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Biological Control 34 (2005) 58–65
Biological Control
www.elsevier.com/locate/ybcon

Horizontal transmission of fungal infection by *Metarhizium anisopliae* in parasitic *Psoroptes* mites (Acari: Psoroptidae)

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Received 23 November 2004; accepted 18 March 2005
Available online 26 April 2005

Abstract

Ectoparasitic mites of the genus *Psoroptes* cause mange in a wide range of domestic and wild vertebrate hosts. The mite infestation may be localised in the ear of the host, or be more generalised over the body. In the latter case, infestation may be debilitating and often fatal, particularly in domesticated cattle, sheep and goats. At present, control depends on the use of organophosphate or pyrethroid plunge dips or injectable endectocides. A series of in vivo bioassays are described here to further evaluate the use of an entomopathogenic fungus, *Metarhizium anisopliae* (Metschnikoff) as a biological control agent for *Psoroptes* mites. Mites were obtained from the ears of rabbits (*Psoroptes ovis* (Hering) syn. *Psoroptes cuniculi*). High levels of mortality (>94%) were observed in all life-cycle stages when exposed to a 1×10^8 conidia ml^{-1} suspension; there were no significant differences in infection between the different life-cycle stages. The treatment of eggs, however, had no effect on hatch rate. The horizontal transmission of the pathogen was considered by placing live uninfected mites in experimental chambers with an infected cadaver, but preventing direct contact using a mesh cage which would allow the passage of conidia but exclude direct physical contact. None of the uninfected mites showed signs of mycosis-induced mortality, indicating that direct contact with the cadaver was required for infection. Uninfected live mites were then brought into direct contact with an infected cadaver by a single touch of the mite to the cadaver (<1 s), or indirectly by allowing the live uninfected mite to walk over a piece of filter paper from which an infected cadaver had been removed. Both treatments resulted in fungal infection in approximately 40% of the live mites. The implications of these results for the transmission of *M. anisopliae* between infected *Psoroptes* mites, when used as a biological control agent, on an infected host is discussed.

Keywords: *Psoroptes*; Biological control; Fungus; Mites; *Metarhizium*; Sheep scab; Horizontal transmission

1. Introduction

Mites of the genus *Psoroptes* (Acari: Psoroptidae) are obligatory ectoparasites that infest a range of mammalian hosts. The genus is distinguished by the presence of relatively long, jointed pre-tarsi (Babcock and Black, 1933; Sanders et al., 2000; Sweatman, 1958). The mites are non-burrowing and feed superficially on a lipid emulsion of skin cells, bacteria and lymph on the host skin, produced as a result of a hypersensitivity reaction to the presence of antigenic mite faecal material (Blake et al., 1978; Sinclair and Kirkwood, 1983). Infestation may be chronic or even sub-clinical and localised, usually in the ear of the host. Or it may be acute and more generalised over the entire body, described as psoroptic mange (Bates, 1999). The taxonomy of the mites in this genus is confused, with mites located in different parts of the body or on different hosts traditionally given different species names; however, little good evidence exists to support this taxonomy (Bates, 1999; Evans, 2004; Zahler et al., 1998, 2000).

The most clinically and economically important infestations of *Psoroptes ovis* (Hering), occur on sheep. The first indication that a sheep may have an infestation is the rough appearance of the wool and abnormal animal

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doi:10.1016/j.biocontrol.2005.03.016

activity, including persistent biting and scratching (Babcock and Black, 1933). Small pustules form which expand and rupture, exuding lymph and serous fluids that rapidly congeal to form yellow crusts. The lesions cause severe irritation to the sheep making them restless and nibble and rub at the infested areas (Berriatua et al., 2001; Corke and Broom, 1999). This self-trauma results in wool loss, skin damage and weight loss and if left untreated can lead to epileptiform fitting when handled and ultimately death due to dehydration, pneumonia or bacterial septicaemia (Downing, 1936; Roberts and Meleney, 1971; Tarry, 1974).

Psoroptic mange is commonly treated using organophosphate or pyrethroid plunge dips or injectable macrocyclic-lactone endectocides. However, concerns over potential health and environmental effects of neurotoxic insecticides and logistic, cost and practical difficulties associated with endectocide use, have stimulated interest in the identification of alternative control methods.

