06g</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt; conidia g&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. bassiana</td>
<td>Short</td>
<td>Codacide&lt;sup&gt;®&lt;/sup&gt;</td>
<td>0.1 ml</td>
<td>1 x 10&lt;sup&gt;8&lt;/sup&gt; conidia ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. bassiana</td>
<td>Short</td>
<td>Diatomaceous</td>
<td>0.009g +</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt; conidia g&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. bassiana</td>
<td>Short</td>
<td>Sunflower oil</td>
<td>0.1 ml</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt; conidia ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. bassiana</td>
<td>Short</td>
<td>Dry conidia</td>
<td>0.009g</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt; conidia g&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. bassiana</td>
<td>Long</td>
<td>Codacide&lt;sup&gt;®&lt;/sup&gt;</td>
<td>0.1 ml</td>
<td>1 x 10&lt;sup&gt;8&lt;/sup&gt; conidia ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. bassiana</td>
<td>Long</td>
<td>Diatomaceous</td>
<td>0.009g +</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt; conidia g&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. bassiana</td>
<td>Long</td>
<td>Sunflower oil</td>
<td>0.1 ml</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt; conidia ml&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>B. bassiana</td>
<td>Long</td>
<td>Dry conidia</td>
<td>0.009g</td>
<td>1 x 10&lt;sup&gt;9&lt;/sup&gt; conidia g&lt;sup&gt;-1&lt;/sup&gt;</td>
</tr>
<tr>
<td>None</td>
<td>Short</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>Long</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>
Chapter 3 – Effects of fungal pathogens on *Psoroptes* mites

Results

The proportion of adult female mites infected and the proportion of adult female mites surviving the 5 day *in vivo* period, were each analysed individually using 3-way ANOVAs with fungal species, formulation and fleece length as the independent factors each time.

When the controls are excluded from the analysis, formulation had a significant effect on the proportion of adults infected ($F_{3,32} = 7.46, P = 0.001$) as those mites exposed to conidia formulated in Codacide® had a lower proportion of infection than all other formulations (Fig. 3.13). There was no significant effect of fungal species or fleece length on the proportion of infected mites and there were no significant interactions.

The species of fungus had a significant effect on the proportion of adult females that were alive when removed from the arenas ($F_{1,32} = 5.56, P = 0.025$) with a lower proportion of mites surviving the 5 day period *in vivo* when exposed to *M. anisopliae* (Fig. 3.14). There was also a significant effect of formulation on the proportion of adult females surviving the 5 day *in vivo* period ($F_{3,32} = 9.76, P < 0.001$; Fig. 3.14). A significantly lower proportion of mites survived the 5 day *in vivo* period when exposed to diatomaceous earth than any of the other treatments ($P < 0.002$), which did not differ significantly from each other ($P > 0.05$).
Fig. 3.13. Mean proportion (± S.D.) of adult female mites showing infection after 5 days exposure, *in vivo*, to *Metarhizium anisopliae* (solid circles), *Beauveria bassiana* (open squares) in various formulations and no treatment (solid triangle).
Fig. 3.14. Mean proportion of adult female mites that were recovered alive from the arenas after exposure to *Metarhizium anisopliae* (solid circles), *Beauveria bassiana* (open squares) and no treatment (black triangle) *in vivo* for 5 days.
3.3 Discussion

The key result presented in this chapter is the demonstration that under controlled conditions it is possible for the fungal pathogens *M. anisopliae* and *B. bassiana* to infect *P. ovis* in vivo. This and previous studies have demonstrated the efficacy of fungal pathogens to *P. ovis* in vitro, but were unable to achieve infection of the mites on a live host. By using rubber arenas attached to the dorsal surface of the sheep, the present study was able to induce lethal infections in *P. ovis* at a level comparable to previous in vitro work.

Several studies have investigated the use of *M. anisopliae* and *B. bassiana* against veterinary ectoparasites in vivo. A $1 \times 10^8$ conidia ml$^{-1}$ suspension of *M. anisopliae* induced mortalities of 30 and 37% in the adult ticks *R. appendiculatus* Neumann and *A. variegatum* Fabricius, respectively, as they fed on the ears of rabbits (Kaaya et al., 1996). In the same study (Kaaya et al., 1996), a $1 \times 10^8$ conidia ml$^{-1}$ suspension of *B. bassiana* was responsible for a mortality of 34% in adult *R. appendiculatus*, although it failed to kill any *A. variegatum*. In field trials, $1 \times 10^8$ conidia ml$^{-1}$ suspensions of *M. anisopliae* and *B. bassiana* caused mortalities of 83% and 77% respectively when applied to *R. appendiculatus* naturally infesting zebu cattle (Kaaya et al., 1996). *In vitro* experiments conducted by Maranga et al. (2005) showed that 100% mortality can be achieved with both *M. anisopliae* and *B. bassiana* against *A. variegatum*. *Metarhizium anisopliae* has also been shown to induce high levels of infection in the cattle louse, *Bovicola bovis* (L.) both in vitro and in vivo (Briggs et al., 2006); 71% of lice exposed to a $1 \times 10^8$ conidia ml$^{-1}$ suspension of *M. anisopliae* in vitro became infected as did 73% of lice exposed to the same concentration in vivo (Briggs et al., 2006).

The first in vivo trial conducted in the present study with a Danish strain of *M. anisopliae* found only 2 infected mites out of 291 recovered. Both of these mites came from the same sheep in a wash sample taken 14 days after application of *M. anisopliae*. Hence, an in vitro trial was set up to assess the relative pathogenicity of the isolate of *M. anisopliae* used in this in vivo trial in comparison with another isolate (the French strain). The Danish
isolate was much less pathogenic than the French isolate with proportions of infected mites of 0.38 and 0.91 respectively. Hence it would appear that the reason for the lack of infected mites found could have been that the isolate used was not as pathogenic as other isolates of *M. anisopliae*.

The second *in vivo* trial used the French strain of *M. anisopliae* rather than the Danish strain and also tried a different approach with the method of exposure. As with the previous trial, the potential for *M. anisopliae* to treat an established infestation was tested but also its potential to act as a preventative treatment. However, no infected mites were recovered from any of the sheep treated with the fungal pathogen. Possible explanations for this were that the temperature at the skin surface may have been too hot for the conidia to germinate and infect the mites. All of the sheep were housed indoors and the trial was run during the summer.

Several studies have shown that grasshoppers inoculated with *M. anisopliae* and *B. bassiana* show reduced levels of mycosis when allowed to bask at temperatures of 35 – 40 °C (Inglis *et al.*, 1996). It has been suggested that grasshoppers and locusts induce a behavioural fever, raising their body temperature to 42 °C in response to fungal infection (Blanford *et al.*, 1998). The increased body temperature becomes too hot for the fungal pathogen, inhibiting its growth within the insect and thus increasing the survival of the infected individual (Inglis *et al.*, 1996, 1999; Blanford *et al.*, 1998). It is not known whether or not behavioural responses may be induced by fungal infection in *P. ovis*. However, it may not be necessary to induce a behavioural response such as basking in *P. ovis* if the body temperature is already high enough to inhibit the infection process.

Another explanation for the lack of infection seen in the *in vivo* trials was considered to be the possibility that there is something present on the skin or fleece of sheep that might inhibit the action of the conidia. The skin secretions consist of two main components; the water insoluble wool wax and the water soluble suint. The wool wax, also referred to as lanolin, is made up of many different compounds including fatty esters (branched and unbranched), free alcohols and alkanols, sterols and ceramides (*Alzaga et al.*, 74).
Suint consists of a complex mixture of metallic ions, peptides, weak bases and organic acids such as acetic and propionic acid (Deane and Truter, 1955). Some skin secretions can inhibit the adherence of fungal spores to epidermal cells (Meyer et al., 2001). It has been shown that saccharide residues that inhibit the binding of fungal spores to epidermal cells are present in the skin secretions of sheep and goats (Meyer et al., 2001). It might be that these chemicals are able to inhibit the action of *M. anisopliae* and *B. bassiana* against *P. ovis* in vivo.

The *in vitro* sheepskin bioassay was designed to test whether or not there was any intrinsic inhibition by the skin and fleece. The results show that 52% of mites exposed to sheepskin and fleece treated with *M. anisopliae* for 24 h, became infected. For mites kept in chambers with fungal treated cloth 34% of mites became infected. Although the difference was not significant, the lower level of infection found in the mites exposed to cloth treated with the fungal pathogen could be due to the ability of the mites to escape the pathogen by climbing on the walls and lid of the chamber. The mites exposed to the pathogen on sheepskin had less free space in the chamber. Clearly therefore, it was not intrinsic inhibition by fleece components that inhibited infection in the first two *in vivo* trials.

The temperature at the skin surface of a sheep has been estimated to be between 31 - 37 °C (Wall et al., 1992). It has already been shown that the French isolate of *M. anisopliae*, also used here, can infect a high percentage (48%) of mites *in vitro* at 37.5 °C (Brooks, 2004). Bioassays were conducted in the present study using isolates of *M. anisopliae* that had been isolated from insects inhabiting hotter climates and thus would be expected to be more tolerant of higher temperatures. However, none of these isolates showed any pathogenicity towards *P. ovis* when tested at 37 °C; hence the French isolate would appear to be unusual.

The strain of *B. bassiana* that was isolated from a *Psoroptes* mite removed from a sheep at the CSL was found to be highly pathogenic against *P. ovis* in the *in vitro* trials with 97% of mites becoming infected at a concentration of $1 \times 10^5$ conidia ml$^{-1}$. At a concentration of $1 \times 10^7$ conidia ml$^{-1}$ all exposed mites became infected. However, the initial
bioassay to assess the pathogenicity of *B. bassiana* using a $1 \times 10^8$ conidia ml$^{-1}$ suspension only caused 58% of mites to become infected. The ability to produce conidia with a consistent level of pathogenicity is an important factor if enough appropriately pathogenic conidia are to be produced to make fungal pathogens a viable alternative treatment for scab. The use of fungal pathogens as a treatment for scab will be discussed later in the chapter.

A third possibility for the lack of infected mites found in the earlier *in vivo* trials was that the fleece was acting as a physical barrier preventing the conidia from coming into contact with the mites. The conidia of the fungal pathogen were applied onto the sheep either in suspension or as dry, unformulated conidia. The conidia would have to pass through the fleece to the surface of the skin where the mites are. The scab itself may also act as a barrier.

Given that the *in vitro* work suggested that fleece was likely to have had no, or little effect, on the pathogenicity of *M. anisopliae*, a third *in vivo* trial was planned. However, rather than simply trying to treat a scab infested sheep as with the previous trials a more controlled approach was used. By using rubber arenas attached to the surface of the sheep it was possible to contain a known number of mites *in vivo* for a set period of time. This method also allowed for a controlled exposure to the fungal pathogens and ensured that the mites came into contact with the conidia as the fleece had been sheared. The double exposure to conidia that some mites were given was used so that some mites would already be infected with the fungi prior to being placed on the sheep. This was done so that possible points of inhibition could be identified. If the mites that had the double exposure failed to show signs of infection it would have demonstrated that inhibition was due to something inherent to the sheep skin environment.

There was no significant difference between the proportion of mites infected from the groups that had the double exposure and the groups that were only exposed to the fungi *in vivo*. This therefore demonstrates that mites are able to acquire lethal infections when exposed to conidia on a live animal. Mites must have been infected internally as they were surface sterilised on removal to kill off conidia present on the external cuticle. However,
mites were only on host for 48 h so signs of mycosis were only seen after they were removed. No mite with external hyphae was ever found on a live sheep. Hence, it is still not known whether or not the mites that became infected on the host would have developed lethal mycoses if they had remained on the host.

The two new strains of *M. anisopliae* tested in the third *in vivo* trial were mixed together to minimise the number of arenas that would be used. Under the guidance of CABI this seemed to be acceptable method. If relatively high levels of infection were obtained, the two strains could be tested separately at another time. A high level of infection was obtained except for the groups exposed to the double exposure regime, where an inexplicably low level of infection was seen. However, all fungal strains tested performed well and so no further investigations were carried out on either of the mixed strains. However, mixing two or more fungal strains may not be the best approach to use as it could be possible that any infections found are the result of only one of the strains. The addition of another strain that may be of lower virulence or pathogenicity would dilute the effect seen.

In the fourth *in vivo* trial (the second trial to utilise the rubber arenas) mites were exposed to conidia for 5 days on a host. Despite the longer time on host, there were still no mites that showed signs of advanced infection when removed from the arenas. A significantly greater proportion of mites exposed to conidia formulated in diatomaceous earth were dead on removal than for any other formulation. Unfortunately, due to the experimental design, it is not possible to determine if this effect was due to an interaction between the conidia and the diatomaceous earth or the diatomaceous earth alone. Also, care needs to be taken when interpreting the data, as a lower number of mites were removed from the arenas treated with diatomaceous earth. There were also fewer mites recovered from the arenas that contained *M. anisopliae*. This can be explained by the physical nature of the fungal treatment. The formulations containing *M. anisopliae* were much darker and thicker than the *B. bassiana* formulations making it much harder to find and recover the mites. Although the difference in numbers was taken into account by the statistical tests, the effect
of treatment on the number of mites recovered may well have been due to the physical properties of the medium in which the fungus was formulated rather than its pathogenicity.

In both of the *in vivo* trials that used the rubber arenas, there was a significant effect of formulation on the proportion of infected mites. The first of these trials found that the conidia formulated in diatomaceous earth induced the lowest level of infection. The second of the arena based trials found a high proportion of infection in the mites exposed to conidia in diatomaceous earth. A possible reason for the increased mortality of the mites exposed to conidia in diatomaceous earth could be due to the action of the earth. Diatomaceous earth is an inert dust which, when applied to an arthropod, adheres to the cuticle and causes desiccation (Ebeling, 1971). It could be that the action of diatomaceous earth requires a longer exposure period to affect enough damage to the mite for it to become susceptible to infection from the conidia. Until this threshold is reached the diatomaceous earth may actually hinder the conidia by binding to the cuticle of the mite and occupying potential binding sites of the conidia. The conidia of *B. bassiana* are much smaller than *M. anisopliae* and may not be so strongly affected by the decreased surface area. This may well explain the different results between the two trials.

Other studies have found that when diatomaceous earth is combined with pathogenic fungi to control stored product pests, greater mortality is achieved at higher temperatures, provided the lethal limit of the fungi is not reached (Lord, 2005; Michalaki et al., 2006; Vassilakos et al., 2006; Michalaki et al., 2007). A study looking at the effects of diatomaceous earth and *B. bassiana* on the lesser grain borer, *Rhyzopertha dominica* (F.) and the rice weevil, *Sitophilus oryzae* (L.) found that the diatomaceous earth caused greater mortality at higher temperatures, although *B. bassiana* was more effective at 26 °C than 30 °C (Vassilakos et al., 2006). A similar study comparing the use of Silicosec® and *M. anisopliae*, alone and in combination found that both were more effective in killing larvae of the confused flour beetle, *Tribolium confusum* du Val at higher temperatures and that *M. anisopliae* achieved the greatest mortality when mixed with Silicosec® (Michalaki et al., 2006).
The exact mechanism by which diatomaceous earth acts as a dessicant is unclear. A recent study examining the effects of three commercially available diatomaceous earths on *A. siro* showed that although the particles of the diatomaceous earth had removed certain cuticular hydrocarbons, there were no signs of abrasion on the mite cuticle when examined by scanning electron microscope after 72 h of exposure (Cook *et al.*, 2008). Despite the conflicting results from the two arena based trials, diatomaceous earth could be an effective medium for the formulation of fungal conidia.

The third and fourth in vivo trials showed that under controlled conditions the temperature at the skin surface of a sheep does not inhibit *M. anisopliae* or *B. bassiana* from infecting *P. ovis*. There is also no inhibition due to any substances present on the skin and fleece on the infectivity of the fungal pathogens. However, a mite showing signs of infection i.e. a cadaver with conidia or hyphae growing on its surface has not yet been observed in vivo. It may be that mites do not develop mycosis while on the host or it may be that infection develops initially but is then suppressed within a short period and thus was not observed in the trials. Alternatively, it may be that infected mites that did develop mature mycosis either decayed rapidly or were lost off the sheep or were otherwise not recovered.

