



Seasonal patterns of persistence and infectivity of *Metarhizium anisopliae* var. *acridum* in grasshopper cadavers in the Sahel

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Abstract

Field-based experiments were conducted to evaluate the fate and infectivity of the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum* (Deuteromycotina: Hyphomycetes) in grasshopper cadavers in the Sahel. Unlike uninfected cadavers, which were rapidly scavenged, those infected with the fungus persisted in the environment for a number of weeks. The environmental factor most associated with cadaver disappearance was rainfall. The high environmental humidity associated with rainfall was also required for sporulation of the fungus on host cadavers, although the likelihood of sporulation differed between microsites. Characteristics of the infection profile from infective cadavers were investigated by the sequential exposure of uninfected hosts to sporulating cadavers in field cages. This experiment revealed that cadavers remained infective for > 30 days, with the net infectivity changing through time. The most likely explanation for these changes is climatic influences on both the fungus and host. High humidity was not required for infection. A measurement of the transmission coefficient between healthy hosts and sporulating cadavers in the field was obtained at a realistic density of infectious cadavers. This revealed a figure of 0.45 m² day⁻¹. Overall, these experiments show that following host death, *M. anisopliae* var. *acridum* can be persistent in the environment, sporulate on host cadavers and reinfect new hosts at a realistically low field density, although at least in arid or semi-arid areas, rainfall may be critical to the horizontal transmission of this pathogen.

Introduction

A mycoinsecticide based on the fungal entomopathogen *Metarhizium anisopliae* var. *acridum* (= *flavoviride*) (Deuteromycotina: Hyphomycetes) is being developed for locust and grasshopper control in Africa (Bateman, 1997). An important consideration in the use of entomopathogenic fungi formulated in microbial sprays is their ability to persist in the environment via horizontal transmission between infective and healthy hosts (Anderson, 1982; Harper, 1987; Roberts & Hajek, 1992). The effect of secondary cycling of *M. anisopliae* var. *acridum* on the seasonal population dynamics of locusts and grasshoppers has

been explored using theoretical population models (Thomas et al., 1995, 1999). These predict that cycles of the disease following death of hosts infected by the initial spray application can contribute significantly to the overall impact of the mycopesticide, primarily by reducing the frequency of treatments required to maintain host density below a given threshold.

In general, fungal entomopathogens must kill their host and then develop and liberate spores or conidia into the environment before they can potentially cause new infections. Two crucial factors in the transmission of fungal pathogens are thus the production of conidia from host cadavers and the ability of conidia to initiate new infections, i.e., germinate on new hosts (see Butt,

1988; Clarkson & Charnley, 1996; Hajek & St. Leger, 1994 and Charnley, 1992, for particular reference to locusts).

Understanding the influence of environmental conditions on transmission of *M. anisopliae* var. *acridum* and, thus, predicting where and when cycling of the fungus is likely to occur, requires knowledge of the persistence and infectivity of the pathogen within the biotic/abiotic environment. Moisture is probably the most widely recognised environmental factor that determines transmission of fungal entomopathogens (Carruthers & Soper, 1987; Hall & Papierok, 1982) although other abiotic factors (e.g., temperature and ultraviolet radiation) are known to be important for pathogen survival (Roberts & Campbell, 1977). Factors that affect transmission of pathogens from infected to healthy insects therefore regulate a critical step in the dynamics of insect diseases. Despite this, few studies have considered how environmental factors influence entomopathogenic fungi following death of the host, especially in tropical or subtropical regions.

This paper reports on field-based experiments investigating the seasonal persistence and infectivity of grasshopper cadavers infected with *M. anisopliae* var. *acridum* in the Sahel. This work was conducted concurrently with a large-scale field trial where a mycoinsecticide based on *M. anisopliae* var. *acridum* was applied against the seasonal Sahelian grasshopper pests (see Langewald et al., 1999 and Materials and methods below).

Materials and methods

Field site. Field studies were conducted in the Maïne Soroa district in south-east Niger Republic, West Africa during the rainy season in August–September 1997. The area comprised lightly wooded open Savannah with mixed forbes and grasses (*Cenchrus biflorus* was the dominant species) together with members of the following gramineous genera: *Aristida*, *Eragrostis*, *Chloris*, *Dactyloctenium*, and *Pergularia* spp. Vegetation was principally annual, ranging from 2–20 cm in height, with 20–60% ground coverage.