The use of entomopathogenic fungi for the control of insect pests was considered around the turn of the 20th century, but with little commercial success. The most commonly investigated entomopathogenic fungi are species of *Metarhizium* and *Beauveria* (Deuteromycotina: Hyphomycetes), as they have a wide geographic spread and host range. These entomopathogenic fungi have been widely considered for biological control of agricultural pests (Gillespie and Moorhouse, 1989; Van der Geest et al., 2000), but only a small number of studies have considered their potential against parasites of animals, particularly ticks (Kaaya et al., 1996; Kirkland et al., 2004). In mites, *Metarhizium anisopliae* (Metschnikoff) Sorokin, has previously been shown to give mortalities of up to 71% in adult female *Psoroptes* mites in vitro (Smith et al., 2000) and fungal infections shown to be transmitted from infected cadavers to live uninfected mites (Brooks and Wall, 2001). This previous work suggested that the fungal pathogen, sporulating on infected cadavers after the death of mites on the ovine host, may serve as an ongoing source of the pathogen and hence sustain the control effect after the initial application of conidia to a sheep. Substantive strain differences in pathogenicity of *M. anisopliae* against *Psoroptes* mites at a range of temperatures has been demonstrated (Brooks et al., 2004). The aim of the present study therefore, was to consider the mechanism through which horizontal transmission of *M. anisopliae* may occur and to extend the previous studies to examine the effects of this pathogen against other life-cycle stages of *Psoroptes*.

2. Materials and methods

2.1. Mites

Mites were collected from scabs removed from the ears of two infected New Zealand white rabbits. These mites are described here as *P. ovis* (syn. *Psoroptes cuniculi*) since genetic studies do not support a distinction between *Psoroptes* isolated from sheep and rabbits (Evans, 2004; Zahler et al., 1998, 2000). After removal from the host, the scabs were placed into clean plastic jars and the mites allowed to wander up the sides of the jars from where they were removed a few minutes later using a fine paintbrush. Different life-cycle stages were identified (Sanders et al., 2000) and separated into sterile 1.5 ml eppendorf tubes until required; all mites were used in experimental trials within 6 h of collection.

2.2. Fungal strain: maintenance and growth

Metarhizium anisopliae, strain ARSEF 4556, which had been isolated originally from a *Boophilus* spp. tick, was obtained from the USDA-ARS collection. The isolate was cultured at 25 °C on Sabouraud dextrose agar plus yeast (SDAY) (Oxoid, Basingstoke, UK). Conidia were collected from 10- to 14-day-old plates by adding 10 ml sterile 0.05% Tween 80 (BDH Chemicals, Poole, UK) and the surface of the culture agitated, using a sterilised loop, to bring the spores into suspension. The conidial suspension was pipetted from the plate and the spore concentration calculated using an Improved Neubauer Haemocytometer (Weber Scientific International, Middlesex, UK). The conidial suspension was diluted using 0.03% Tween 80 to a concentration of 1×10^8 conidia ml^{-1}. The lower concentration of Tween 80 was used in the final dilutions to reduce any toxic effects of the detergent on the mites.

2.3. Pathogenicity to different life-cycle stages

Mites were maintained and monitored in experimental chambers constructed from $35 \times 75 \times 6$ mm glass blocks with a 20 mm diameter hole through the centre. Two pieces of 42.5 mm diameter Whatman No. 1 filter papers (Whatman International, Maidstone, UK) were placed centrally over the 20 mm diameter hole in the glass block and pressure used to create a shallow indentation. 500 µl of ovine serum was then pipetted onto the filter papers and either 500 µl of a 1×10^8 conidia ml^{-1} suspension or 500 µl of 0.03% Tween 80 was also added. Twenty mites were selected at random from the eppendorf tubes and placed in the centre of the indentation in the filter paper and a glass microscope slide was placed over the top and clamped at either end using foldback clips. The chambers were maintained at 30 °C and 95% r.h. and 400 µl serum was added each day to the filter paper to prevent the filter papers drying out. Experimental chambers were set-up containing adult females, adult males, or male nymphs. In addition adult male *Psoroptes* mites attach and guard female nymphs; it is assumed that this allows copulation at the point of adult moult. Twenty attached pairs of adult males with female protonymphs and twenty attached pairs of adult males with

with female tritonymphs were also placed into separate chambers. For each life-cycle stage, two chambers, one control and one with fungal-treated mites were set up at the same time, giving a total of 10 chambers; this proptocol was then repeated on eight separate occasions.