The effects that the fungal pathogens have on exposed mites prior to death are not known in vivo. In the fourth in vivo trial, live protonymphs were found at the end of the 5 day in vivo period. Larvae and eggs were also present. This suggests that oviposition and subsequent development had taken place in the presence of the fungal pathogens. More work is therefore required to find out how the reproductive output and the development of juvenile stages are affected in vivo. If the fungal pathogen does not kill or debilitate the mite, allowing it to continue to develop and oviposit, the advantages of using fungi may be limited. If the reproductive output of the females is largely unaffected, along with the subsequent development of the juvenile stages, the mite population will still be able to increase. The scab would spread across the body of the sheep as the infection rate of the fungal pathogen in the mites will struggle to keep pace.
A further complication facing the use of fungal pathogens as a control for sheep scab is the problem of formulating the conidia. Several formulations were tested for their pathogenicity against *Psoroptes* both *in vitro* and *in vivo*. As has been demonstrated with the diatomaceous earth formulations, the results can be very variable. A formulation that may be suitable for *B. bassiana* may not be suitable for *M. anisopliae* and vice versa. *In vitro* bioassays showed that fewer mites became infected when exposed to *M. anisopliae* suspended in Output® and sunflower oil (29% and 25%, respectively) than when suspended in vegetable oil or Codacide® (100% and 99%, respectively). Mites exposed to *B. bassiana* however, showed very low levels of infection regardless of formulation. The highest level of infection was found from conidia suspended in sunflower oil (6%). However, when mites were exposed to conidia in Codacide® and in sunflower oil *in vivo*, much greater levels of infection were seen.

In the last two *in vivo* trials the fleece was shorn to under 20 mm in length. This ensured contact of the treatments with the mites. However, on a scab infested sheep, the fleece will be longer, approximately 100 mm. This can act as a physical barrier preventing the conidia from reaching the mites. A suitable formulation and application method will need to be devised that not only takes into account the potential effects on pathogenicity and viability of the conidia but also delivers the conidia to the skin surface. The initial *in vivo* trials may have failed to produce a significant number of infected mites because the mites were simply not exposed to enough conidia.

The inconsistency seen in the results of this chapter are a clear indication of how many variables and problems are facing the development of a commercially available fungal treatment of sheep scab. This will be considered further in Chapter 6.


CHAPTER 4

LIFE HISTORY AND POPULATION DYNAMICS OF

ACARUS SIRO

The primary aim of this thesis was the study of the biological control of sheep scab and its causative agent, *P. ovis*. However, as an obligate parasite, this mite is particularly difficult to use in long-term *in vitro* studies and *in vivo* studies are also difficult, expensive and compromise animal welfare. Therefore *in vivo* studies need to be used sparingly. It was decided that when attempting to investigate the transmission of entomopathogenic fungi through populations, to use an alternative, model mite species, *Acarus siro*.

*Acarus siro* shares some key similarities with *P. ovis* that make it a useful model species (more detail is provided on *A. siro* in the following sections). Both species of mite are astigmatic and therefore potentially very susceptible to infection with the fungal pathogens used in this study. The life-cycles of both mites are also very similar with the same number of life stages. However, an important difference is that *A. siro* are free living and relatively easy to rear under laboratory conditions that closely resemble the conditions that the mites are naturally found in. This means that the work presented in Chapter 5 will be able to show the effects of a fungal pathogen on a healthy population of mites.

4.1 Introduction to *Acarus siro*

The astigmatid flour mite, *A. siro* is a major pest of grain stores (Solomon, 1946; Griffiths, 1962; Cunnington, 1965; Lukáš *et al.*, 2007). It is distributed across Europe, North America, Russia and Australia (Cunnington, 1985). The range of the mite is limited to regions of temperate climate; the development of the mite requires temperatures of between
4 and 32 °C, depending on relative humidity (Solomon, 1962; Cunnington, 1965). A study of 514 storage units from 147 grain stores in the Czech Republic between 1996 and 1998, showed that 67% were infected with pest mites (Lukáš et al., 2007). It is easily cultured and makes a good model species.

4.1.1 Life-cycle

Adult females are morphologically the largest stage. They are approximately 0.5 mm in length with a rounded appearance. The adult males are slightly smaller and flatter and can also be distinguished by a spur on the trochanter of each fore leg. The adult mites have a pale yellow/brown body with dark brown legs. Juvenile stages are paler and lack the brown colouration in the legs.

There are 5 stages in the lifecycle: egg, larva, protonymph, tritonymph and adult. The juvenile stages, apart from the egg, show two phases; an active phase and a resting (moulting) phase. The active phase is the period that the mite is mobile and feeding. The resting phase occurs before the mite moults into the next stage of the lifecycle. It can be distinguished by the change in behaviour as the mite becomes still and the distance between the anterior two pairs of legs and posterior two pairs of legs increases and the distal legs are tucked under the body. The mite also becomes much more round in appearance. The resting phase, during which apolysis occurs, usually lasts 48 hours (Cunnington, 1984). At ecdysis the mite then moult into the active phase of the next stage in the lifecycle. Under certain conditions it is possible that a sixth stage in the lifecycle can occur. This is the formation of a deutonymph, also known as the hypopus, between the protonymph and the tritonymph stages (Solomon, 1962; Knülle, 1987; Corente and Knülle, 2003). The hypopus is a stage in the lifecycle that is physiologically suited for dispersal or for resisting conditions that are not suitable for continued development and is particularly common in the astigmata (Knülle, 1987; Corente and Knülle, 2003). However, the production of a hypopus is very rare in A. siro and not seen at all in P. ovis (Knülle, 1987; Sanders, 2000). Although hypopi have been observed and induced in other astigmatic species, less success has been achieved with
Chapter 4 - Life history and population dynamics of *Acarus siro*

-attempts to induce the formation of hypopi in *A. siro* (Knülle, 1987). Experiments designed to induce hypopus formation in *A. siro* reared on poor quality food have only sporadically yielded the development of a hypopus. A hypopus forming line of *A. siro* has not been established as it has been for other astigmatic mites. It may be that the predominant conditions under which *A. siro* has evolved have not applied strong enough selection pressures to retain formation of hypopi (Knülle, 1987).

Several studies have previously been conducted investigating the life history and population dynamics of *A. siro* (Cunnington, 1985; Kawamoto *et al.*, 1991; Ždárková and Voracek, 1993; Fejt and Zdarkova, 2001). However, these studies show a large degree of variation in the development times of the lifecycle stages and also in the conditions used to rear the mites, since the development of *A. siro* is highly dependant on the environmental conditions, particularly temperature and relative humidity. Under favourable conditions the complete development can occur in 14 days (Fejt and Ždárková, 2001).

4.1.2 Effects of infestation

*Acarus siro* infests a wide range of food stuffs such as grain, cheese and various cereal-based products found in the supermarket and people's homes (Thind and Clarke, 2001). An infestation of mites can cause considerable economic loss through the consumption of and damage to the food and the treatment necessary to remove the mites (Dunn *et al.*, 2005). Mites infesting grain stores feed on the wheatgerm of the grain, removing it completely but leaving the rest of the grain intact (Solomon, 1946). The grain has to be damaged for the mites to gain access to the germ as they are unable to penetrate the grain-coat, however, usually less than 10% of stored grain is undamaged (Solomon, 1946).

The mites also have the potential to cause skin and respiratory problems in humans and animals. Several studies have shown that extracts from storage mites including *A. siro* can induce allergic reactions when administered to humans in a skin prick test (Calvo *et al.*, 2005; Ruoppi, *et al.*, 2005; Vidal *et al.*, 1997).
A study in Chile demonstrated a prevalence of 56% of child asthma sufferers reacted to extracts of *A. siro* (Calvo *et al*., 2005). A study of university employees involved in animal handling, showed that 14 out of 40 people tested (35%) were sensitive to storage mites (Ruoppi *et al*., 2005). Of those 14 people sensitised to storage mites, only one person tested positive for a reaction to house dust mites, suggesting that it is not cross-reactivity causing false positives. Even dogs show sensitisation to *A. siro* extracts when injected intradermally; 28% of atopic dogs tested showed positive reactions (Bensignor and Carlotti, 2002). However these studies were conducted only on people or animals that were known to have respiratory problems or skin disorders at the time of the trials. There is no evidence to show that *A. siro* or other storage mites were the initial cause of the problem.

A study of the general population of the municipality of A-Estrada in Spain showed that 24.4% of people tested positive for sensitisation to the storage mites *Tyrophagus putrescentiae* (Schrank) and *Lepidoglyphus destructor* (Schrank) even though they had no occupational exposure to the mites (Vidal *et al*., 1997). For the people who showed sensitivity to mites (either storage mites or house dust mites), 50% were sensitive to both types of mite but 44.6% were sensitive to storage mites only. Only 5.4% were sensitive to house dust mites only. The study did not pick test subjects who were already suffering from an allergic disorder. It was also found that there was no significant link between allergic sensitisation and profession (Vidal *et al*., 1997).

### 4.2 Materials and methods

#### 4.2.1 Longevity, fecundity and development of *Acarus siro*

Previous studies investigating the life history of *A. siro* have shown that there can be considerable variation in the development time, longevity and fecundity under different conditions. The aim of the work presented in this section was to confirm the development times, longevity, fecundity and mortality rates of *A. siro* when reared under the constant laboratory conditions to be used for later fungal infection studies.
To measure the oviposition rates, tritonymphs were collected from culture, placed into individual incubation chambers (Fig. 2.4) and checked every 24 h (Cunnington, 1985). Once the mites had moulted and emerged as adults, pairs of adult females and males were placed into incubation chambers with 0.003g (deemed to be an excess) of food and incubated at 20 °C and 70% r.h. This was recorded as Day 0. Each subsequent day, the pair was checked and carefully removed from the chamber with a paint brush and placed into a new clean chamber with the same amount of food. This was done so that any eggs that had been laid in the previous 24 h period could be counted. If one of the pair died, the dead individual was replaced with another mite of the same sex, although not necessarily the same age. The replacement mite was added to the chamber so that the remaining original mite was still kept in a pair. No recordings were taken from the replacement mite. The number of eggs laid each day by the females and the day of death for each mite was recorded. In total, recordings were taken from 40 mites of each sex. Adult mites were paired because a previous study had found that this gave the highest mean egg output per female (Cunnington, 1985).

To determine the development times for each stage of the life-cycle, mites from each of the juvenile stages were taken from the general culture and placed individually into incubation chambers (Fig. 2.4) with approximately 0.003g of food. Daily observations were made. Mites to be observed were placed into chambers during the life-cycle stage prior to the stage to be examined and the day that the mites emerged into the required life-cycle stage was noted as Day 0. The time spent in the active phase and the resting phase was recorded for each stage. The day that the mites entered the resting phase was recorded when the mites showed no signs of movement when touched with a paintbrush. Dead mites could be distinguished from mites in the resting phase by their appearance. In the resting phase, each mite becomes characteristically more rounded and the legs curl underneath the ventral surface of the mite (Solomon, 1962). Dead mites do not show the curling up of the legs and also appear desiccated. Each stage of the life-cycle was deemed to have ended at ecdysis, i.e. once the mites had completed the moult into the next life-cycle stage. Eggs for
observation were collected from the paired adults. Observations were taken from 40 mites of each stage of the lifecycle incubated at 20 °C and 70% r.h. Sex was not determined for any of the juvenile stages since there are no descriptions for morphological differences.

4.2.2 The population dynamics of Acarus siro

Sampling technique

A technique for sampling laboratory populations was developed and tested for its accuracy before being used to sample the experimental populations. Cuvettes (Fig. 4.1) containing known numbers of mites in 0.3 g of wholemeal flour and yeast in a 3:1 (w/w) ratio were sampled using a capillary tube with an internal diameter of 4 mm which was gently pushed through the culture of food and mites until the tube reached the bottom of the cuvette. The content of the capillary tube was then tapped out into a worm egg counting chamber (Improved McMaster, Weber Scientific International Ltd. Teddington, U.K.). A saturated salt solution (NaCl) was then added to the chamber to float the mites up from the food and allow them to collect on the underside of a coverslip that was placed over the chamber. The mites were then counted under a binocular microscope. Each cuvette was sampled 5 times and the samples pooled. The amount of substrate removed from the cuvette was recorded each time a sample was taken by measuring the weight of the cuvette after the sample was taken and subtracting it from the weight of the cuvette before the sample. The number of mites in each sample with a known weight could then be multiplied to give the number of mites present in the cuvette (0.3g). Samples were taken from 5 replicates of 5 cuvettes seeded with 100, 200, 300, 400 or 500 mites.

Population dynamics

To examine the population dynamics of A. siro, groups of mites were set up in cuvettes, each containing 0.3 g of wholemeal flour and yeast in a 3:1 (w/w) ratio. The initial population consisted of 50 adult females and 50 adult males, all of a similar age, taken from the same stock culture and placed into the food in the cuvette. The cuvettes were then sealed
with non-absorbent cotton wool and incubated at 20 °C and 70% r.h. The cuvettes were agitated every 48 h by inverting the cuvette 3 times in quick succession to prevent the food from clumping and to ensure similar conditions were present in all the cuvettes. The cuvettes were sampled using the technique described and mites of all life-cycle stages were counted. Sex was also recorded for the adult mites. Only mites that appeared to be alive were counted. These were mites which showed movement or had no signs of the discolouration of the cuticle or desiccation associated with death. Over an 80 day period, 5 different cuvettes were sampled every 48 h. Once a cuvette had been sampled it was removed from the experiment.
Fig. 4.1. Cuvette used to house populations of *Acarus siro* into which 0.3 g of wholemeal flour and yeast (3:1, w/w) was placed.
4.2.3 Modelling the population dynamics of *Acarus siro*

The population dynamics of *Acarus siro* has been studied previously, usually with the aim of creating models, yet very few of these studies have used complete sets of data. Hence, the analysis presented here, uses data gathered in the previous section of this chapter allowing parameters which are consistent with each other to be used. These data were used to create a simple Leslie matrix model (Leslie, 1945; Begon and Mortimer, 1981; Wall et al., 1999) to simulate the dynamics of *A. siro* populations. The aim of using the Leslie matrix approach to modelling was not to create a comprehensive simulation but rather to allow basic hypotheses to be generated relating to expected mite abundance against which the experimental data could be compared. This approach has been successfully employed with mite populations previously (Wall et al., 1999).

A Leslie matrix model (Fig. 4.2) consists of a column vector that describes the starting age structure of the population at time $t_1$. A suitable transition matrix is then constructed using the rules of matrix multiplication to create values for the age-structure at time $t_2$. The parameters used in the transition matrix are the survival rates for each stage of the life-cycle and the birth rate. The transition matrix is then multiplied by the age distribution at $t_1$ to give the age distribution at time $t_2$. The values used in the transition matrix for this study are the proportion of mites surviving at each stage of the lifecycle and the number of eggs laid per adult over a 6 day period. A time-step of 6 days was chosen for the model as an approximation of the duration of each juvenile stage of *A. siro* (see Results section 4.3.1). The stage survival values used for the Leslie matrix model were: 75% for eggs, 65% for larvae, 82.5% for protonymphs and tritonymphs and 51.6% for adults. The survival value for the adults represents the survival rate for adult mites of both sexes over a 6 day period. The number of eggs laid per day per adult female was 1.25, thus the number of female eggs per time step per adult female mite was 3.75.

Once the model had been created, using the data gathered previously in this chapter, the model was run with a range of different adult reproductive outputs. The increases in the reproductive output were made by multiplying the original value by 2, 3 and 4 times. To
check the fit between the models and the laboratory population an index of estimation ratio (Q) was obtained for each model/population comparison. The Q value is similar to the $r^2$ value used in regression and has been used in previous studies that have compared models of *A. siro* with actual population data (Kawamoto *et al.*, 1991; Pekar and Ždárková, 2004).