Climatological data were recorded throughout the study period using a 1000 series Squirrel data logger (Grant Instruments Ltd, UK) and representative results from both clear dry and more rainy and overcast conditions are shown in Figure 1. During the study temperatures ranged from $\approx 20^\circ\text{C}$ (night-time minimum)

to ≈ 40 , ≈ 50 , and $\approx 65^\circ\text{C}$ (daytime shade, sun air, and sun ground maximum, respectively). Humidity levels during dry weather ranged from $<20\%$ during the day to $\approx 90\%$ during the night. This increased to $>96\%$ during wet weather, although humidity remained slightly (2–5%) higher in shaded locations near the ground.

Source of infected cadavers. In early August 1997, an aerial spray treatment of an oil miscible flowable formulation of *M. anisopliae* var. *acridum* conidia was applied to an 800 ha area of Savannah. The volume application rate was 0.5 l ha^{-1} and the concentration adjusted to deliver 2.5×10^{12} spores ha^{-1} . The overall density of grasshoppers (pre-spraying) was $\approx 10 \text{ m}^{-2}$, although the treatment was effective at reducing grasshopper densities by about 90% after 16 days. Full details of this field trial are given in Langewald et al. (1999). Infected grasshopper cadavers used in the studies described below were obtained from grasshoppers that were collected from inside the treated area at various periods in the weeks following spraying and incubated in laboratory cages under ambient shade conditions. Under these conditions, most ($>80\%$) grasshoppers died in 5–8 days with a red mycosis, distinctive of *Metarhizium* infection. These ‘mycosed cadavers’ were stored under cool dry conditions ($<20^\circ\text{C}$ and $<50\%$ r.h.) to maintain fungus viability but prevent sporulation. Where specified, ‘sporulating cadavers’ were obtained by incubating mycosed cadavers in petri-dishes at 100% r.h. for $\approx 72 \text{ h}$ to encourage fungal sporulation (under these conditions both internal and external sporulation occurred). Grasshoppers used in these studies were *Acrotylus blondeli* Saussure, *Pyrgomorpha cognata* Krauss, and *Oedaleus senegalensis* Krauss, the most abundant species throughout the study period. All studies were conducted in an untreated area $>2 \text{ km}$ from the area treated with the mycoinsecticide.

Persistence of infected and non-infected grasshopper cadavers. A study plot was established to investigate the environmental persistence of grasshoppers killed by *M. anisopliae* var. *acridum*. Cadavers of *A. blondeli* and *P. cognata* in both pre-sporulated ‘mycosed’ and sporulating states were used. Non-infected cadavers, obtained from grasshoppers of the same species collected from untreated areas and killed with a pyrethroid-based aerosol, were included for comparison. Mycosed and non-infected cadavers were used within 36 h of death. Four cadavers of the three

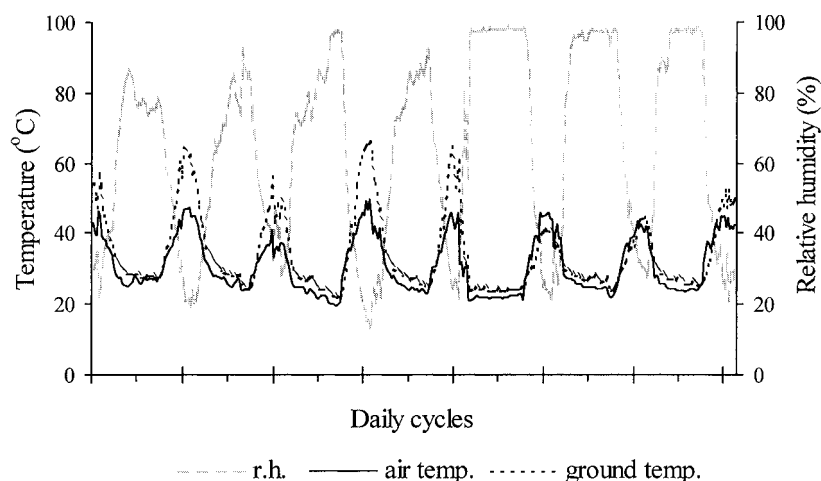


Figure 1. Example of climatic data recorded from the Sahel showing daily fluctuations of temperature (air and ground) and r.h. during a period of dry clear conditions (first four days) and more rainy/overcast conditions (latter three days).

types (i.e., mycosed, sporulated, and uninfected) were placed singly at 5 m intervals in a randomly chosen sequence along a line transect, and their positions and co-ordinates marked to enable relocation. This was repeated at 11 parallel equidistant locations, forming a 12×12 grid. Each cadaver was revisited at 24 h intervals over 30 days and categorised as either 3 (intact excluding appendages); 2 (damage restricted to one body section); 1 (extensive damage over > one body section/hollowed out); 0 (complete visual removal). The scoring system was adopted to reflect the rate of decay of cadavers. The cumulative rate of cadaver disappearance was assessed using Kaplan–Meier survival analysis (SPSS for Windows 6.1). Differences in the mean persistence between types of cadaver were tested using a log rank test with significance levels of multiple comparisons adjusted using a Bonferroni correction.