The chambers were inspected for dead mites every 24 h until all the mites were dead. Death was defined as the point when there was no movement from the mite when touched with the bristles of a paintbrush. Dead mites were removed from the experimental chambers and surface sterilised in 2%(w/v) sodium hypochlorite solution (Sigma–Aldrich, Dorset, UK) for 30–60 s and then washed in sterile water for 30–60 s. Sterile 6 mm discs of filter paper were placed in the wells of a 96-well microtitre plate. Drops of sterilised purity water were added to the pieces of filter paper prior to use. Each individual dead mite was transferred to the filter paper in an individual well after surface sterilisation. For the paired mites, usually both mites were found to be dead at the same inspection. In this case, pairs were left together and placed in the same well. Sometimes, however, the pairs of adult males with female nymphs separated and one died before the other; attached pairs with one dead and one live mite were never observed. Where separation had occurred, individual males and females were placed in separate wells. The microtitre plates were then sealed with Parafilm M (Pechiney Plastic Packaging, Chicago, USA) and incubated at 30 °C. Experimental chambers and microtitre plates containing the cadavers were incubated in the dark.

The median survival time was calculated for each chamber. For mites placed in the chambers as attached pairs, the number that died on each day was calculated separately for the male and female life-cycle stages.

The microtitre plates were inspected for mites with fungal infections identified by external hyphae observed protruding through the cuticle of a mite. The pieces of filter paper were kept damp by adding drops of sterile water when needed. If no infections were observed in the three weeks following death, mites were considered to have been uninfected.

To study the effect of *M. anisopliae* on the eggs of *P. ovis*, two chambers containing 20 eggs placed onto black filter paper were used. One was a treatment chamber, to which 300 µl of fungal suspension was added and one was a control, to which 0.03% Tween 80 was added. The chambers were maintained in the dark at 95% r.h. and 30°C and 50 µl sheep serum was added to each chamber every day. The number of eggs that hatched was recorded. This experiment was repeated four times.

2.4. Horizontal transmission between infected cadavers

To consider the degree of contact with an infected cadaver required to induce fungal mycosis, live mites were exposed to infected cadavers by various methods. To produce infected cadavers of the required age adult female mites were immersed in a 1×10^8 conidia ml^{-1} suspension for 10 min and then transferred to serum-soaked incubation chambers. Dead mites were removed from the chambers, surface sterilised and placed on damp sterile filter paper at the base of the wells in a 96-well microtitre plate. These dead mites were then checked every 24 h for fungal infections detected by the presence of external hyphae. The day on which the infection first appeared was noted. Cadavers were used 5 days after the initial observation of hyphae protruding from the cuticle of the mite. At this time the fungus is sporulating on the cadaver and has been shown to be more infective than older cadavers (Brooks and Wall, 2001).

Experimental chambers were set-up as described previously, with the exception that rather than filter paper bases, a square of fine cotton was glued securely covering the underside of the hole in each glass block. A single infected cadaver with a 5-day-old infection was placed in the centre of the cotton cloth. The infected cadaver was then covered by a mesh cage which was glued in place (Fig. 1). The cage was constructed from stainless steel mesh (Advent Research Materials, Oxon, UK) with plain weave wire diameter 0.050 mm and aperture size 0.085 mm (open area ~36%). The cages were approximately 7 mm in diameter and 3 mm high with a 2 mm wide rim (Fig. 1). The glue was allowed to set for 1–2 h and then 500 µl serum was pipetted gently onto the cotton base. Twenty live adult female mites, selected at random, were then placed into the chamber and the upper entrance of each chamber was again sealed by a glass microscope slide clamped in place with foldback clips. The chambers were incubated in the dark at 30 °C and 95% r.h. Five chambers were set-up with infected cadavers and, as a control, five chambers were set up with a dead, non-infected mite sealed in the central cage. For this experiment, all ten chambers were set-up at the same time. The chambers were checked every 24 h for dead mites, which were removed and surface sterilised in 2% sodium hypochlorite, as described previously. The dead mites were placed on damp filter paper in individual wells of a 96-well microtitre plate and monitored for fungal infections.