The equation for Q is:

$$Q = 1 - \frac{\sum (Y_{obs} - Y_{est})^2}{\sum (Y_{obs} - Y_{null})^2},$$

where $Y_{obs} = \log (N_{obs} + 1)$ and $Y_{est} = \log (N_{est} + 1)$. $Y_{null} = \log \sum N_{obs} / n$ which acts as the null model. $N_{obs}$ is the number of observed mites in the population. $N_{est}$ is the number of mites estimated by the model. A Q value of 1 indicates a perfect fit between the model and the population. Q cannot be greater than 1 but can be negative which indicates that the estimated values from the model give a worse fit to the data than the null model (Kawamoto *et al.*, 1991; Pekar and Ždárková, 2004).
Fig. 4.2. Leslie matrix model for *Acarus siro* fed on a diet of wholegrain flour and yeast in a 3:1 (w/w) ratio and maintained at 20 °C and 70% r.h. The column vector (first set of brackets) shows the initial age distribution of the population. The transition matrix (second set of brackets) shows the age specific survivorship and birth rate. The third set of brackets shows how the transition matrix is applied to the column vector to give the age distribution at time $t_2$ (fourth set of brackets). Each time step, $t$, represents six days.

\[
\begin{align*}
\text{Eggs} & \quad \begin{bmatrix} t_1 & 0 \end{bmatrix} \quad \begin{bmatrix} 0 & 0 & 0 & 0 & 0 & 3.756 \\ \text{Larvae} & \quad \begin{bmatrix} t_1 & 0 \end{bmatrix} \quad \begin{bmatrix} 0.75 & 0 & 0 & 0 & 0 \\ \text{Protonymph} & \quad \begin{bmatrix} t_1 & 0 \end{bmatrix} \quad \begin{bmatrix} 0 & 0.65 & 0 & 0 & 0 \\ \text{Tritonymph} & \quad \begin{bmatrix} t_1 & 0 \end{bmatrix} \quad \begin{bmatrix} 0 & 0 & 0.825 & 0 & 0 \\
\text{Adult} & \quad \begin{bmatrix} t_1 & 0 \end{bmatrix} \quad \begin{bmatrix} 0 & 0 & 0 & 0.825 & 0.516 \\
\end{align*}
\]

\[
\begin{bmatrix}
(0 \times 0) + (0 \times 0) + (0 \times 0) + (0 \times 0) + (3.756 \times 100) \\
(0.75 \times 0) + (0 \times 0) + (0 \times 0) + (0 \times 0) + (0 \times 100) \\
(0 \times 0) + (0.65 \times 0) + (0 \times 0) + (0 \times 0) + (0 \times 100) \\
(0 \times 0) + (0 \times 0) + (0.825 \times 0) + (0 \times 0) + (0 \times 100) \\
(0 \times 0) + (0 \times 0) + (0 \times 0) + (0.825 \times 0) + (0.51 \times 100)
\end{bmatrix} =
\begin{bmatrix}
\begin{bmatrix} t_2 & 375.6 \\
\begin{bmatrix} t_2 & 0 \\
\begin{bmatrix} t_2 & 0 \\
\begin{bmatrix} t_2 & 51.6
\end{bmatrix}
\end{array}
\end{array}
\end{array}
\]
4.2.4 Analysis

All data were tested for normality using the Kolmogorov-smirnov test prior to statistical analysis and subjected to log_{10} transformation when required.

4.3 Results

4.3.1 Longevity, fecundity and development of *Acarus siro*

There was little adult mortality over the first 5 – 10 days of adult life but subsequently mortality occurred at a constant rate over time (Fig. 4.3). Adult females survived for a mean (± S.D.) of 23 ± 10.5 days and adult males survived for a mean of 26 ± 10.2 days (Table 4.1). However, there was a large degree of variation in survival times as the shortest survival period was 1 day for an adult male and 2 days for an adult female. The longest survival times were 43 days and 40 days for an adult male and adult female respectively.

Adult females produced a mean (± S.D.) of 29 ± 17.1 eggs. The egg production reached a peak at day 7 with 2.18 ± 1.2 eggs being laid per female with a mean (± S.D.) egg output per female per day of 1.25 ± 0.4 eggs. Egg production gradually declined with the age of the female (Fig. 4.4). Unsurprisingly, females which lived longer laid more eggs \( F_{1, 36} = 412.02, P < 0.001, r^2 = 92\% \).

The length of each juvenile life-cycle stage varied significantly (ANOVA; \( F_{3, 118} = 24.7, P < 0.001 \); Fig. 4.5). Tukey post-hoc tests showed that although there was no significant difference in the duration of egg and larval stages, both took significantly longer than both the protonymph and tritonymph stages \( P < 0.001 \). There was no significant difference between the duration of the protonymph and tritonymph stages.

The duration of the active phase was significantly different between the various pre-adult life-cycle stages (ANOVA; \( F_{2, 89} = 39.8, P < 0.001 \); Fig. 4.5) with larvae having a significantly longer active phase than both protonymphs and tritonymphs \( P < 0.001 \). There was no significant difference in the duration of the two nymphal stages. There was no
significant difference in the time spent in the resting phase between any of the life-cycle stages.

Using the mean values for longevity of each stage of the life-cycle, the mean (± S.D.) egg to adult time is 25.64 ± 5.05 days. The quickest possible egg to adult time is 17 days and the longest possible time is 38 days.

Of the 40 eggs examined, 10 failed to hatch (25% mortality). The mean levels of mortality for larvae, protonymphs and tritonymphs were: 35%, 17.5% and 17.5% respectively. The cumulative level of mortality suggests that only 33.2% of eggs are likely to hatch and mature into adult mites.
Table 4.1. The mean duration (± S.D.), range of duration and stage-specific survival for each stage of the life-cycle of *Acarus siro*. For each stage in the life-cycle and for both sexes of adult mites, 40 individuals were maintained on wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h.

<table>
<thead>
<tr>
<th>Life-cycle stage</th>
<th>Mean duration (days) ± S.D.</th>
<th>Range (days)</th>
<th>Stage-specific survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult female</td>
<td>23 ± 10.53</td>
<td>2 - 40</td>
<td>-</td>
</tr>
<tr>
<td>Adult male</td>
<td>26 ± 10.22</td>
<td>1 - 43</td>
<td>-</td>
</tr>
<tr>
<td>Tritonymph</td>
<td>5.55 ± 1.39</td>
<td>4 - 10</td>
<td>0.825</td>
</tr>
<tr>
<td>Protonymph</td>
<td>5.33 ± 1.16</td>
<td>4 - 7</td>
<td>0.825</td>
</tr>
<tr>
<td>Larva</td>
<td>7.73 ± 1.28</td>
<td>5 - 11</td>
<td>0.65</td>
</tr>
<tr>
<td>Egg</td>
<td>7.03 ± 1.22</td>
<td>4 - 10</td>
<td>0.75</td>
</tr>
</tbody>
</table>
Fig. 4.3. The number of adult female (solid circles) and adult male (open squares) *Acarus siro* alive over time (days) when maintained on wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h.
Fig. 4.4. Mean number of eggs (± S.D.) laid per day per mite, by 40 adult female Acarus siro reared on wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h. Fitted line is a 3rd order polynomial ($y = 0.0002 x^3 - 0.0158 x^2 + 0.2788 x + 0.3616$). Dashed line shows the mean egg output per female for the 40 day period (1.25 ± 0.41).
Fig. 4.5. Mean duration (± S.D.) of the larval, protonymph and tritonymph stages of *Acarus siro* split into time spent in the active phase (solid bars) and time spent in the resting phase (open bars) when maintained on wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h.
4.3.2 The population dynamics of Acarus siro

Sampling technique

In the calibration of the capillary tube sampling technique there was a highly significant relationship between the calculated number of mites and the actual number of mites that had been placed into a cuvette ($F_{1, 23} = 146.2, P < 0.001, r^2 = 86.4\%$; Fig. 4.6). This demonstrates that the sampling technique used gives an appropriate estimation of population size.

Population dynamics

The laboratory populations show a steady and synchronous rise throughout the life-cycle stages until approximately day 60 when the population begins to rapidly decline. This decline is due to a shortage of food and space and increased competition (Fig. 4.7). For this reason, analysis of the population was conducted up to day 60.

The laboratory populations showed no significant periodicity in the mean abundance of any stage of the lifecycle when analysed with an autocorrelation. Eggs were the most abundant stage throughout the growth of the population (Fig. 4.7). The eggs per female peaked at day 18 with a mean of 33.3 ($\pm$ 22.7) eggs counted per female and a maximum of 58 eggs per female (Fig. 4.8). The number of eggs per female then decreased over time after reaching the peak at day 18.

The mean sex ratio for the 60 day period was 1.3:1, female: male (Fig. 4.9). However the ratio fluctuated in cycles of approximately 20 to 25 days ranging from 0.8:1, female: male to 2.1:1, female: male.

4.3.3 Comparison of the modelled and observed population dynamics of Acarus siro

The model estimates that there should be a peak in mite numbers at day 48 with a total number of mites, of all life-cycle stages, of 8965 mites (Fig. 4.10). The estimated mean number of mites taken from samples of the populations reared under laboratory conditions
show that the peak population is 10080 mites at day 54. The model predicts that at day 54 the population should be 8827 mites.

When the various life-cycle stages are distinguished, the final model indicates that the initial cohort of adults would be expected to decline steadily during the first 24 days (Fig. 4.8). However, oviposition followed by the development of the juvenile stages would lead to an increase in adult numbers by day 30 (Fig. 4.10). The population would then be expected to move through a period of unstable growth until a stable age structure is reached, approximately 300 days after the introduction of mites to the food source. However, the laboratory populations only ran for 80 days, reaching a peak of 10080 mites at day 54. The stable age structure was not reached by the laboratory population.

Comparison between the model and the laboratory population was conducted up until day 60. The original Leslie matrix model parameterised using the data collected from the current study was found to underestimate the total number of mites observed in the laboratory populations. Sensitivity analysis showed that overall, tripling the number of eggs laid per adult in the model was the single change that gave the best improvement in fit between the total number of mites predicted by the model and the laboratory populations \((Q = 0.76; \text{Fig. 4.11})\). However, while the overall fit was better, tripling the number of eggs did not always give the best fit when each stage of the life-cycle was considered separately (Table 4.2). Nevertheless, for the purpose of this study, the model population with a reproductive output of 11.25 eggs per female per six day period was used for comparison with the laboratory populations.

Comparing the distribution of mites across the life-cycle stages (Fig. 4.12) showed that while the general pattern of abundance was similar there was a significant difference between the prediction of the final model and the laboratory population \(\left(\chi^2 = 797, P < 0.01; \text{Fig. 4.13}\right)\).
Fig. 4.6. Number of adult female *Acarus siro* estimated in 0.3g of wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h. when known numbers of mites were introduced. Samples were taken from 5 replicates at densities of 100, 200, 300, 400 and 500 mites per cuvette. 

$Y = 0.75x + 17.95; r^2 = 86.4\%$. 

![Graph showing the relationship between estimated number of mites and number of mites introduced, with a linear regression line and data points.](image-url)
Fig. 4.7. The 3 point sliding mean of the log number of mites +1 for each stage of the life-cycle of *Acarus siro* sampled at different times from initial populations of 50 adult female and 50 adult male mites maintained on 0.3g of wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h. Eggs are shown by the pink line; larvae, green; protonymphs, blue; tritonymphs, red and adults, black. Dashed line indicates the point of decline for the total number of mites.
Fig. 4.8. Number of eggs per adult female *Acarus siro* sampled from 5 replicates of 40 populations of various ages reared on wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h. The fitted line is the 3 point sliding mean.
Fig. 4.9. Mean sex ratio (± s.d.) for adult *Acarus siro* sampled from 5 replicates of 40 populations of various ages maintained on wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h.
Fig. 4.10. The number of *Acarus siro* of all lifecycle stages as predicted by a Leslie matrix model over time. Eggs are shown by the pink line; larvae, green; protonymphs, blue; tritonymphs, red and adults, black.
Fig. 4.11. The number of *Acarus siro* as predicted by a Leslie matrix model over time (solid circles) and the mean number of mites sampled at different times from initial populations of 50 adult female and 50 adult male mites maintained on 0.3g of wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h. (open squares).
Table 4.2. Index estimation ratio showing the goodness of fit for the numbers of mites of each life-cycle stage between populations of *A. siro* maintained at 20 °C and 70% r.h. and a Leslie matrix model when the egg output per adult is unadjusted, doubled, tripled and quadrupled.

<table>
<thead>
<tr>
<th>Life-cycle stage</th>
<th>Goodness of fit (Q) between the model and populations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unadjusted</td>
</tr>
<tr>
<td>Egg</td>
<td>-2.46</td>
</tr>
<tr>
<td>Larva</td>
<td>0.69</td>
</tr>
<tr>
<td>Protonymph</td>
<td>0.72</td>
</tr>
<tr>
<td>Tritonymph</td>
<td>0.43</td>
</tr>
<tr>
<td>Adult</td>
<td>0.38</td>
</tr>
<tr>
<td>All mites</td>
<td>-0.23</td>
</tr>
</tbody>
</table>
Fig. 4.12. The number of *Acarus siro* predicted by the adjusted Leslie matrix model over time (solid circles) and the mean number of mites sampled at different times from initial populations of 50 adult female and 50 adult male mites maintained on 0.3g of wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h. (open squares).
Chapter 4 - Life history and population dynamics of *Acarus siro*

**Protonymphs**

- Number of protonymphs
- Time (days)

**Larvae**

- Number of larvae
- Time (days)

**Eggs**

- Number of eggs
- Time (days)
Fig. 4.13. The mean proportion of mites at each stage of the lifecycle of *Acarus siro* during the 60 day period post establishment of an initial population of 50 adult females and 50 adult males. Solid bars represent mean values (± S.D.) of the adjusted Leslie matrix model and open bars represent mean values (± S.D.) of 5 populations maintained on 0.3g of wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h.
4.4 Discussion

In this study the mean time required to go from egg to adult was 25.6 ± 5 days. However there was a large amount of variation as the minimum time calculated for complete development was 17 days and the maximum time required for complete development was 38 days. The results of the present study support the findings of previous studies that found that the protonymphs and tritonymphs had similar durations, which were shorter than the egg and the larval stages (Fejt and Ždákrová, 2001). However the previous studies found that the egg and not the larva was the longest stage.

In the present study there was no significant difference between adult female and adult male longevity. However, a previous study had found that adult males lived longer than adult females across all the temperature and humidity regimes examined (Cunnington, 1985). At 20 °C and 70% r.h. it was found that the mean duration of adult life for males was 52 days but was only 36.2 days for females (Cunnington, 1985). Similar results were found at 15 °C and 90% r.h. with adult males sometimes living up to 90 days longer than the females (Parkinson et al., 1991). However, when the diet was changed from yeast and wheatgerm (considered to be optimal) to a fungal diet the disparity between the sexes decreased as the females were able to live longer than on the control diet whereas the male longevity decreased.

As expected, the length of the oviposition period increased with female longevity. However, females did not lay eggs up until the day they died but stopped a few days before. The number of eggs a female laid in her lifetime was determined more by the length of time available to the female to oviposit rather than the ability to lay more eggs per day. It was also found in this study that there is a mean pre-oviposition time of 2 days (± 1.4 S.D.) before the female lays her first egg regardless of the longevity of the female. A previous study showed that at low temperatures (5 and 10 °C) the mean number of eggs laid per female per day stayed at a lower and more consistent rate throughout the oviposition period whereas at higher temperatures a sharp increase in egg production during the first week led
to a peak at approximately 7 days (Cunnington, 1985). This study also shows the sharp rise over time in mean eggs laid per day per female (Fig. 4.2) with the peak rate being achieved 7 days into the adult female’s life, approximately 5 days into the oviposition period.

The number of eggs laid each day by females paired with a male and kept in chambers, was much lower in the present study than that found in previous studies. Also, a much higher number of eggs per female was counted in the laboratory populations, than when mites were kept in pairs (Figs. 4.4 and 4.8). It is possible that some dead eggs were counted as these cannot be distinguished from live eggs.

The number of eggs per female decreased as the number of mites increased. It could be that the presence of conspecifics at high densities inhibits oviposition. However, the decrease in oviposition at the higher density seen in the present study could be due to the diminished resources rather than the density of the mites. Previous studies have been conducted on various foodstuffs but have not used the same food as the work presented here. The most similar food used in a previous study is wheatgerm and yeast but the study that used this food source was conducted at 15 ºC and 90% r.h. (Parkinson et al. 1991). Another study, conducted on wheatgerm flakes found that at 20 ºC and 70% r.h. the mean fecundity was 133 eggs in a female’s lifespan (Cunnington, 1985). This finding was supported by a later study also using wheatgerm at 20 ºC and 70% r.h. which found similar results, with females laying a mean of 140 eggs in a lifetime (Fejt and Ždárková, 2001). The lower fecundity of the mites used in the present study could have been due to the different food substrate that the mites were reared on or a strain difference.

In many naturally occurring populations of arthropods it might be expected that a cyclical pattern of growth and abundance of the lifecycle stages would be observed. The periodicity explained by the development time of the juvenile stages and the longevity of the adult stages. An initial population of a few adults will lay eggs which hatch into larvae, the larvae moult into protonymphs and so on until the next generation of adults emerge, at which point the initial population of adults has died. As each stage of the lifecycle progresses into the next, the composition of the population changes in a synchronous, periodical manner as
the generations rarely overlap, although this would only happen initially. The Leslie matrix model created in the present study is a clear example of how this process leads to a stable age structure and exponential growth after an initial unstable period of growth as the population establishes itself. The original model, constructed using the life-history data collected previously in this study, shows that a stable age structure will not be reached for approximately 162 days after the introduction of 50 adult females and 50 adult male mites. However when the egg output is tripled the population does not begin to stabilise until about 306 days post establishment.

Early on in the growth of the laboratory populations, the emergence and abundance of the lifecycle stages are synchronised, as expected, and there is a slight periodicity noticeable in the adult females. However, there was no significant periodicity when analysed by autocorrelation. This lack of periodicity is largely due to the adult females living for a long period of time, up to 40 days found in the present study. This means there is a steady production of eggs and a consistent transition of mites between life-cycle stages.

The populations in the present study were maintained free from predation and disease and early on in the study there would have been an abundance of resources. As shown by the life-history data collected in this study and confirmed in the population dynamics experiment, *Acarus siro* can survive for a long time as adults and continue to lay eggs for the majority of their adult life. Adult female longevity is not the only likely cause of the lack of periodicity seen in this study. There is large variation in the development times of the life-cycle stages and the time taken to reach adulthood ranged from 17 to 38 days.

The synchronicity between the lifecycle stages is largely maintained. However the abundance of the tritonymphs was greater than the protonymphs. This could be due to the difficulty in identifying the nymphal stages when floating in a salt solution. However due to the relatively low mortality of protonymphs and tritonymphs it would be expected that the abundance for the nymphal stages and even the adult stages would be fairly similar.

As the resources of space and food start to diminish, the population begins to decline (Fig. 4.7). This is seen first in the larvae and closely followed by the eggs. The larvae are
morphologically the smallest stage in the life-cycle and may be more likely to suffer the
effects of starvation and therefore less likely to survive in a highly competitive environment.
The egg abundance starts to decline as the female abundance begins to plateau (Fig. 4.7). At
the same time, resources are beginning to run low. Fewer females may be laying eggs and
those females that are still able to, may not be laying as many eggs as they potentially could,
due to the lack of resources. The decrease in the number of eggs will also contribute to the
decline in the abundance of larvae and both lines representing the eggs and larvae in Fig.
4.7 show parallel rates of decline from day 50 onwards. The adult and nymphal stages do
not show any signs of decline until much later, around day 70. However growth in the
abundance of each stage stops between day 48 and day 54. Once these stages begin to
decline, it is the smaller life cycle stage, the protonymph, that decreases in abundance the
most rapidly. This could be for similar reasons that the decline is first noticed in the larval
stage; that the smaller stages are not able to compete for resources as well as the larger
stages. The larger stages may also be more tolerant of unfavourable conditions such as the
build up of waste products as well as starvation.

In previous studies, there has been some debate about the sex ratio in populations of
*Acarus siro*. One study calculated that females made up 64.5% of populations maintained on
wheatgerm and yeast (1:3) at 15 °C and 90% r.h. and used the assumption of 60% females in
a simple model of population growth (Berreen, 1974). However, it was found that a model
using 46.8% females gave the best estimation for the data collected by Solomon (1969) for
15 °C and 70% r.h. (Berreen, 1974). A sex ratio of 1:1, was found by Ignatowicz (1986,
1987), and was used for the construction of the population model by Pekar and Ždárková
(2004). The findings from the present study demonstrate that the sex ratio is not always
constant throughout the growth of the population. Here the mean sex ratio for the 60 day
period was 1.3:1, female: male. However the ratio fluctuated in cycles of approximately 20
to 25 days ranging from 0.8:1, female: male to 2.1:1, female: male (Fig. 4.9). The initial
population of 50 females appear to survive for longer than the initial population of 50 males.
As the population begins to decline the sex ratio becomes more variable but also biased
towards females. It could be that the larger females are able to survive poorer conditions for longer than the males. They may be more resistant to starvation or the build of waste products. A study on *Hemisarcoptes coccophagus* Meyer, a mite parasitic to armoured scale insects, showed that females survived longer than males in the absence of food (Izraylevich and Gerson, 1995). Another study showed that the spider mite *Tetranychus medanielli* McGregor, produces more female offspring in response to deteriorating conditions and extreme temperatures (Roy et al., 2003). The authors of this study suggested that it was an evolutionary response as the females are better suited to dispersal than the males due to their ability to develop into a mobile hypopus during times of poor conditions. *Acarus siro*, however, very rarely develop hypopi and none were found in the present study. *Tetranychus medanielli* mites are arrhenotokous, therefore females can produce male progeny by the oviposition of unfertilised eggs (Roy et al., 2003). Sex determination in *A. siro* is currently unknown, although arrhenotoky has not been observed in this mite. This does not necessarily preclude *A. siro* from adjusting the sex ratio of its progeny. Many animals, particularly invertebrates, are able to adjust the sex ratio of their progeny regardless of the method of sex determination (Toyoshima and Amano, 1998; Roy et al., 2003; West et al., 2005). However, it is not possible from the present study to determine if the fluctuation in the sex ratio has been caused by adjustment of the sex ratio in the offspring or by different mortality levels between the sexes or a combination of these two factors. It can however be seen in this study that the sex ratio does fluctuate and favours a female bias.

Density-dependant effects were not taken into account with the Leslie matrix model used in the present study. This means that oviposition was consistent at all population densities which is unlikely to be the case for the laboratory populations. The lower egg count per adult at day 6 is due simply to there being fewer eggs in the laboratory populations than predicted by the model even though the number of adults present were very similar. However, by day 12 there were a greater number of eggs in the laboratory population but also a greater number of adults than predicted by the original model. From day 12 up until day 72, the laboratory population consistently had a much greater number of eggs than
predicted by the model and between days 30 and 42 had a greater number of eggs per adult female.

Multiplying the number of eggs laid per adult in the model by a factor of 3 gave a better fit between the model and the population with regards to the number of eggs counted. This increase in fecundity still falls within the range found in previous studies (Boczek and Davis, 1985; Cunnington, 1985; and Fejt and Žďárková, 2001) and could be a more accurate representation of the fecundity of *A. siro* within the laboratory populations than the paired mites in chambers.

The model used in this study, although useful for generating broad hypotheses, seems to be too simplistic to accurately predict the dynamics of *A. siro* populations. Relatively good fits were achieved between the final model (when the egg output was tripled) and the laboratory populations for the larval, protonymph and tritonymph stages but were not so good for the egg and adult stages (Table 4.2). However if the final model is lagged by 2 time steps the fit between the model and the laboratory populations for the adults is greatly improved, $Q = 0.58$. Lagging the final model did not improve the fit for the other pre-adult stages.

The model, even with adjustments, is unable to cope with variation over time or density-dependant effects. The model relies upon mean data collected from life-history studies which themselves showed a wide range of variation. Factors such as the sex ratio, egg production and development time are not always constant over time in real populations. A more complex model may be needed to accurately predict the dynamics of *A. siro* populations. The simple Leslie matrix model used in the present study was however a useful tool for interpreting and highlighting the complexities of populations of *A. siro*.

The results presented in this chapter are similar to those found in previous studies. However, clearly *A. siro* is very sensitive when reared under different conditions and even when reared under the same conditions in the same study. Temperature, relative humidity and the food substrate on which the mites are reared on are the major factors in determining the growth and composition of populations. When all of these factors are considered, it is
evidently important to refer the life history parameters for *A. siro* to the specific conditions under which they are maintained.
5.1 Introduction

The entomopathogenic fungus *M. anisopliae* has been used before to control many pest species but has yet to be tested on *A. siro*. Research has focused on the effects that fungal pathogens have on individuals or on the reduction in the amount of damage done to the crop by the target pest. Not so much is known about the effects of an introduced fungal pathogen on population dynamics. Key questions include whether or not the fungus can be transmitted through the mite population, whether dead infected mites will act as a reservoir of disease and whether the pathogen and host will establish stable or cyclical co-existence patterns or will the disease die-out.

A recent study using the entomophthoralean fungal pathogen, *Neozygites tanajoae* Delalibera, Hajek and Humber, against the cassava green mite, *Mononychellus tanajoa* Bondar, found that the fungal pathogen can spread throughout a population from an inoculum of live infected mites (Hountondji et al., 2007). It was shown in the same study that the number of mites on plants inoculated with the fungal pathogen was lower than on the uninoculated control plants and that there was a decreased growth rate (Hountondji et al., 2007). However, the fungal pathogen used was an entomophthoralean fungus which, once it has sporulated on an infected host, forcibly discharges the spores. This would help to spread the pathogen and increase the chances of a mite coming into contact with the infective spores. The entomopathogenic fungi *M. anisopliae* and *B. bassiana* do not discharge their conidia but rely upon direct contact between the infected mites and uninfected mites. It has
already been shown that *M. anisopliae* is pathogenic to astigmatid mites with high levels of infection achieved against *Psoroptes* mites (Smith et al., 2000; Brooks and Wall, 2002; Chapter 3).

Hence, the work presented in this chapter aimed to investigate the pathogenicity of, *B. bassiana* and *M. anisopliae* against *A. siro* when mites were directly exposed to unformulated conidia and conidia suspended in silicone oil. The chapter also examines the transmission of infection between mites under controlled conditions and then, following on from this, the effect a fungal pathogen may have when introduced to naïve populations.

### 5.2 Materials and methods

#### 5.2.1 Pathogenicity of *Metarhizium anisopliae* and *Beauveria bassiana*

To test the pathogenicity of *M. anisopliae* and *B. bassiana* against *A. siro*, adult female mites were taken from the stock culture and divided into 5 groups of 20 mites. The groups were then treated, using the method described in Chapter 2, with one of the following: silicone oil, conidia of *M. anisopliae* (IMI386697) formulated in silicone oil, unformulated *M. anisopliae* (IMI386697), unformulated *B. bassiana* (CSLI) conidia and no treatment (control). Mites were exposed to each treatment for 1 minute. The *M. anisopliae* formulated in silicone oil was at a concentration of $1 \times 10^8$ conidia ml$^{-1}$ and 2ml of this suspension was used to inoculate the mites. The same volume, 2ml, was used for the silicone oil only treatment. The unformulated conidia of *M. anisopliae* and *B. bassiana* were weighed so that the mites would be exposed to a similar number of conidia as when the fungi were formulated in 2 ml of silicone oil at $1 \times 10^8$ conidia ml$^{-1}$. There are approximately $1.8 \times 10^{10}$ conidia per gram for *M. anisopliae* and $1.2 \times 10^{11}$ conidia per gram for *B. bassiana*. Therefore, 0.011g was measured for *M. anisopliae* and 0.017g was measured for *B. bassiana*. Following exposure, the mites were placed inside clean chambers (Fig. 2.4) with 0.03g of food and incubated at 20 °C and 70% r.h. The chambers were inspected every 24 h and dead mites were removed, surface sterilised and then placed into individual wells of a 96
well microtitre plate with a drop of distilled water. Dead mites were checked every 24 h for signs of infection.

5.2.2 Horizontal transmission of fungal infection

Prior to the horizontal transmission experiments, a trial was carried out to determine the effects, if any, of a permanent black ink applied to the idiosoma of the mites. For this, 20 adult female mites were selected from the stock culture. A small spot of black ink (Staedtler, Germany) was applied to the dorsal surface of the idiosoma using a fine grade paintbrush. The ink was applied so that it covered approximately one quarter to a third of the dorsal surface of the idiosoma. The mites were then placed into chambers with 0.03g of food and incubated at 20 °C and 70% r.h. The control mites had no ink applied but were touched with a fine grade paintbrush that had been dipped in distilled water. The experiment was repeated 5 times. Chambers were inspected every 24 h for the presence of dead mites.

To find whether transmission can occur between mites treated with a fungal pathogen and live untreated mites, adult females which had been exposed to dry conidia of *M. anisopliae* (IMI386697) were placed into incubation chambers (Fig. 2.4) containing 0.03g of food along with live unexposed adult females in various ratios. Five groups of 10 treated adult females and five groups of 5 treated adult females were placed into chambers. Each chamber then had live naïve mites added to make a total of 20 mites per chamber. Therefore, in the chambers containing 10 treated mites, 10 naïve, adult, female mites were added and for the chambers containing 5 treated mites, 15 naïve, adult, females were added. The mites that had been treated with the fungal pathogen were marked on the dorsal surface of the idiosoma with the black permanent ink as described above, to distinguish them from the mites that had not been inoculated. As a control, 10 more chambers containing mites in the same ratios were set up, replacing the inoculated mites with mites that had only been marked with the permanent black ink. Each chamber was inspected every 24 h for the presence of dead mites, which were treated and checked for signs of infection, as described in Chapter 2. This experiment was then repeated but with the treated live, mites replaced
with dead mites. Cadavers with 5 to 10 day-old infections of *M. anisopliae* were used and placed into the chambers in set spatial patterns that were constant for all the chambers (Fig. 5.1). Each cadaver was marked on the idiosoma with permanent black ink. Uninfected cadavers were used as controls.
Fig. 5.1. Position of *Acarus siro* cadavers placed into the experimental chambers used in experiments to test horizontal transmission between cadavers infected with *Metarhizium anisopliae* and live, naïve mites when in the ratio of 5:15, infected cadavers: live, naïve mites (top) and 10:10, infected cadavers: live, naïve mites (bottom).
5.2.3 Effect of a fungal pathogen on the population dynamics of *Acarus siro*

**Small-scale, high density**

To find out whether or not a fungal pathogen could transmit between mites within one generation in a small but dense population, populations consisting of 50 adult female and 50 adult male, *A. siro* were set up in 4 incubation chambers (Fig. 2.4) each containing 0.03g of food. The mites were selected at random from the stock cultures. To these chambers, 20 cadavers marked with permanent black ink (as described previously) and with 5 - 10 day old infections of *M. anisopliae* (IMI386697) were added. The chambers were then incubated at 20 °C and 90% r.h. for 4, 8, 12 or 16 days. At the end of this period, each chamber was placed into a freezer at -12 (± 0.5) °C for 48 h. It had previously been confirmed that freezing would not alter the subsequent expression of mycosis of an infected mite. The chambers were then removed from the freezer and the adult mites were removed from the chamber, surface sterilised and checked for signs of infection every 24 h as previously described. Five replicates were used for each time period. For controls, five chambers received only uninfected cadavers.

**Large-scale, low density**

This experiment was set up to find out if a fungal pathogen could transmit between mites and persist within larger populations of *A. siro* over several generations. Populations of *A. siro* were set up in cuvettes (Fig. 4.2) in the same way as described in Chapter 4. The cuvettes were sealed with non-absorbent cotton wool and incubated at 20 °C and 70% r.h. for 20 days before the addition of 20 *A. siro* cadavers which had been infected with *M. anisopliae* (IMI386697). The infection for each cadaver was between 5 and 10 days of age. A previous study which had examined the infectivity of cadavers of *P. ovis* found that 5 day-old infections were the most infective to live mites (Brooks and Wall, 2002). The cuvettes were agitated every 48 h by inverting them 3 times in quick succession, to prevent the food from clumping and to ensure similar conditions were maintained in all the cuvettes.
Samples were taken from the cuvettes and the mites were counted as described in Chapter 4. The number of live mites, life-cycle stage and sex of the adults were recorded. Over an 80 day period, 5 different cuvettes were sampled every 4 days. Five samples were taken from each cuvette and the samples were pooled. Once a cuvette had been sampled it was removed from the experiment and frozen at -12 (± 0.5) °C. At the end of the experiment, cuvettes containing populations aged 40 and 60 days post-establishment were defrosted, 79 and 71 days after they were frozen, respectively. A saturated salt solution (NaCl) was added to the cuvettes to float the remaining mites from the food. These mites were carefully removed using a paint brush, surface sterilised and then placed onto a damp piece of filter paper in a 100 mm diameter petri dish. The petri dish was sealed with parafilm and incubated at 20 °C. The mites were checked every 24 h for signs of fungal infection.

5.2.4 Analysis

Data were checked for normality using the Kolmogorov-Smirnov test and proportions were arcsine transformed before analysis. However, for clarity the untransformed mean (± S.D.) data are plotted in the figures presented.

5.3 Results

5.3.1 Pathogenicity of Metarhizium anisopliae and Beauveria bassiana

There was a significant effect of treatment on the arcsine proportion of infected mites (ANOVA; $F_{4,25} = 204.3, P < 0.001$; Fig. 5.2). As expected, there were no infected mites in the control group or the group that was treated with silicone oil only. All three groups exposed to conidia were significantly different to each other (Tukey post hoc test; $P < 0.01$; Fig. 5.2).