To assess the effects of direct contact between infected cadavers and uninfected mites, an infected cadaver, 5 days after the initial observation of infection, was placed on the end of a mounted needle and touched very briefly (<1 s) on the dorsal surface to five live uninfected mites in turn (Fig. 2A). Each individual live mite was then placed in a polystyrene cuvette ($45 \times 10 \times 10$ mm) (Sigma–Aldrich, Poole, UK) with a 10×10 mm piece of fine cotton cloth and 100 µl sheep serum. This was repeated 16 times. For controls, dead uninfected mites were used in place of infected cadavers, and four repeats were used. The cuvettes were incubated at 30 °C and the mites were checked every 24 h. After death the mites were surface sterilised as described previously, placed on damp filter paper in the wells of a 96-well microtitre plate, and monitored for infections.

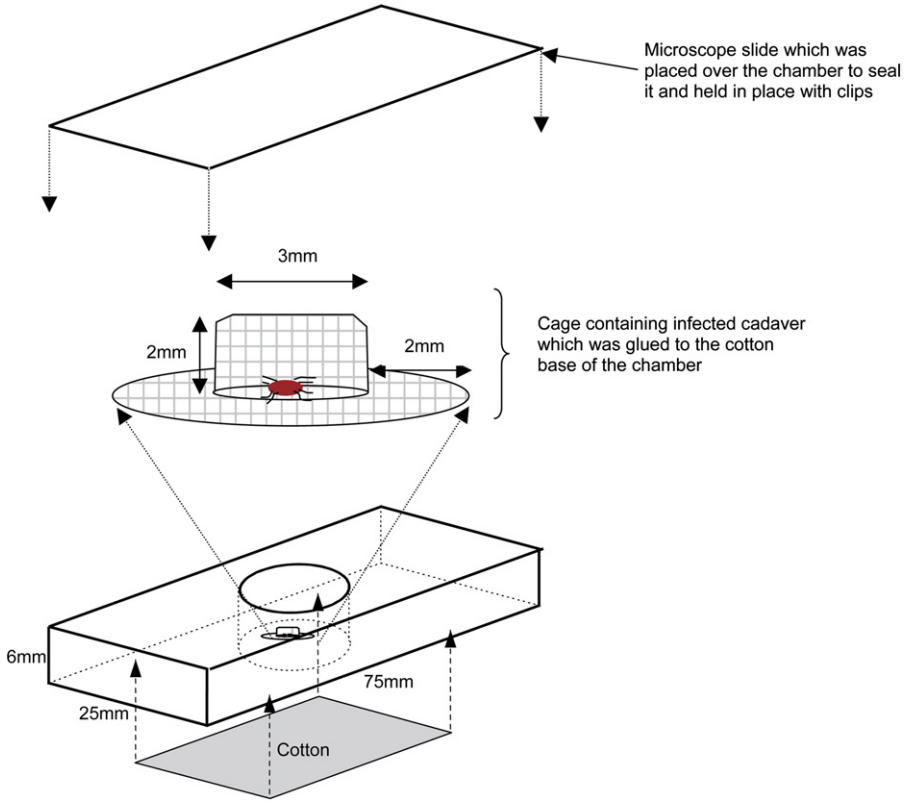

Fig. 1. The experimental chamber used to expose live uninfected *P. ovis* derived from rabbit hosts (syn. *P. cuniculi*) to a cadaver infected with *M. anisopliae* separated by a mesh cage to prevent the live mites coming into direct contact with the infected cadaver. Twenty live mites were placed in the chamber, which was sealed and incubated at 30°C and 95% r.h.