There was also a significant effect of treatment on the LT$_{50}$ values (ANOVA; $F_{4,25} = 12.62, P < 0.001$; Fig. 5.3). There was no significant difference in the LT$_{50}$ between the
groups treated with silicone oil, silicone oil and *M. anisopliae*, unformulated *M. anisopliae* or unformulated *B. bassiana* at between 3 – 5 days (*P* > 0.05). However, there was a significant difference between all of the groups and the untreated controls (*P* < 0.05) with the untreated controls having a significantly longer LT$_{50}$ than all the other groups at 9.5 days (± 2.8).
Fig. 5.2. Proportion of adult female *Acarus siro* infected (± S.D.) after exposure to no treatment (1), silicone oil only (2), *Metarhizium anisopliae* in silicone oil (3), unformulated *Metarhizium anisopliae* (4), or unformulated *Beauveria bassiana* (5) at 20 °C and 70% r.h.
Fig. 5.3. Mean LT$_{50}$ values (± S.D.) for adult female *Acarus siro* after exposure to no treatment (1), silicone oil only (2), *Metarhizium anisopliae* in silicone oil (3), unformulated *Metarhizium anisopliae* (4), or unformulated *Beauveria bassiana* (5) at 20 °C and 70% r.h. Letters indicate groups between which there is no significant difference.
5.3.2 Horizontal transmission of fungal infection

For the experiment testing for any effects of the black ink on *A. siro* survival, there was no significant difference for the LT$_{50}$ between the mites marked with black ink (5 days) and the control mites (6 days) (ANOVA; $F_{1,8} = 0.20, P > 0.05$).

There was a significantly greater proportion of infections (0.33 ± 0.16) in the naïve mites exposed to *M. anisopliae* (either via infected cadavers or exposed live mites), than the naïve mites that were not exposed to *M. anisopliae* (controls), (ANOVA; $F_{1,38} = 81.15, P < 0.001$; Fig. 5.4).

There was no significant difference in the proportion of infected mites regardless of whether or not the fungal pathogen was introduced via live, exposed mites or via infected cadavers (two-way ANOVA; $F_{1,19} = 2.49, P = 0.13$; Fig. 5.4) with 0.38 (± 0.18 S.D.) and 0.28 (± 0.11 S.D.) of the mites becoming infected, respectively. 0.34 (± 0.15 S.D.) of mites became infected when exposed to *M. anisopliae* in the ratio of 15:5 (naïve: exposed) and 0.32 (± 0.17 S.D.) of mites became infected when exposed in the ratio of 10:10. There was no significant difference in the proportion of infected mites, regardless of the ratio that the mites or cadavers were introduced to the naïve mites (two-way ANOVA; $F_{1,19} = 0.61, P = 0.81$; Fig. 5.4). There was no significant interaction ($F_{1,19} = 0.77, P = 0.39$).

When grouped together, mites exposed to *M. anisopliae* had a significantly lower mean LT$_{50}$ of 12.7 (± 8, S.D.) days whereas the control mites had a mean LT$_{50}$ of 18.4 (± 7.5, S.D.) days (ANOVA; $F_{1,38} = 5.4, P = 0.03$; Fig. 5.5).

Mites exposed to *M. anisopliae* in the ratio 15:5 had a mean LT$_{50}$ of 12.5 (± 6) days and showed no significant difference to the mites exposed to *M. anisopliae* in the ratio 10:10, which had a mean LT$_{50}$ of 12.9 (± 10) days (two-way ANOVA; $F_{1,19} = 0.02, P = 0.89$; Fig. 5.5). There was also no significant difference for the LT$_{50}$ between the groups of mites housed with live, exposed mites (15.1 days ± 8.2 S.D.) and the mites housed with infected cadavers (10.3 days ± 7.4 S.D.) (two-way ANOVA; $F_{1,19} = 2.9, P = 0.105$; Fig. 5.5). There was however a significant interaction between ratio and the type of introduced mites ($F_{1,19} = 12.3, P = 0.003$).
There were no significant differences in the LT$_{50}$ for the controls regardless of treatment.
Fig. 5.4. Mean proportion of naïve *Acarus siro* (± S.D.) infected with *Metarhizium anisopliae* (IMI386697) when 5 or 10 exposed live mites, or infected cadavers, were introduced to 15 or 10 naïve live mites, to give a total of 20 mites in an experimental chamber (Fig. 2.4) with 0.03g of food. Open squares show the mean proportion of naïve mites infected when housed with mites which had been exposed to or infected with the fungal pathogen. Solid circles show the mean proportion of mites infected when housed with mites that had not been exposed or infected with the fungal pathogen (controls).
Fig. 5.5. Mean LT$_{50}$ of naïve *Acarus siro* (± S.D.) when 5 or 10 exposed live mites or infected cadavers were introduced to 15 or 10 naïve live mites to give a total of 20 mites in an experimental chamber (Fig. 2.4) with 0.03g of food. Open squares show the mean LT$_{50}$ of naïve mites when housed with mites which had been exposed to or infected with the fungal pathogen. Solid circles show the mean LT$_{50}$ of mites when housed with mites that had not been exposed or infected with the fungal pathogen (controls).
5.3.3 Effect of a fungal pathogen on the population dynamics of *Acarus siro*

**Small-scale, high density**

There was no significant difference in the number of infected mites regardless of the exposure time (4, 8, 12, 16 days) to the infected cadavers (ANOVA; $F_{3,13} = 0.62, P = 0.61$; Fig. 5.6). The mean number of infected mites (± S.D.) found in the groups of 100 mites exposed to 20 infected cadavers for 4, 8, 12 and 16 days, were: 8 (± 6.2), 8.2 (± 4.8), 4.4 (± 4.6) and 6 (± 5.6) respectively, i.e. less than 10%.

However, one replicate, exposed to infected cadavers for 4 days, had 51 mites which became infected (Fig. 5.6). This was considered as an outlier for the purpose of statistical analysis. There were no infected mites found in the control groups.

**Large-scale, low density**

The abundance of mites increased over time, in a similar manner to the pathogen-free population (Fig. 5.7). Using the index of estimation ratio (Q) there was a strong correlation between both populations (0.90). When the separate life-cycle stages were compared there were close fits between all stages (Table 5.1). However, in the later stages of population growth there were a greater number of mites in the population with the introduced pathogen than the pathogen-free population (Fig. 5.7). When the distribution of mites across the life-cycle stages over the 60 day period was analysed with Chi square, there was a significant difference between the two populations ($\chi^2 = 636, P < 0.01$; Fig. 5.8). This difference was mainly due to the much greater number of eggs counted in the population with the pathogen. The peak number of eggs counted, were 7612 at day 52 and 4493 at day 46 for the population with pathogen and the pathogen-free population, respectively. The mean number of eggs per female was slightly greater in the population with the pathogen (11.1 ± 10.2, S.D.) than the pathogen-free population (9.7 ± 7.3) although this was not a significant difference after a log transformation to normalize the data (ANOVA; $F_{1,55} = 0.234, P = 0.63$; Fig. 5.9).
The mean sex ratio for the 60 day period was 0.93:1, female: male (Fig. 5.10). However the ratio ranged from 0.75:1, female: male to 1.5:1, female: male. The mean sex ratio for the pathogen free population was 1.4:1, female: male (Fig. 5.10). There was a significant difference in the mean sex ratio between the exposed and pathogen-free populations (ANOVA; $F_{1,38} = 17.03$, $P < 0.001$).
Fig. 5.6. Number of *Acarus siro* infected with *Metarhizium anisopliae* (IMI386697) after various time periods of exposure to 20 infected cadavers when maintained on 0.03g of wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h. Each point represents the number of infected mites from one replicate consisting of approximately 100 adult mites.
Fig. 5.7. The calculated mean number of mites sampled at different times post-establishment from populations of *Acarus siro* initially consisting of 50 adult female and 50 adult male mites maintained on 0.3g of wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h. Open squares represent populations which had 20 cadavers infected with *Metarhizium anisopliae* added at day 20, soild circles represent pathogen-free populations which were not exposed to the fungal pathogen. Each data point is the mean of 5 replicates (standard deviations not shown for clarity).
Table 5.1. Index estimation ratio showing the goodness of fit for the numbers of mites of each life-cycle stage between the population that was exposed to *Metarhizium anisopliae* at day 20 and the pathogen-free population that was not exposed to *M. anisopliae*. Both populations were maintained on wholemeal flour and yeast, 3:1 (w/w) at 20 °C and 70% r.h.

<table>
<thead>
<tr>
<th>Life-cycle stage</th>
<th>Goodness of fit (Q)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All stages</td>
<td>0.89</td>
</tr>
<tr>
<td>Egg</td>
<td>0.82</td>
</tr>
<tr>
<td>Larva</td>
<td>0.76</td>
</tr>
<tr>
<td>Protonymph</td>
<td>0.92</td>
</tr>
<tr>
<td>Tritonymph</td>
<td>0.92</td>
</tr>
<tr>
<td>Adult</td>
<td>0.89</td>
</tr>
</tbody>
</table>
Fig. 5.8. The mean number of mites at each stage of the lifecycle of *Acarus siro* during the 60 day period post-establishment of an initial population of 50 adult females and 50 adult males. Solid bars represent mean values (± S.D.) of 5 pathogen-free populations that had no exposure to *Metarhizium anisopliae* and open bars represent mean values (± S.D.) of 5 populations which had 20 cadavers infected with *Metarhizium anisopliae* added 20 days post establishment. Populations were maintained on 0.3g of wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h.
Fig. 5.9. The mean number of eggs per adult female *Acarus siro* calculated from samples taken at various times, post-establishment, from initial populations of 50 adult female and 50 adult male mites maintained on 0.3g of wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h. Solid circles represent pathogen-free populations that were not exposed to *Metarhizium anisopliae*; open squares represent populations that had 20 cadavers infected with *M. anisopliae* added 20 days post-establishment. Each point is the mean of 5 replicates (standard deviations not shown for clarity).
Fig. 5.10. Mean sex ratio (± S.D.) for adult *Acarus siro* maintained on wholemeal flour and yeast (3:1, w/w) at 20 °C and 70% r.h. Open squares represent populations that had 20 cadavers infected with *Metarhizium anisopliae* (IMI386697) introduced at day 20. Solid circles represent pathogen-free populations that were not exposed to *Metarhizium anisopliae*. Each point represents the mean of 5 replicates.
5.6 Discussion

The results from the present study show that *A. siro* is susceptible to infection by both of the fungal pathogens *M. anisopliae* and *B. bassiana*. However, in contrast to the data shown in Chapter 3 on the effect of fungal pathogens on *Psoroptes* mites, infected *A. siro* survive for longer after infection than *P. ovis*. *Acarus siro* had a LT$_{50}$ of approximately 5 days when directly exposed to conidia whereas *P. ovis* had a LT$_{50}$ of about 3 days (Brooks and Wall, 2001; Chapter 3). However, the LT$_{50}$ for mites not exposed to fungal conidia was also higher for *A. siro* than *P. ovis*, approximately 10 and 6 days respectively. This difference could be due to the conditions at which the experiments were conducted in relation to the conditions that the mites are adapted for.

Previous studies have shown that *A. siro* can act as a vector of non-pathogenic fungi, carrying conidia in their gut and on the surface of their cuticle (Hubert et al., 2003, 2004). The results from the present study show that horizontal transmission of *M. anisopliae* from exposed live mites and infected cadavers to live, naïve mites is possible. A greater number of infections was achieved in the naïve mites that were housed with the exposed live mites than those housed with infected cadavers, particularly when the ratio was 15:5, naïve to exposed mites. However there was no significant effect of ratio, or the type of introduced mites on the survival of the naïve mites. The mites exposed to the conidia but still alive are able to move around the chamber and would come into contact with the naïve mites, transferring conidia as they touch. The contact rate between the infected cadavers and live, naïve mites would not be as high. However, there was no significant effect of ratio on the level of infection which would be expected if this was the case.

The fungus *M. anisopliae* is clearly pathogenic to *A. siro* and able to transmit from one mite to another but with varying degrees of success. The levels of infection found in the horizontal transmission part of the present study are relatively low (33%) in comparison to a previous study using *P. ovis* (Brooks and Wall, 2001). It was shown that when 5 cadavers of *P. ovis* infected with the same strain of *M. anisopliae* were introduced to 15 naïve mites,
over 50% of mites became infected. This increased to over 60% when 10 infected cadavers were introduced to 10 naïve mites (Brooks and Wall, 2001).

It could be that the species or strains of fungi used are more pathogenic to *P. ovis* than *A. siro* but high levels of infection were recorded in *A. siro* that were directly exposed to conidia. However, it could be that the high levels of infection seen in the Brooks and Wall (2001) paper are partly attributable to the *Psoroptes* mites being removed from their natural environment and thus more susceptible to infection. The levels of infection and survival seen in the present study with *A. siro*, may be more indicative of what might be seen with *P. ovis* when exposed to a fungal pathogen on host. The results presented in Chapter 3 show that while *P. ovis* is highly susceptible to infection *in vitro*, the mites are still able to lay eggs and develop to at least the protonymph stage while on a sheep even when exposed to fungal pathogens.

The small-scale, high density populations showed significant prevalence of infection in the populations exposed to the fungal pathogen. However, surprisingly there was no significant effect of exposure time on the prevalence of infection. This suggests that after 4 days of exposure the fungal conidia are either no longer infectious or there are no more susceptible mites left to become infected. It is unlikely that there is a lack susceptible mites, as the previous work has shown that most mites exposed to conidia become infected. It could be that the conidia only remain viable for a few days and that contact with conidia after this point does not lead to infection. A previous study on the pathogenicity of *M. anisopliae* to *P. ovis* showed that the most infectious conidia were those that were 5 days-old and pathogenicity decreased at 10 days and older (Brooks and Wall, 2001).

Although the smaller populations were allowed to reproduce and develop without interference, only the initial population of 100 adults was used for analysis and therefore the effects of the fungal pathogen were only studied for one generation. The large-scale, low density populations were studied for several generations and all life-cycle stages were examined. However, there seemed to be no effect of the introduced pathogen on the dynamics of those populations. Only one infected mite was found from all the mites
removed from the cuvettes at the end of the bioassay. This mite came from a population sampled 40 days post-establishment (20 days after the introduction of the infected cadavers). Apart from this infected mite, the fungal pathogen did not show signs of persisting within the population of *Acarus siro*.

The growth of the population followed the same trends as for the pathogen-free population with no significant periodicity. The mean egg output per female peaks at a similar time and the same relationships are seen between eggs laid per female and the number of adult mites as well as the changes in sex ratio. However, there was less variation in the sex ratio in the populations exposed to the fungal pathogen than in the pathogen-free population. There was also a significant difference in the mean sex ratio of the two populations with female bias in the pathogen-free population but a more even sex ratio in the population with the pathogen. However, there was considerable variation in the sex ratio of both populations across the sampling period.

Although there was a greater number of mites in the population with the pathogen than in the pathogen-free population, the change in abundance over time for the various life-cycle stages were very similar when analysed with the index of estimation ratio (Q). However, Chi square analysis on the mean abundance of each life-cycle stage showed that there was a significant difference between the two populations. This seems to be due mainly to the increased number of eggs counted in the population with the pathogen. Previous studies have found that fungal pathogens such as *M. anisopliae* and *B. bassiana* can lower the fecundity of infected females (Wekesa *et al.*, 2006; Roy *et al.*, 2008). However, no such effect was seen in this study.

The numbers of larvae and nymphal stages match very closely between the two populations. This would suggest that although more eggs are being laid in the population with the added pathogen, there is also a greater mortality for the eggs. Conversely, it seems that the mortality of tritonymphs is less in the population with the added pathogen as the number of tritonymphs is very similar between the two populations, yet there are a greater number of adults in the population with the added pathogen. A previous study on the use of
M. anisopliae on P. ovis showed that there was no significant difference for the levels of infection between the different life-cycle stages and also that the exposure of eggs to the conidia had no effect on hatching (Brooks and Wall, 2005).

Given the lack of any other significant effect of the fungal pathogen on the dynamics of the population and the lack of infection present in the population exposed to M. anisopliae, it could be that the differences seen between the two populations are due to natural variation for a species of mite that has highly variable life-history parameters.

It may be that the conditions within the cuvettes are not suitable for the pathogen to remain viable and therefore failed to infect enough mites to elicit a noticeable effect. Another possibility is that the fungus did infect the mites but did not induce mycosis and therefore did not inhibit the normal behaviour or effect the reproduction and longevity of the mite to a significant extent. Infected mites that did not exhibit signs of mycosis may not have produced conidia after the death of the mite and therefore the propagation of infection from mite to mite would not occur and the population was able to grow in a normal manner.

The fungal pathogen used in the present study relies upon direct contact between the conidia and the mites to transmit infection. The small-scale populations would have had a greater contact rate between mites and the infected cadavers than the large-scale populations. Although the large-scale populations increased in density over time, it may be that the fungal pathogen was no longer viable and was not able to infect mites once the contact rate had increased to a comparable level to the small-scale populations. It would seem probable that there is a threshold density, below which the pathogen is not able to transmit and persist within a population. The small-scale populations may have exceeded this threshold and achieved horizontal transmission, while the large-scale populations did not.
CHAPTER 6

DISCUSSION

The work presented in this study shows that under tightly controlled conditions it is possible to infect *Psoroptes ovis* on a live animal with *M. anisopliae* and *B. bassiana*. However, there are still many problems to overcome if either of these two pathogens are to be used as a practical treatment for sheep scab.

*Psoroptes* mites live on the skin surface of the host where they are protected by the fleece. In the third and fourth *in vivo* trials conducted in the present study where a high level of infection was achieved, the fleece was removed or reduced in length. This meant that it was possible to apply the fungal conidia in a position that ensured contact with the mites. Although the results presented here suggest that there is no intrinsic inhibition by the fleece, it can still act as a physical barrier preventing the conidia from reaching the mites. It is unlikely to be practical or desirable to simply shear sheep that are infested with scab on ethical or welfare grounds. Therefore, a treatment is required that can be applied to a fully-fleeced sheep. This places a high degree of importance on the formulation and application of the conidia.

The work in the present study demonstrated that different excipients had different effects on the conidia of *M. anisopliae* and *B. bassiana*. A large decrease in the efficacy of *M. anisopliae* was observed when conidia were formulated in sheep dip excipient and diatomaceous earth, compared to the unformulated conidia, which gave the highest level of infection. Although the same trend was observed for *B. bassiana* there was no significant difference between the treatments and a high level of infection was achieved for all formulations. There was less variation between the formulations tested, although one formulation, Codacide®, gave significantly fewer infections. However, when the same formulations were tested *in vitro*, there were significant differences in the proportion of infected mites between the different formulations and the two fungal pathogens. When *M. anisopliae* was formulated in Codacide®
and vegetable oil, a large proportion of mites became infected but few mites became infected when exposed to conidia in Output® and sunflower oil. When the same formulations were tested with *B. bassiana* instead of *M. anisopliae*, the proportion of infected mites was so low that there was no significant difference to the controls that had no infected mites. The only formulation that gave a significantly greater proportion of infected mites than the controls, was conidia in sunflower oil with 6% of mites infected. Clearly formulation can make a significant difference to the pathogenicity of conidia.

The different exposure times may explain the difference observed for the formulation of diatomaceous earth that was tested in the *in vivo* trials. In one *in vivo* trial, the diatomaceous earth formulation was highly pathogenic with both fungal pathogens but was less so in another, especially with *M. anisopliae*. It could be, as discussed in Chapter 3, that diatomaceous earth requires a longer period of exposure to the mites before having an effect. Until then, the competition between the diatomaceous earth particles and conidia for attachment sites on the cuticle, inhibits the pathogenicity of the formulation.

The *in vivo* trials described here have shown that for most of the formulations there is no significant difference in the proportion of mites infected, provided that the mites come into contact with the conidia for a long enough time period. This suggests that the most important aspect of the excipient is whether or not it will be able to penetrate the fleece and carry the conidia to the mites. Therefore, an excipient that may not be as effective as others *in vitro*, may be just as effective or even more so *in vivo*, if it is better able to penetrate the fleece.

Of note was the large degree of variation between the pathogenicity of the various formulations of the two fungal pathogens *in vitro* and *in vivo*, but the reasons for this difference are unclear. One possible explanation is the longer time of exposure to the formulations *in vivo*. The fourth *in vivo* trial had an on host exposure period of 5 days. Therefore, less efficacious formulations would have a longer time to infect mites. The *in vitro* trials exposed the mites for 1
min to the fungal pathogen. This might not be long enough for the less pathogenic formulations to infect all of the mites.

Another possible explanation for the variation seen between the trials could be variation between the batches of conidia used. The properties of *M. anisopliae* and *B. bassiana* can be highly variable between batches, even when reared under the same conditions (Dr Belinda Luke, personal communication). The nutritional properties of the culture media that fungal pathogens are reared on have been shown to affect the growth and virulence of the conidia (Shah *et al.*, 2005; Safavi *et al.*, 2007). Repeated subculturing has also been shown to affect the growth and virulence of fungal pathogens, with repeated subculturing weakening the virulence of the pathogen (Shah *et al.*, 2007). It could be possible that even slight changes in the culture media or the storage of conidia could affect the level of mycosis produced in the target organism.

Conidia can be damaged by exposure to high temperatures, low humidities and ultraviolet light (McClatchie *et al.*, 1994; Alves *et al.*, 1998; Arthurs and Thomas, 2001; Braga *et al.*, 2001a,b,c). Therefore, the storage and formulation of conidia will be particularly important to minimise the effects of exposure to sub-optimal conditions.

Conidia of *B. bassiana* and *M. anisopliae* applied to cowpea foliage (*Ceratosanthes hilariana* Cogniaux) and exposed to sunlight in Brazil, were found to have lost viability after one week (Daoust and Pereira, 1986a). Conidia that were protected from sunlight remained viable for up to 3 weeks (Daoust and Pereira, 1986a). Conidia of *B. bassiana* present on the surface of cadavers of the cowpea pest, *Chalcodermus aeneus* Boheman, were able to survive for at least 16 weeks in PVC cylinders in outdoor conditions when protected form direct sunlight and rain (Daoust and Pereira, 1986b). Cadavers kept in cylinders that were not protected form direct sunlight or rain had much fewer conidia present although there was no loss in the viability of the remaining conidia (Daoust and Pereira, 1986b). The damaging effects of sunlight are likely to be due to UV-A and UV-B radiation. These have been shown to cause delays in germination (Braga *et al.*, 2001a,b). A 1h exposure of *M. anisopliae* conidia to UV-B radiation caused a
delay in germination, with only 15% of conidia germinating after 12h (Braga et al., 2001a). However, 95% of conidia germinated after 48h (Braga et al., 2001a). Certain isolates may be more tolerant of UV radiation than others. A previous study found that there was a correlation between latitude and tolerance to UV-B exposure (Braga et al., 2001c). Conidia of strains of *M. anisopliae* from increasing latitudes were more susceptible to damage by UV-B radiation than those from nearer the equator (Braga et al., 2001c).

Suspending the conidia in oils and chemical sunscreens can increase the tolerance to UV exposure (Moore et al., 1993; Alves et al., 1998). A previous study found that 22% of conidia of *M. anisopliae* suspended in peanut oil, germinated 24h after a 6h exposure to UV radiation compared to 0.5% germination for conidia suspended in 0.05% Tween 80 (Alves et al., 1998). Another study used sunscreen added to a groundnut oil and Shellsol K mixture to suspend conidia of *Metarhizium flavoviride* Gams and Rozsypol (Moore et al., 1993). The use of sunscreen was found to increase the germination of conidia after UV exposure, with oxybenzone providing the most protection; 82% of conidia germinated 48h after a 3h exposure (Moore et al., 1993). Conidia formulated in oil without the sunscreen only had 28% germination, 48h after a 3h exposure to UV radiation (Moore et al., 1993).

Formulation can also be important in limiting the effects of temperature on the viability of conidia. The addition of silica gel to conidia stored in oil gave a germination of 81% at 36 °C, 5h after a 1h exposure to 60 °C (McClatchie et al., 1994). In contrast, only 25% of conidia germinated when suspended in oil without the silica gel (McClatchie et al., 1994). The same study also found that increasing temperatures decreased the viability of conidia stored in oil and silica gel over time. Only 20% of conidia stored at 55 °C remained viable after 42 days whereas 50% remained viable at 45 °C and 80% were viable at 17 °C (McClatchie et al., 1994).

Freeze drying and spray-drying have also been investigated for the storage of conidia. Spray-drying uses a flow of compressed air to dry the conidia at temperatures between 60 and 80 °C (Stephan and Zimmerman, 1998; Horaczek and Viernstein, 2004). A study investigating the
effect of spray-drying on *M. anisopliae* and *B. bassiana* found that there was no significant loss in viability of conidia after they had been spray-dried (provided that skimmed milk powder was added as a protective agent) compared to the controls that had not been spray-dried (Stephan and Zimmerman, 1998). Skimmed milk acts as membrane stabiliser during cryopreservation and also acts as an emulsification agent (Horaczek and Viernstein, 2004). However, when other protective agents were used there was a significant decrease in germination after spray-drying (Stephan and Zimmerman, 1998). Another study using skimmed milk as a protective agent observed 93% germination for spray-dried *M. anisopliae* (Kassa et al., 2004). However, another study found that spray-drying caused a loss in viability, even when skimmed milk was used as a protective agent (Horaczek and Viernstein, 2004). Conidia of *M. anisopliae* that had not been spray-dried had a germination of 96% after 24h incubation, whereas only 34% of spray-dried conidia germinated. The same trend was observed for *Beauveria bronniartii* (Saccardo) Petch; control conidia had 96% germination while spray-dried conidia had 37% germination (Horaczek and Viernstein, 2004).

Freeze-drying uses liquid nitrogen to freeze the conidia (approximately -80 °C) before they are placed into an oven at temperatures between -35 °C and 40 °C (Horaczek and Viernstein, 2004; Kassa et al., 2004). As with spray-drying, mixed results have been found. Germination levels of 68% were found for *B. bronniartii* but only 4% for *M. anisopliae* (Horaczek and Viernstein, 2004). Another study found a germination level of 66% for freeze-dried *M. anisopliae* (Kassa et al., 2004).

Conidia that have been spray-dried or freeze-dried successfully, can be stored for long periods of time at low temperatures (Horaczek and Viernstein, 2004; Kassa et al., 2004). Freeze-dried conidia of *B. bronniartii* showed 78% germination after 8 weeks of storage at 6 °C, although the spray-dried conidia only had 28% germination under the same conditions (Horaczek and Viernstein, 2004). Freeze-dried conidia of *M. anisopliae* had a lower germination level than *B. bronniartii*; 35% after 8 weeks at 6 °C (Horaczek and Viernstein,
Chapter 6 — Discussion

2004). However, another study found that over 75% of freeze-dried \textit{M. anisopliae} conidia germinated after 45 days storage at 10 °C (Kassa et al., 2004).

Freeze-drying or spray-drying conidia may provide a potential storage method for conidia but more work needs to be done to identify suitable strains of fungi and appropriate methods of drying that will not damage the conidia. The ability for long-term storage of conidia will be an important feature for a potential sheep scab treatment. A product that can be easily stored by farmers or vets that will remain viable through the autumn and winter months, if not several years, would be required so that treatment could be readily applied when necessary. A product with a short shelf-life would probably not appeal to most farmers. A long shelf-life would also prevent the misapplication of a product that has lost viability.

There have been many studies conducted on the effect that fungal pathogens could have on non-target organisms (Burgner et al., 1998; Revankar et al., 1999; Dromph and Vestergaard, 2002; Ginsberg et al., 2002; Milner et al., 2002; Stolz et al., 2002; Zimmerman, 2007a, b).

The isolate of \textit{M. anisopliae} used in the present study for the control of \textit{P. ovis} has also been found to be pathogenic against the cattle louse \textit{B. bovis} (Briggs et al., 2006) and the blowfly \textit{Lucilia serricata} (Meigen) (Wright et al., 2004). This could be beneficial for a treatment of sheep scab as it may be able to provide protection against other ectoparasites. However, the potential for these fungal pathogens to infect non-target organisms could also cause problems if large quantities are released into the environment via run-off. Both \textit{M. anisopliae} and \textit{B. bassiana} are naturally present in the environment. However, their use in the control of sheep scab could considerably increase the number of conidia in certain parts of the environment at certain times of the year.

A strain of \textit{M. anisopliae} used to infect the tick, \textit{I. scapularis} was also pathogenic to the ladybird beetle, \textit{Hippodamia convergens} Guerin-Meneville and the house cricket, \textit{Acheta domesticus} L. (Ginsberg et al., 2002). Infection of hymenopteran parasitoids exposed to the same strain of \textit{M. anisopliae} used in the commercial product Green Muscle\textsuperscript{®} has also been
shown (Stolz et al., 2002). The survival time for adult females of the wasp, *Apoanagyrus lopezi* De Santis, was reduced by 24% when exposed to conidia of *M. anisopliae* (Stolz et al., 2002). However, direct exposure for 10s, of three collembolan species to various isolates of *M. anisopliae* and *B. bassiana* conidia at a concentration of $1 \times 10^7$ conidia ml$^{-1}$ had no effect on mortality (Dromph and Vestergaard, 2002). Continual exposure to *M. anisopliae* conidia in sphagnum moss resulted in an increased mortality for the collembolan, *Folsomia fimetaria* (L.) after 14 days, with 42% mortality for exposed *F. fimetaria* and only 17% for controls (Dromph and Vestergaard, 2002).

A study conducted on the risk of *M. anisopliae* to aquatic ecosystems found no significant difference between the mortality of mayfly nymphs (*Ulmerophlebia* spp.) exposed to $2 \times 10^6$ conidia ml$^{-1}$ and nymphs that were not exposed to conidia (Milner et al., 2002). There was also no effect of conidia on 8 week-old rainbow fish (*Melanotaenia duboulayi* Castelnau), although it was not reported in the paper what parameters were being tested (Milner et al., 2002). At concentrations of $1.3 \times 10^6$ conidia ml$^{-1}$, 100% mortality of the cladoceran, *Ceriodaphnia dubia* Richard occurred within 48h of exposure (Milner et al., 2002). However these concentrations are likely to be much greater than they would experience in the field. Samples taken from six water bodies, 48h after field spraying, showed low levels of contamination, with only 18 of 96 agar plates inoculated with the sampled water, growing colonies of *M. anisopliae* (Milner et al., 2002). However the size of the water bodies or their location in relation to the field spraying was not reported.

The effect of entomopathogenic fungi on vertebrates has also been investigated. A study that fed laboratory reared mice with food inoculated with *M. anisopliae* conidia, found no clinical signs of infection or harm to the mice (Toriello et al., 2006). Conidia of *M. anisopliae* and *B. bassiana* also appear to be harmless to reptiles, amphibians and birds when ingested or via skin contact (Zimmerman, 2007a, b). However, a case of a domestic cat with sinusitis was shown to have an infection of *M. anisopliae* (Muir et al., 1998). The cat was successfully
Chapter 6 – Discussion

treated with itraconizole, administered orally (Muir et al., 1998). Two human cases of sinusitis have also been attributed to *M. anisopliae* (Revankar et al., 1999). One occurred in a 36 year-old male and the other case was a 79 year-old female. Both humans were immunocompetent and were treated surgically without the use of antifungal therapy (Revankar et al., 1999). One case exists of an immunocompromised child with a disseminated infection of *M. anisopliae* (Burgner et al., 1998). Lesions were found in the patient’s lungs, skin and brain. Although the patient’s death was not caused by the infection, it was concluded that the infection contributed to his death by preventing further chemotherapeutic treatment (Burgner et al., 1998).

Although it has been shown that both of the fungal pathogens used in the present study are able to infect non-target organisms, it is unlikely that these organisms would become infected under field conditions. The studies mentioned previously have all concluded that while it was possible to infect the study organism in the laboratory, the concentrations of conidia required to induce those infections are much greater than that which the organism would be exposed to in the wild. The fungal pathogens *M. anisopliae* and *B. bassiana* are still considered to be safe to vertebrates and non-target organisms and are unlikely to pose a threat to the environment.

It has been shown that non-target organisms could even be utilised as dispersal agents of entomopathogenic conidia (Butt et al., 1998). Pollen beetles (*Meligethes aeneus* (F.)) were maintained on flowers in cages, in the presence of honeybees (*Apis mellifera* L.). Trays of *M. anisopliae* conidia were placed at the entrance of the hive so that the bees would pick up conidia as they walked across the surface. Mortality levels for pollen beetles kept in cages with bees and conidia reached 99% for samples taken 7 days after the start of the trial (Butt et al., 1998). The mortality of pollen beetles maintained in the presence of bees but no conidia were only 23%, 7 days post-establishment (Butt et al., 1998). The authors concluded that honey bee mediated transport of conidia to flowers were pollen beetles are present, could be incorporated into a crop management strategy (Butt et al., 1998).
Although the work in Chapter 3 has shown that it is possible to infect *Psoroptes* mites on a live animal, it has yet to be seen if mycosis will fully develop while the mite is on the host. All infected mites were removed from the sheep before they showed any exterior signs of mycosis. It was observed in the fourth *in vivo* trial that oviposition continued in the presence of the conidia as did the subsequent development of the eggs up to the protonymph stage. At this point the trial ended so it is not known whether or not the mites would continue to develop into reproductive adults.