To assess the effects of indirect contact between infected cadavers and uninfected mites, an infected cadaver 5 days after the initial observation of infection, was removed from the filter paper on which it had been placed after death. Live uninfected mites, selected at random from the mites collected that day, were placed adjacent to the spot from which the infected mite had been removed and were allowed to walk once across the piece of filter paper, carefully guiding the mite to ensure that it walked over the place from which the cadaver had been removed (Fig. 2B). This was repeated for five mites for each filter paper. The experiment was repeated 16 times. For controls dead uninfected mites were used in place of the infected cadavers and repeated four times. After walking over the filter paper, the live mites were placed immediately in cuvettes with a 10×10 mm piece of cotton cloth and 100 µl sheep serum and incubated at 30 °C in the dark and inspected daily. After death the mites were surface sterilised as described previously, placed in individual wells of a microtitre plate and monitored for infections.

2.5. Analysis

The normality of the data was determined using the 1 sample $K-S$ test and homogeneity of variance was checked using Levene's test. The life-cycle stage mortality data followed a binomial distribution and so were analysed using a generalised linear model modelling binomial proportions, with the life-cycle stage and treatment as factors (Genstat 5 Committee, Release 3, Oxford, UK). Results for differences in the number of eggs that hatched into larvae after exposure to the fungal pathogen were analysed using an independent t test assuming equal variances (SPSS 11.0, SPSS Chicago, USA). Time taken for mites to die was analysed using univariate general linear models and Tukey multiple range tests, (Minitab Statistical Software v13, Minitab, PA., USA). The ranked median survival time was treated as the dependent variable and the life-cycle stage and treatment type, fungal suspension or aqueous Tween-80, as factors. The data relating to transmission were analysed using χ^2 tests with the order of touch and the treatment type as the different categories.

3. Results

3.1. Pathogenicity to different life-cycle stages

Exposure to *M. anisopliae* resulted in the acquisition of fatal infections for all life-cycle stages. Within 5 days

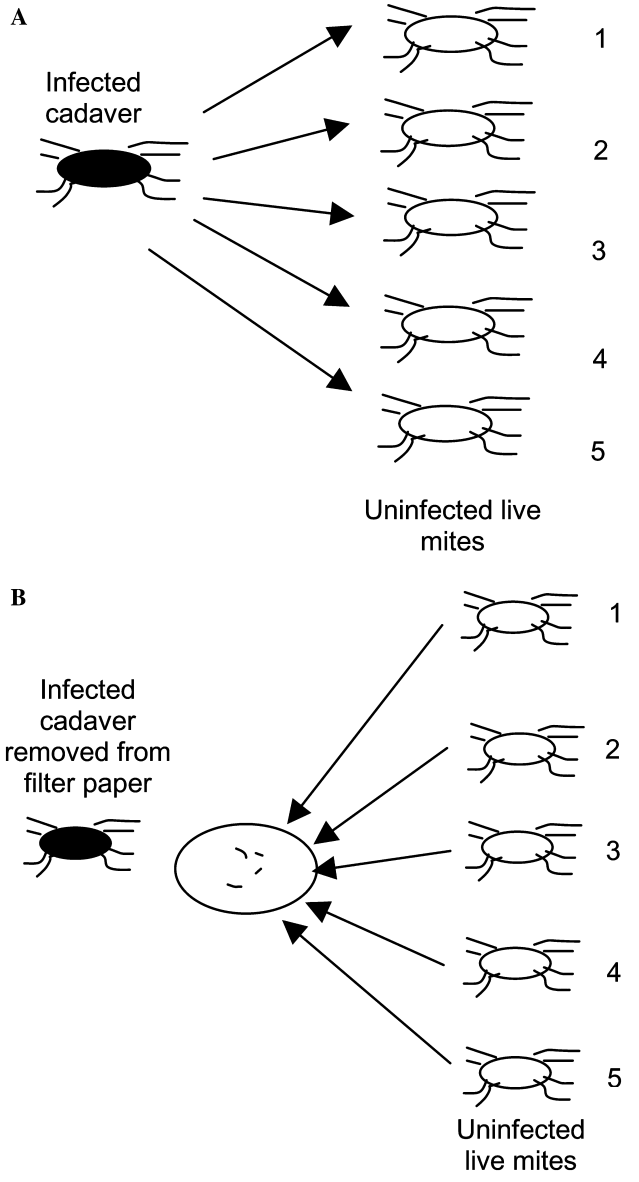

Fig. 2. Schematic representation of the method of direct contact of live uninfected mites with an infected cadaver (A) or indirect contact of live uninfected mites with the residue from an infected cadaver left behind a piece of filter paper (B).