*In vitro* experiments using *Psoroptes* mites exposed to conidia, produced low LT$_{50}$ values. This was due to the sub-optimal conditions for the *Psoroptes* mites that are unable to survive for long periods off host at the conditions used in the bioassays (30 °C, 90% r.h.). However, the *in vitro* bioassays conducted with *A. siro* rather than *P. ovis* had larger LT$_{50}$ values. These bioassays were conducted at conditions that were highly suitable for the survival and development of the mites (20 °C, 70% r.h.). Although the conditions that *P. ovis* and *A. siro* are exposed to are considerably different, the *A. siro*/fungal pathogen system provides useful information as to what could be expected in populations of *P. ovis* exposed to a fungal pathogen *in vivo*. The laboratory populations did not reach a stable age structure, which was predicted to occur approximately 300 days post-establishment. However, this is not a problem when using the system to gain an insight into what might occur when a fungal pathogen is introduced to a population of *P. ovis* on a live host. The infestation of *P. ovis* will rapidly grow but is unlikely to reach a stable-age structure before the point at which intervention is required. Therefore the transient state of the *A. siro* populations should give a close representation of a growing *P. ovis* population at a point at which the fungal pathogens would be applied.

Previous studies, investigating the horizontal transmission of fungal infection from infected cadavers to live, naïve *Psoroptes* mites, showed a high level of transmission and subsequent infection; 50 - 60% (Brooks and Wall, 2001). The current study (Chapter 5) showed that levels of transmission and infection from infected cadavers to live, naïve *A. siro* were lower,
only 33%. When the pathogen was introduced via infected cadavers to small but dense populations it was able to induce between 22% and 42% infection within the adult mites. However, when the pathogen was introduced to larger, less dense populations, there were no signs of infection or of any effect on the development of the populations. This suggests that there could be a density threshold, below which the contact rate is not sufficient to transmit the disease through the population. Although *M. anisopliae* induces high levels of infection in *A. siro* with controlled exposures, it seems that the fungus lacks enough pathogenicity and transmissibility to persist within growing populations of *A. siro*. The initial density of the population is too low for a pathogen that is unable to infect every individual that makes contact with conidia or infected cadavers. By the time the *A. siro* populations reached a density that could support the transmission of the pathogen, the conidia were no longer viable. It could also be that any individual that became infected was not killed before reproducing and ovipositing.

The transmission and persistence of pathogens in many cases depends on host density, with important implications for the control of pests, parasites and disease (Swinton et al., 1998; McCallum et al., 2001). The effect of density is complicated in populations that are not constrained to particular geographical areas. Many pest species are characterised by their ability to disperse and colonise new areas. This migration offers the population a chance to escape the pathogen (Loehle, 1995; Bradley and Altizer, 2005). If the proportion of infected individuals within a population is low, it is unlikely that any new colony that is formed by migration will contain enough infected individuals for the pathogen to be maintained within the new population (Anderson and May, 1979). Some viruses are unable to persist at sub-threshold host densities (Swinton et al., 1998) and the same principle might apply to fungal pathogens. Even if the level of infection is high within an original population, migration would still provide an opportunity to escape the disease. New populations formed from migration of a few individuals would most likely exist, initially, at low densities which would be below the density threshold required for the persistence of the disease (Anderson and May, 1979). Therefore, populations, such as *A.*
siro, infected with a pathogen like M. anisopliae, would be able to evade infection through migration. This could also be the case for P. ovis. Populations of P. ovis grow rapidly on a host and spread from the initial point of infestation to cover a large part of the host body. This growth may occur too rapidly for a fungal pathogen to persist within the expanding element of the population. Even if an infected mite is able to migrate with the expanding population, it will not become infectious to other mites until it has died and sporulated. The growth of conidia on the surface of a dead mite does not usually occur until 48h after death. By this point the rest of the developing population may have moved on (Bradley and Altizer, 2005).

One of the possible benefits of biological control using entomopathogenic fungi is the potential that only one treatment needs to be applied following which the infection will spread and produce new conidia. This would reduce the initial volume of treatment required. The work in this thesis has shown that this is not necessarily the case. It may be necessary to reapply the treatment at certain times after the initial application to ensure that there is a fresh supply of viable conidia in an area in contact with the pest. It may also be necessary to ensure that all pests come into direct contact with the treatment and not rely on the transmission from one individual to another.

There is still potential for use of entomopathogenic fungi for the treatment of sheep scab. However, the work presented in this thesis suggests that it is unlikely that either M. anisopliae or B. bassiana will provide an effective treatment for sheep scab in the near future. Although it has been shown that both fungal pathogens tested can infect P. ovis on a live animal, it remains to be seen whether or not these infections are lethal to the mites in vivo. The work using A. siro as a model species highlights the problems of using a pathogen with low virulence in populations of a low density or an ability to migrate. The work presented in Chapter 3 also demonstrates the difficulties in the application of a fungal treatment to control scab. Even if a highly pathogenic strain of fungus is found that is highly efficacious against Psoroptes mites in vivo, a suitable method of application is still needed.
Perhaps the use of fungal pathogens against scab mites of cattle or other ectoparasites such as lice, where fleece is not a barrier to the treatment, would be more effective. However, a previous study on the use of *M. anisopliae* against *B. bovis* showed that although there was a high degree of infection in the lice exposed to conidia *in vivo*, not all lice exhibited signs of infection (Briggs *et al.*, 2006). When conventional insecticides are used to treat against pest or parasite species, complete control is usually the expected outcome. However, the use of alternative treatments such as entomopathogenic fungi, will require different goals and expectations will need to change. Rather than using biological control as a replacement for conventional control methods, they could be used in conjunction with each other. The biological controls could be used for suppression, to maintain infestations at levels below a predetermined welfare and economic threshold (Wall, 2007). If the disease or infestation rises above that threshold then conventional treatments could be used selectively. Although this strategy incorporates the use of chemical pesticides, it would decrease the amount used and thus minimise the exposure of potentially harmful chemicals to humans and the environment.

Entomopathogenic fungi have been combined with chemical pesticides in previous studies. The addition of imidacloprid (a neonicotinoid insecticide) to *B. bassiana* significantly increased the mortality of the aphids *Myzus persicae* (Sulzer) and *Macrostephonella sanborni* (Gillette) (Ye *et al.*, 2005) compared to the unexposed controls and aphids exposed to *B. bassiana* only. The volume of imidacloprid added to the conidia was below the lethal dose required for imidacloprid alone. The authors concluded that the combination would be an effective treatment and reduce the volume of pesticide used to control these pests (Ye *et al.*, 2005). The same trend was observed in another study combining imidacloprid with *M. anisopliae* (Jaramillo *et al.*, 2005). The combination of the insecticide with the fungal pathogen increased mortality compared to the use of the conidia alone when applied to the subterranean burrower bug, *Cyrtomenus burgi* Froeschner (Jaramillo *et al.*, 2005). *Metarhizium anisopliae* has also been combined with the pyrethroid, deltamethrin for the control of the cattle tick,
Boophilus microplus (Canestrini) (Bahiense et al., 2006). As with the previous studies, it was found that mortality was greater for the ticks exposed to the conidia combined with deltamethrin than for the ticks exposed to conidia alone (Bahiense et al., 2006). It was also found that the combination of conidia and deltamethrin induced greater mortality than deltamethrin only (Bahiense et al., 2006). Clearly there is the potential for the combination of entomopathogenic fungi and conventional pesticides for the control of sheep scab. A combination like this would potentially increase the efficacy of the fungal pathogen while decreasing the volume of insecticide used. Further research into the effects of combining entomopathogenic fungi with the conventional treatments of sheep scab such as diazinon and the macrocyclic lactones would provide useful data. Combination of the fungal pathogens with a dip or a pour-on treatment would probably involve a straight-forward mixture. However, use of the pathogens with the injectable avermectins would require two separate applications of treatment to the sheep; an injection for the avermectins and a dip or pour-on for the fungal pathogen. This may prove to be too time consuming and too costly to be a practical treatment.

Future work must identify an excipient which can deliver conidia to the skin surface, where the mites are present. This might preclude many of the excipients currently used in agriculture and veterinary medicine, and require the development of untested compounds which would need to be tested for the safety to humans and other non-target organisms. Alongside the development of new formulations, in vivo trials examining the efficacy of fungal pathogens to Psoroptes mites need to be conducted. These should progress from the small-scale arena trials described here to larger-scale tests, perhaps using larger arenas on the skin of sheep with fleece that has not been sheared. Lower concentrations of conidia should also be tested to find the minimum threshold of conidia required to produce infected mites. Most importantly, trials conducted over a longer time period need to be carried out to see whether infected mites will develop mycosis on a host. Using a careful, staged approach, trials could eventually move onto
whole sheep trials using a potential fungal-pathogen-based treatment to control an infestation of sheep scab.

There is still potential for use of fungal pathogens for the control of sheep scab but there are many problems that need to be overcome before a marketable product can be produced. Although *M. anisopliae* and *B. bassiana* have been shown to be effective against many pest arthropods, doubts still remain about its efficacy against *Psoroptes* mites *in vivo*. 
References


Control of the sheep scab mite *Psoroptes ovis* in vivo and *in vitro* using fungal pathogens

S. Abolins\textsuperscript{a,}\textsuperscript{*}, B. Thind\textsuperscript{b}, V. Jackson\textsuperscript{b}, B. Luke\textsuperscript{c}, D. Moore\textsuperscript{c}, R. Wall\textsuperscript{a}, M. A. Taylor\textsuperscript{b}

\textsuperscript{a}School of Biological Sciences, University of Bristol, Woodland Road, Bristol BS8 1UG, UK
\textsuperscript{b}Central Science Laboratory, Sand Hutton, York YO41 1LZ, UK
\textsuperscript{c}CABI Europe-UK, Silwood Park, Buckhurst Road, Ascot SL5 7TA, UK

Received 5 April 2007; received in revised form 1 June 2007; accepted 4 June 2007

**Abstract**

As part of a research programme designed to identify biological agents for the control of sheep scab, the pathogenicity of the fungus *Metarhizium anisopliae* to *Psoroptes* mites in the presence of sheepskin and wool was examined in the laboratory. No inhibitory effects of skin and wool were observed and high levels of infection were recorded. Subsequently the pathogenicity of formulations of both *M. anisopliae* and *Beauveria bassiana* to *Psoroptes ovis* was studied in vivo. For this, 36 batches of 20 adult female *Psoroptes* mites were confined in 25 mm diameter chambers which were attached to the backs of 6 scab-naive sheep. In some treatments, mites were exposed to the fungal pathogens for 48 h *in vitro* prior to being placed on the host, while other treatments involved mites with no prior exposure placed directly onto the skin of a host treated with a fungal pathogen. After 48 h on the host, mites were removed, incubated individually and all fungal infections were recorded. Fungal infection was observed in all treatments, except untreated controls. However, *B. bassiana* infected a significantly greater number of mites than *M. anisopliae* with all the formulations examined. Infection rates were highest when mites were exposed to dry conidia (>90%) and lowest with *M. anisopliae* in diatomaceous earth. Overall, the infection rate was not affected by whether or not the mites were given prior exposure to the conidia, before being placed on the sheep. The results demonstrate that *Psoroptes* mites can become infected by entomopathogenic fungi on the skin of sheep and provides a first demonstration of the potential of this technology for the control of sheep scab.

© 2007 Elsevier B.V. All rights reserved.

**Keywords:** *Psoroptes ovis*; Sheep scab; Fungus; *Metarhizium anisopliae*; *Beauveria bassiana*; Biological control

1. Introduction

In the UK, in 1989 when compulsory national plunge-dipping for the control of psoroptic mange in sheep (sheep scab) was reduced from two to one annual treatment, there were approximately 40 reported outbreaks of the disease in that year (French et al., 1999). In 1991, when the final compulsory dip was abolished, there were approximately 120 outbreaks (French et al., 1999). Following that year, sheep scab was treated by farmers on a case-by-case basis. However, by 1997 up to 3000 outbreaks were estimated to have occurred nationally (Lewis, 1997) and by 2004 a national prevalence of 9% of flocks was established, equating to approximately 6750 cases of scab per year (Bisdorff et al., 2006). This represents a steady and concerning increase. The disease appears to be most prevalent in Wales, Scotland and...
Northern England (Bisdorff et al., 2006), suggesting an association with upland farms, the movement of stock and the use of common grazing (O’Brien, 1999).

Throughout most of this period, the only insecticides registered for use in dips were the organophosphates prophamphos and diazinon, and the pyrethroids flumethrin and later high cis-cypermethrin. However, because of environmental concerns the pyrethroids are currently unavailable for scab control in the UK. Prophamphos was withdrawn for economic reasons and following temporary withdrawal to allow the development of safer packaging, diazinon remains the only available insecticide for use in dips in the UK, although pressure for its removal remains because of concerns over human exposure. Currently, only the injectable avermectins (moxidectin, ivermectin and doramectin) are available as therapeutic alternatives to dips, although their use is constrained by the relative application difficulty by farmers, lack of residual activity (ivermectin) and expense (Bates, 2004).

As a result, there has been considerable recent interest in the development of alternative approaches to scab treatment, particularly the use of entomopathogenic fungi (Brooks and Wall, 2001; Brooks et al., 2004). High levels of infection and mortality in Psoroptes ovis (Herring) (Acari: Psoroptidae) has been demonstrated in vitro, using the fungus Metarhizium anisopliae (Metschnikoff) Sorokin (Deuteromycotina: Hyphomycetes) following immersion in a 1 x 10^7 conidia ml^-1 suspension (Smith et al., 2000). Subsequently, the ability of mites to acquire a fatal infection following contact with a treated surface (Brooks and Wall, 2001) and the ability of infected mites to transfer infection to other in-contact individuals, were demonstrated (Brooks and Wall, 2005). However, preliminary attempts to use the fungal pathogen applied to infested sheep initially gave unpromising results (Brooks, 2004).

One possible problem was that the lack of initial success in vivo might have been due to naturally occurring fungicides present in the sheep fleece. A second possibility is that the temperature at the sheep skin surface is too high; at the skin temperature found on a fully fleeced sheep, around 35 °C, most strains of M. anisopliae are close to their upper lethal limits (Brooks et al., 2004; Polar et al., 2005). A third alternative was that the method of application failed to deliver fungal spores to the skin surface. The aims of the work reported here, were to assess the first two possible obstacles to in vivo application by examining the pathogenicity of the two fungal pathogens, M. anisopliae and B. bassiana (Balsamo) Vuillémin (Deuteromycotina: Hyphomycetes), when applied to sheep skin and undertaking quantitative trials of the pathogenicity of the fungus under carefully controlled experiments on live hosts.

2. Materials and methods

2.1. Sheep skin bioassay

The skin of a freshly killed lamb with no history of treatment for P. ovis was obtained from an abattoir. A series of 20 mm diameter circles were cut from the skin and the fleece was cut to a length of 20 mm. These discs of skin were placed into chambers constructed from 6 mm x 25 mm x 75 mm glass blocks. Each block had a 20 mm diameter hole drilled through its centre. The bottom of the block was sealed with a fine grade cotton cloth glued to the glass with epoxy resin (Araldite®, Bostik Findlay Ltd., Leicester, UK). Once a disc of skin and fleece, with its appropriate treatment, had been placed into the chamber created in these blocks, the chamber was sealed using a glass microscope slide with a 5 mm diameter hole drilled through its centre, which was also closed with cotton cloth. The hole in the upper glass slide was to allow for regulation of the humidity of the air within the chamber.

Four treatment groups were used: untreated fleece only, fleece to which conidia of M. anisopliae suspended in silicone oil had been added, fleece to which only silicone oil had been added and, as a positive control, cotton cloth to which conidia suspended in silicone oil had been added.

Treatments containing conidia used a suspension of 1 x 10^6 conidia ml^-1 of M. anisopliae (isolate IMI386697) in silicone oil (dimethylpolysiloxane hydrolyzate, Sigma-Aldrich, Dorset, UK). The isolate had been cultured on potato dextrose agar plus yeast (PDA). Conidia were collected from 10 to 14 day-old plates, by scraping a culture of sporulating M. anisopliae with a sterile loop, into 4 ml of silicone oil. The concentration of the conidial suspension was calculated using a haemocytometer and diluted further with silicone oil to produce the desired concentration.

The silicone oil or silicone oil with its suspended conidia was applied using a household plant sprayer (Hozelock Group Ltd., Aylesbury, UK) fixed 20 cm directly above the skin or cloth using a clamp stand. This distance between the nozzle of the sprayer and the discs of skin or cloth was chosen to give an even application of the treatment across the skin or cloth and to simulate a possible application method for use on sheep. The sprayer delivered a standard 0.08 ml of conidia suspended in silicone oil to each surface resulting in the application of 8 x 10^6 conidia per disc.
After application, the skin and cloth were placed into the chambers and left for 1 h before the addition of 20 adult, female *Psoroptes* mites, collected from the ears of experimentally infested rabbits on the same day that they were used in the experiment.