of death, colonisation of the cadaver was extensive, hyphae could be observed protruding through the cuticle and in some cased sporulation had begun. Less than 1% of the controls became infected compared to 94% of the treated mites ($\chi^2 = 128.1$, $df = 1$, $P < 0.001$, Fig. 3). There was no significant effect of life-cycle stage on the proportion of mites acquiring fatal infections ($\chi^2 = 0.73$, $df = 6$, $P > 0.1$), and there was no significant interaction between life-cycle stage and treatment type ($\chi^2 = 0.33$, $df = 6$, $P > 0.1$). However, there was no significant effect of the fungal pathogen on the number of eggs that hatched into larvae ($t = 0.68$, $df = 6$, $P > 0.1$). When exposed to the fungal pathogen the median survival times were between 1 and 2 days, which was significantly shorter than those

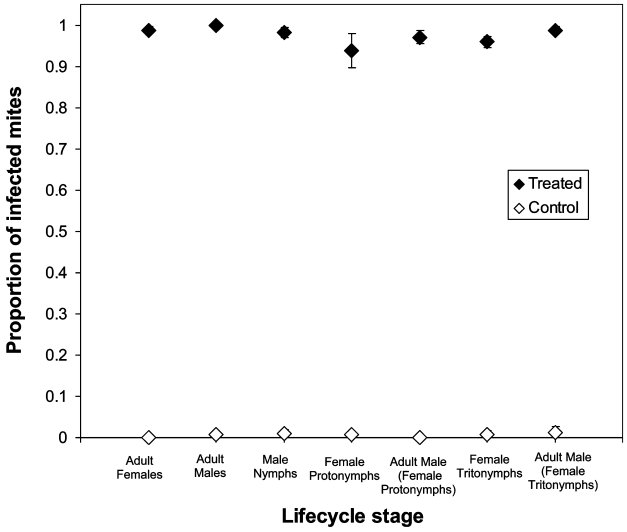

Fig. 3. The proportion (±SE.) of *P. ovis* ($N = 8$) derived from rabbit hosts (syn. *P. cuniculi*) of different life-cycle stages with fungal infections after exposure to *M. anisopliae* suspended in 0.03% Tween 80 (solid diamond). For controls mites were exposed to 0.03% Tween 80 (open diamond). The chambers were incubated at 30 °C and 95% r.h.

of controls ($F_{1,91} = 214.1$, $P < 0.001$, Fig. 4), with the exception of the adult males attached to female protonymphs in copulatory pairs. There was a significant difference in median survival time between the different life-cycle stages for the controls ($F_{6,91} = 3.39$, $P < 0.05$) and multiple range tests showed the survival of adult males was significantly lower than that of female tritonymphs.

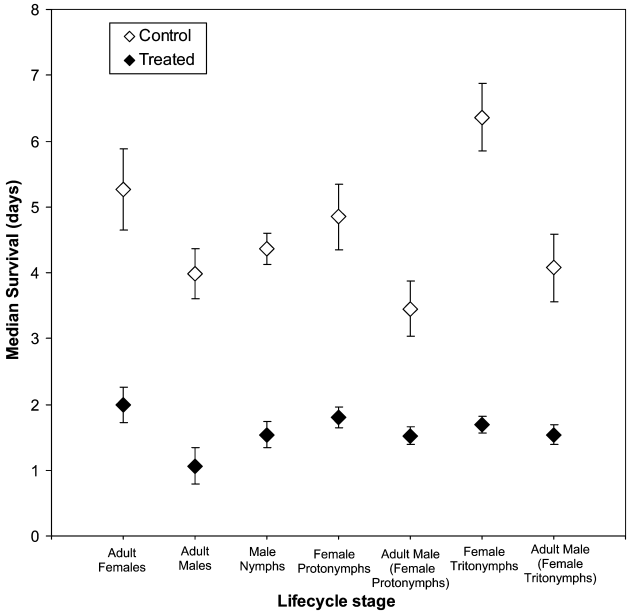

Fig. 4. The median survival time (±SE) in days of different life-cycle stages of *P. ovis* derived from rabbit hosts (syn. *P. cuniculi*) after exposure to *M. anisopliae* suspended in 0.03% Tween 80 (solid diamond). For the controls mites were exposed to 0.03% Tween 80 only (open diamond). The chambers were incubated at 30 °C and 95% r.h.