In the cloth treatment, 500 µl of ovine serum was also added (Sigma-Aldrich, Poole) before the addition of the mites. Five replicates of each treatment were set up. The mites were then left in their treatment chambers for 24 h at 30 °C and 90% relative humidity. After 24 h, all mites were removed from the chambers and transferred in their respective treatment groups to clean, cloth-based chambers to which 500 µl of sheep serum had been added. They were then incubated at 30 °C and 90% relative humidity. A further 200 µl of sheep serum was added every 48 h until all mites had died.

The chambers were inspected every 24 h for the presence of dead mites. Death was defined as the point when there was no movement from the mite when touched with a paintbrush. Different paint brushes were used for each of the treatments. The brushes were washed in 70% ethanol and wiped dry between each sample. Any dead mites were removed from the chambers and surface sterilised in 2% (w/v) sodium hypochlorite solution for 30 s and then washed in sterile water for 30 s to remove any conidia that had not penetrated the mite cuticle before the point of death. Each individual dead mite was then transferred to a damp piece of sterile filter paper (Whatman® No. 1, Whatman International Ltd., Maidstone, UK) at the base of a well in a 96-well microtitre plate (Bibby Sterilin, Stone, UK). The microtitre plates were then sealed with Parafilm® M (Pechiney Plastic Packaging, Chicago, USA) and incubated at 30 °C. The plates were inspected for mites with fungal infections every day for 2 weeks following the observation of the first infected corpse. Fungal infections were recorded when external hyphae could be observed protruding through the cuticle of a mite.

### 2.2. In vivo trial

Six pathogen-free poll Dorset sheep aged between 6 and 12 months were used in this trial. The sheep were maintained indoors as a single group, on straw in approved containment facilities, at the Central Science Laboratory (CSL), York. All animal procedures were conducted under UK Home Office licence and approved by appropriate ethics committees.

Each sheep had six circular rubber arenas glued to the skin on its back, three either side of the midline, giving a total of 36 arenas. Prior to attachment, the fleece was shorn close to the skin and the arenas were attached with surgical glue. The arenas had an internal diameter of 25 mm and were 10 mm high. They were sealed with fine mesh cotton held in place with a tight fitting rubber washer placed in the inside of the arena.

Treatments were allocated to specific sheep/arenas at random, with the constraint that neither sheep had more than one treatment of any particular type. The skin in six arenas acted as controls and had no treatment applied. The skin of six arenas was treated with 0.01 g of dry spores of *M. anisopliae* (isolate IMI386697) at a concentration of $1.8 \times 10^{10}$ conidia g$^{-1}$, three arenas were treated with 2 ml of the same isolate of *M. anisopliae* conidia suspended at $1.5 \times 10^9$ conidia ml$^{-1}$ in sheep dip excipient (70% octyl oil plus stabilisers, diluted to 15%, w/v, in water) and three arenas were treated with 0.01 g of the same isolate of *M. anisopliae* conidia mixed in 0.001 g of diatomaceous earth giving a concentration of $1.6 \times 10^{10}$ conidia g$^{-1}$. A further six arenas were treated with 0.01 g of a mixture of two different isolates of *M. anisopliae* (isolate ARSEF3297) and (isolate ARSEF4556) at a concentration of $8 \times 10^9$ conidia g$^{-1}$. A second fungal species, *B. bassiana*, was also examined in this trial. This isolate had been obtained previously from a naturally infected *Psoroptes* mite isolated from a sheep at the CSL and will be referred to as CSL1. For this, six arenas were treated with 0.01 g of dry spores of *B. bassiana* (1.2 $\times 10^{11}$ conidia g$^{-1}$), three arenas were treated with 2 ml of *B. bassiana* conidia suspended at $1 \times 10^{10}$ conidia ml$^{-1}$ in sheep dip excipient and three arenas were treated with 0.01 g of *B. bassiana* conidia mixed in 0.001 g of diatomaceous earth (1.1 $\times 10^{11}$conidia g$^{-1}$). The skin in each of the arenas was treated 1 h before mites were applied.

For the trial, 25–30 adult female *Psoroptes* mites were placed inside each arena. The mites had been removed from infested sheep 72 h prior to being placed on the experimental sheep. Prior to application to the experimental sheep, half of the groups of mites were maintained in clean chambers at 90% relative humidity and 30 °C and offered 500 µl of sheep serum.

Other groups of mites were exposed to formulations of either *M. anisopliae* or *B. bassiana*. Among these groups, the mites to be exposed to dry conidia on the host were first dipped in suspensions of $1 \times 10^{10}$ conidia ml$^{-1}$ of either *M. anisopliae* or *B. bassiana* in Tween 80 for 1 min. The mites were then transferred to a dry piece of filter paper for 1 min to allow any excess fluid to run off before being placed into a clean incubation chamber. The mites to be exposed on the sheep to conidia in sheep
dip excipient or conidia mixed in diatomaceous earth were placed onto filter paper in a 45 mm diameter Petri-dish which had been treated with the same volume and concentration of the treatment to which they were to be exposed on the sheep for 48 h.

Hence, on the experimental sheep, half the arenas containing dry spores, contained mites that had been pre-exposed, while half contained mites that had not. All the arenas treated with conidia in dip excipient and all the arenas treated with conidia in diatomaceous earth contained mites that had been pre-exposed. Table I summarises the treatment methods.

At the end of the 48 h period on host, the sheep were euthanised by a schedule 1 method and the skin with the chambers was excised. The skin and arenas were then packed into individual sealable plastic bags and refrigerated over night. The following day the mites were removed from the arenas, surface sterilised with 2% sodium hypochlorite solution, placed into clean incubation chambers with 500 µl of sheep serum and incubated at 30 °C and 90% relative humidity. A further 200 µl of sheep serum was added every 48 h until all mites had died. The chambers were inspected every 24 h for the presence of dead mites. Dead mites were treated as described previously and fungal infections were recorded when external hyphae could be observed protruding through the cuticle.

2.3. Analysis

The proportion of mites infected in each treatment was arcsin transformed prior to statistical analysis using analysis of variance (Statgraphics Plus, Statistical Graphics corporation, VA, USA). For clarity however, figures display non-transformed mean proportions ± their standard deviation.

Table 1
Summary of the fungal pathogens, formulations and concentrations applied to the arenas on the experimental sheep and the exposure method used to inoculate adult, female P. ovis mites

<table>
<thead>
<tr>
<th>Fungal pathogen</th>
<th>Formulation of fungal pathogen</th>
<th>Concentration</th>
<th>Exposure method</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. anisopliae</em> (IM1386697)</td>
<td>Dry conidia</td>
<td>1.8 × 10^10 conidia g⁻¹</td>
<td>48 h on host</td>
</tr>
<tr>
<td><em>M. anisopliae</em> (IM1386697)</td>
<td>Dry conidia</td>
<td>1.8 × 10^10 conidia g⁻¹</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td><em>M. anisopliae</em> (IM1386697)</td>
<td>Sheep dip excipient</td>
<td>1.47 × 10^6 conidia ml⁻¹</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td><em>M. anisopliae</em> (IM1386697)</td>
<td>Diatomaceous earth</td>
<td>1.6 × 10^10 conidia g⁻¹</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td><em>B. bassiana</em> (CSL1)</td>
<td>Dry conidia</td>
<td>1.2 × 10^11 conidia g⁻¹</td>
<td>48 h on host</td>
</tr>
<tr>
<td><em>B. bassiana</em> (CSL1)</td>
<td>Dry conidia</td>
<td>1.2 × 10^11 conidia g⁻¹</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td><em>B. bassiana</em> (CSL1)</td>
<td>Sheep dip excipient</td>
<td>1.1 × 10^11 conidia g⁻¹</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td><em>B. bassiana</em> (CSL1)</td>
<td>Diatomaceous earth</td>
<td>8 × 10^9 conidia g⁻¹</td>
<td>48 h on host</td>
</tr>
<tr>
<td><em>M. anisopliae</em> (ARSEF3297 + ARSEF4556)</td>
<td>Dry conidia</td>
<td>8 × 10^9 conidia g⁻¹</td>
<td>48 h in lab and 48 h on host</td>
</tr>
<tr>
<td><em>M. anisopliae</em> (ARSEF3297 + ARSEF4556)</td>
<td>Dry conidia</td>
<td>n/a</td>
<td>48 h on host</td>
</tr>
<tr>
<td>Control (no fungal pathogen)</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
</tr>
</tbody>
</table>

Fig. 1. Mean proportion of mites (±S.D.) showing signs of fungal infection after 24 h exposure to untreated fleece, fleece treated with silicone oil, fleece treated with silicone oil plus *M. anisopliae* conidia (1 × 10^8 conidia ml⁻¹) and cloth treated with silicone oil plus *M. anisopliae* conidia (1 × 10^8 conidia ml⁻¹).

3. Results
3.1. Sheep skin bioassay

These trials aimed to determine whether mites would be infected in vitro, when exposed to conidia on sheepskin. In the groups that received no treatment or silicone oil only, there were no infected mites (Fig. 1). In the group that was exposed to silicone oil and conidia on the skin, 52.2% (±13.8) of mites became infected.
and for the group exposed to silicone oil plus conidia on the cloth, 34.1% (±14.1) of mites became infected. The difference between the two groups exposed to conidia and the two groups not exposed to conidia (control groups) was highly statistically significant ($F_{3,19} = 8.4$, $P < 0.001$; Fig. 1). There was no significant difference in the proportion of infected mites in the two groups exposed to silicone oil and conidia whether on fleece or cloth (Tukey HSD post hoc test, $P > 0.05$).

3.2. In vivo bioassay

These trials aimed to determine whether (a) mites infected in vitro could sustain and develop infections if then placed onto a living host immediately after infection and (b) whether mites could become infected in vivo, after exposure to the skin of treated sheep. The results showed that there was no significant difference between the group of mites that were exposed to the fungi for 48 h in vitro followed by 48 h exposure in vivo and the group of mites that only had the 48 h exposure in vivo (Fig. 2). There was, however, a significant interaction, as those mites exposed to the mixed strains of $M$. anisopliae for 48 h in vitro prior to exposure for 48 h in vivo showed fewer infections than those mites exposed to the fungus for only 48 h in vivo ($F_{2,12} = 11.15$, $P = 0.002$; Fig. 2).

Overall, $B$. bassiana infected a significantly greater number of mites than $M$. anisopliae (IMI386697) when compared in different formulations ($F_{2,12} = 9.93$, $P = 0.003$; Fig. 3). But formulation also had a significant effect on the level of infection ($F_{1,12} = 24.42$, $P < 0.001$; Fig. 3). Overall, dry conidia were significantly more infectious than conidia formulated in sheep dip excipient or diatomaceous earth for both $M$. anisopliae and $B$. bassiana (Tukey HSD post hoc test, $P < 0.05$). However, this difference was due almost entirely to the $M$. anisopliae, because for $B$. bassiana the level of infectiousness remained high when in both sheep dip excipient and in diatomaceous earth, whereas for $M$. anisopliae the level of infection observed with these two formulations was significantly lower.

4. Discussion

Entomopathogenic fungi have been used successfully as treatments for a variety of arthropod pests. Several studies have investigated the use of $M$. anisopliae and $B$. bassiana against ectoparasites. A $1 \times 10^8$ conidia ml$^{-1}$ suspension of $M$. anisopliae induced mortalities of 30 and 37% in adult Rhipicephalus appendiculatus Neumann and Amblyomma variegatum Fabricius (Acari: Ixodidae), respectively.
and diatomaceous earth formulations reducing its mortality than B. bassiana with the sheep dip excipient naturally infesting zebu cattle (Kaaya et al., 1996). In vitro experiments conducted by Maranga et al. (2005) showed that 100% mortality could be achieved with both M. anisopliae and B. bassiana against A. variegatum. M. anisopliae has also been shown to induce high levels of infection in the cattle louse, Bovicola bovis (Linnaeus) (Trichodectidae: Ischnocera) both in vitro and in vivo (Briggs et al., 2006); 71% of lice exposed to a 1 x 10^8 conidia ml^-1 suspension of M. anisopliae in vitro became infected as did 73% of lice exposed to the same concentration in vivo (Briggs et al., 2006).

In contrast, previous attempts to apply fungal pathogens to P. ovis on living sheep have given disappointing results (Brooks, 2004). In this previous work, fungal suspensions were simply pored onto infested hosts but little curative effect was observed. Here, a much more controlled approach was adopted. First, the laboratory trials were used to confirm that there was nothing intrinsic in the pelt that inhibited infection. Subsequently, on the living host the experiment demonstrated that conidia of both M. anisopliae and B. bassiana could infect P. ovis on the skin. The purpose of pre-exposure of some groups of mites, prior to being placed on the sheep, was to allow possible points, where inhibition of infection might occur to be identified. Infection in pre-exposed mites would have already been initiated when they were placed on the sheep, so any subsequent lack of infection would have demonstrated inhibition of the infection after penetration of the mite cuticle by something inherent to the sheep skin environment. However, no such inhibition was found and similar levels of inhibition were observed in the pre-exposed and naive groups of mites. Because mites were surface sterilised when they were collected from the skin at the end of the trial, any infections must have been acquired before being retrieved from the sheep. Hence it can be concluded that conidia placed on the sheep, survived and remained infectious within the skin microclimate, attached, germinated and then successfully penetrated the mite cuticle, allowing the development of subsequent lethal infections.

Notably, M. anisopliae showed greater variability than B. bassiana with the sheep dip excipient and diatomaceous earth formulations reducing its pathogenicity significantly. Choosing a suitable formulation will clearly play a key role in maximising the ability of any fungal pathogen to infect mites in vitro. Diatomaceous earth has previously been shown to be able to increase the pathogenicity of M. anisopliae and B. bassiana to some pests of stored products. It is thought that diatomaceous earth may improve the adherence of the conidia to the cuticle and act as an abrasive and dessicating agent, easing the penetration of the pathogen (Lord, 2005; Michalaki et al., 2006, 2007; Vassilakos et al., 2006). However, this was not observed here. Some studies have shown that the insecticidal properties of diatomaceous earth decrease with increasing humidity (Cook and Armitage, 1998; Fields and Korunic, 2000; Vayas and Athanassiou, 2004; Kavallieratos et al., 2006). It could be possible that the high moisture levels at the skin surface of the sheep interfere with the action of the diatomaceous earth, inhibiting the process of infection.

Various forms of sheep dip excipient are widely available and familiar carriers for insecticides used in the farming industry; their ability to spread across the body makes them good potential candidates as carriers for entomopathogenic fungi. The results presented here show that some excipients may have adverse effects; M. anisopliae in the dip excipient used here performed poorly with low levels of infection. In contrast, B. bassiana seemed unaffected and even when in sheep dip excipient, was able to infect a high proportion of mites.

In terms of pathogenicity alone, this study has shown that the fungi can induce high levels of mycosis in Psoroptes mites in vivo, in excess of the level required to suppress a mite population (Wall et al., 1999). However, it has also been shown that M. anisopliae has sub-lethal effects against ticks by reducing fecundity, egg mass and hatchability (Kaaya et al., 1996). It is possible that fungal pathogens would also result in sub-lethal reductions in fecundity when on an ovine host, and therefore, increase the effectiveness of the fungal pathogen further. However, not only must the pathogenicity of the fungal formulations be taken into account, several other key requirements need to be considered, such as the period of residual activity, the practicalities of applying the treatment and the effect on the environment and human safety.

P. ovis can survive off the host for up to 18 days and if the treatment does not provide protection for this period the sheep will be highly susceptible to re-infestation and the flock will have to be moved and maintained in a clean environment after treatment. Previous work has suggested that M. anisopliae conidia can persist on the skin or in the fleece of the sheep for up to 4 weeks.
Brooks, A.J., Aquino de Muro, M., Buffee, E., Moore, D., Taylor, Brooks, A.J., Wall, R., 2005. Horizontal transmission of fungal pathogens to vertebrates and are considered unlikely to cause human or animal disease; hence their safety to vertebrates makes them a good candidate for pest arthropod control. However, precautions will still need to be taken when using the fungal pathogen and further investigations into its safety will have to be conducted before it can be used as a commercial product. In practical application the fungal pathogen would be exposed to several constraints, such as extremes of temperature and humidity, ultra violet radiation and rainfall. In the current work, the sheep were housed indoors, and therefore, were not exposed to solar radiation or rain. Future work would also need to address these potential problems.

Acknowledgements

We are grateful to the Department for Environment, Food and Rural Affairs of the UK government for funding this research and to Dr. Tariq Butt of the University of Wales for supplying us with isolates ARSEF 3297 and ARSP 4556.

References

against *Rhyzopertha dominica* and *Sitophilus oryzae* on stored wheat. Biol. Control 38, 270-281.