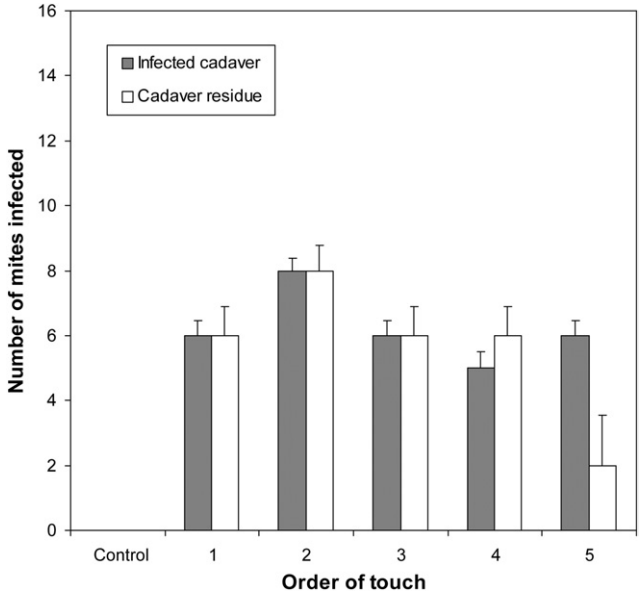

Fig. 5. The number (±pooled SE of the group) of adult female *P. ovis* ($N = 16$), derived from rabbit hosts (syn. *P. cuniculi*), with fungal infections after being touched with an infected cadaver, with the infected cadaver touching five live uninfected mites in turn, or after walking over the filter paper after the infected cadaver had been removed, with five live mites used for each piece of filter paper. Infected cadavers were used 5 days after the initial observation of infection with the pathogenic fungus *M. anisopliae*. For controls ($N = 4$) uninfected cadavers were used.

3.2. Horizontal transmission between infected cadavers

None of the control mites showed signs of fungal infection. None of the live mites acquired fatal infections when the infected cadavers were protected by a mesh cage. Fungal infections were observed in the mites that were touched against infected cadavers or the filter-paper surface on which the cadaver had been placed after death (Fig. 5). However, there was no significant effect of the order of touch, despite an apparent ultimate decline in the number of mites infected when allowed to contact the contaminated filter paper for the fifth time. An average of between 6.2 and 5.6 mites became infected in the two treatment groups, equivalent to 38.7 and 35% of the mites, respectively. There was no significant difference in the mortality-induced by the two contact treatments ($\chi^2 = 0.15$, $df = 1$ $P > 0.1$).

4. Discussion

Previous investigations of the effect of *M. anisopliae* and *Beauveria bassiana* against the ticks *Rhipicephalus appendiculatus* (Neumann) and *Amblyomma variegatum* Fabricius by immersion for a few seconds, showed 78–80% mortality in adults, 80–95% mortality in nymphs and 100% mortality in larvae (Kaaya and Hassan, 2000). Exposure was also shown to reduce fecundity, egg mass and hatch rate (Kaaya et al., 1996). Gindin et al. (2002) found that unfed immature stages of three species of ticks, *Boophilus annulatus* (Say), *Hyalomma excavatum* (Koch), and *R. appendiculatus*, were more susceptible to *M. anisopliae* than the engorged stages. A similar higher susceptibility of immature compared to adult *A. americanum* and *A. maculatum* was reported by Kirkland et al. (2004). In the present work there was no significant difference between different life-cycle stages treated with a 1×10^8 conidia ml^{-1} suspension, with all stages exhibiting over 94% fungal-induced mortality. This difference observed between the different stages of ticks could be due to changes in the degree of sclerotization of the exoskeleton in the later stages providing a protective barrier to penetration of the fungus. In contrast, all stages of *Psoroptes* mites remain relatively unsclerotised. There were differences in the survival times of the different life-cycle stages of the untreated mites with female nymphs living longest, followed by adult females, and adult males having the shortest survival time. These survival times are similar to those reported previously by Smith et al. (1999); determination the cause, for example of the lower male survival relative to other life-cycle stages, is beyond the scope of this study.

No effect on the hatch rate of *Psoroptes* eggs was observed in the present study. However, *B. bassiana* (Balsamo) Vuillemin, *M. anisopliae* and *Paecilomyces fumosoroseus* (Wize) Brown and Smith were shown to cause significant mortalities in the eggs of the carmine spider mite, *Tetranychus cinnabarinus* (Boisduval) (Shi and Feng, 2004). Exposure to both *M. anisopliae* and *B. bassiana* reduced egg laying prior to death and caused 70–98% mortality of treated eggs of *B. annulatus* and the fungal pathogens were also shown to reduce the weight of the eggs laid and the egg laying capacity of adult female ticks (Gindin et al., 2002).

Exposure to *M. anisopliae* has also been shown to reduce the survival time of *Psylliodes chrysocephala* Linnaeus, the cabbage stem flea beetle, from 10 to 4 days (Butt et al., 1994). Smith et al. (2000) was able to reduce the mean survival time of *Psoroptes* mites from 3.6 to 2.7 days when treated with a fungal pathogen. In the present study, exposure of mites to conidia reduced off-host survival significantly to between 1 and 2 days for all life-cycle stages, compared to 4–6 days for the control mites.

The horizontal transmission of *B. bassiana* and *M. anisopliae* between tsetse flies, *Glossina morsitans morsitans* Westwood has been demonstrated (Kaaya and Okech, 1990). Equal numbers of treated and untreated flies were mixed in cages 1 day after treatment for 32 days. Fungal-induced mortality in the untreated flies was between 62 and 75% and 48 and 55% for *B. bassiana* and *M. anisopliae*, respectively. Similarly, it has been suggested that once applied to a sheep, the effect of the fungal pathogen could accumulate over time, rather than

decay as with chemical insecticides, by proliferating in the cadavers of dead infected mites, which could act as a source of conidia to produce secondary infections (Smith et al., 2000). It has been shown that uninfected *Psoroptes* mites may acquire infections from infected cadavers in the same experimental chamber, with only one infected mite was required to infect 15–36% of other mites (Brooks and Wall, 2001).

The data presented here show that contact is required either with an infected cadaver or any residue left behind on the substrate for this infection to occur. Where infected cadavers were separated from live mites using a mesh cage none of the live mites became infected. The conidia of *M. anisopliae* are approximately 9 μm in length (Humber, 1997) and therefore, the holes in the mesh were large enough for conidia to pass through. However, the conidia of *M. anisopliae* are not actively dispersed, unlike some species of fungi such as the Entomophthorales (Papierok and Hajek, 1997), and clearly the conidia did not pass through the mesh. This could be due to the lack of air movement within the experimental chambers, but when the infected cadavers are on an ovine host the movement of the sheep may help disperse conidia throughout the fleece. However, cadavers produced enough spores to infect other live mites and even leave behind a residue of conidia that could infect passing mites. In this way uninfected mites could still be exposed to conidia even if an infected cadaver has fallen off the sheep as it may leave behind a residue of conidia on the skin or in the fleece.

Simulation modelling has suggested that mortality of adult female *Psoroptes* mites of over 50% every 2 days would be required to prevent population growth (Wall et al., 1999). The data presented here shows that this level of mortality is achievable in vitro, with greater than 95% mortality of all life-cycle stages within 2 days. However, it is notable that the pathogen used here did not result in the infection of every mite exposed. This inevitably raises questions, firstly about why some mites do not acquire infections and secondly about its potential for use as a control agent against a livestock parasite, since even a small residual mite population remaining on a sheep after treatment could rapidly reinfect the host and transmit infection to other naive sheep. If this result indicates that there is genetic variation in susceptibility to infection, the potential for the rapid selection for resistance is highlighted.

Acknowledgments

We are grateful to Prof. Mike Taylor at the Central Science Laboratory and and Drs Dave Moore and Marilena Aquino de Muro at CABI. Dr Karen Spencer provided invaluable statistical advice. We thank Dr Richard Humber at USDA-ARS for the supply of the fungal isolate. We gratefully acknowledge the financial support of the Department for the Environment Food and Rural Affairs of the UK Government (Grant code CSA 3503).

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