Sporulation of infected cadavers. Mycosed non-sporulating *A. blondeli* cadavers were laid out in different microsites in the field within 48 h of death (12–68 per microsite). Cadavers were positioned with different levels of exposure to the prevailing environment, comprising \pm vegetation (within a dense patch of vegetation or exposed) and \pm ground contact (on soil surface or supported 10–15 cm above the ground by tying cadavers to small sticks). At various stages, both prior to rainfall and following variable exposure to rainfall, cadavers were broken apart and inspected for signs of sporulation. The significance of microsite and rainfall for the numbers of cadavers in which

sporulation was visible in the field was analysed using contingency tables utilising Pearson χ^2 tests for independence (Phi and Cramer's V) with a Bonferroni correction used in multiple comparisons.

Measurement of infection profile from cadavers in field cages. The potential for sporulating cadavers to infect healthy grasshoppers in the field was determined by sequential exposure of uninfected hosts to an ageing population of sporulating cadavers. The experimental procedure followed that of Thomas et al. (1995) and used foldable open bottomed field cages (68×68 cm) with polyester mesh sides, supported by corner posts and dug into the ground around an area of typical patchy vegetation. At the beginning of the study, six sporulating cadavers of *A. blondeli* were placed into each cage in a regular pattern (20 cm apart from one another). Fifty healthy grasshoppers (mainly adults and final nymphal instars of *A. blondeli* and *P. cognata* sweep-netted nearby) were then introduced into each cage through a top flap. After 48 h, live insects were removed and infection rates determined by incubating grasshoppers in wooden framed ($30 \times 30 \times 30$ cm) nylon mesh cages under ambient shade conditions in a laboratory. Grasshoppers dying within 21 days were allowed to sporulate under moist conditions to confirm infection by *M. anisopliae* var. *acridum*. To reduce the risk of contamination all field and laboratory cages were washed in bleach solution prior to and after use. Six replicate cages were used and the process of introducing healthy grasshoppers into cages and determining resulting infection

levels was repeated after 2, 4, 8, 12, 16, 23, and 32 days. On each occasion, a number of field-collected grasshoppers were taken back to the laboratory to check background infection levels.

Estimating the transmission coefficient in the field. While the experiment described above provides information about temporal changes in the infectivity of cadavers under field conditions, the experimental cages may influence host behaviour and possibly cadaver infectivity and hence absolute rates of disease transmission. A preliminary assessment of the horizontal transmission parameter in the open field, at a given pathogen density, was therefore done.

An open area of Savannah was selected late in the season during hot dry weather when the resident grasshopper populations were low (the density was estimated to be $0.94 \pm 0.2 \text{ m}^{-2}$). A sample of ≈ 40 grasshoppers was collected to estimate background infectivity. A square plot ($38 \times 38 \text{ m}$) was delimited with string and 360 sporulating cadavers were placed singly at regular 2 m intervals within this plot giving a fixed density of $0.25 \text{ cadavers m}^{-2}$. This corresponds to the maximum density of grasshopper cadavers that was observed in the mycopesticide treated plot following spraying (Arthurs, 2000). The plot was revisited 48 and 72 h later and a sample of ≈ 100 grasshoppers was collected from the central area and subsequently incubated in a number of 2.5 l sterilised plastic bottles (15 grasshoppers per bottle) for 21 days under ambient shade conditions to determine infection levels. Grasshoppers were provided with fresh grass daily during incubation.

Results

Persistence of infected and non-infected grasshopper cadavers. The cumulative pattern of decay of infected and uninfected cadavers, expressed as a percentage of the accumulated total representing no damage/removal, is shown in Figure 2. The average time to disappearance of grasshopper cadavers (restricted to 30 days) was significantly affected by infective state ($F_{1,87} = 74.9$, $P < 0.0001$). Field observations suggested that the rapid removal of uninfected cadavers was mainly due to scavenging by the numerous ground dwelling arthropods, largely ground-dwelling ants, notably *Monomorium* spp. (Formicidae), and beetles, notably *Zophosis* spp. and *Erodius* spp. (Tenebrionidae). By contrast, such scavengers were commonly

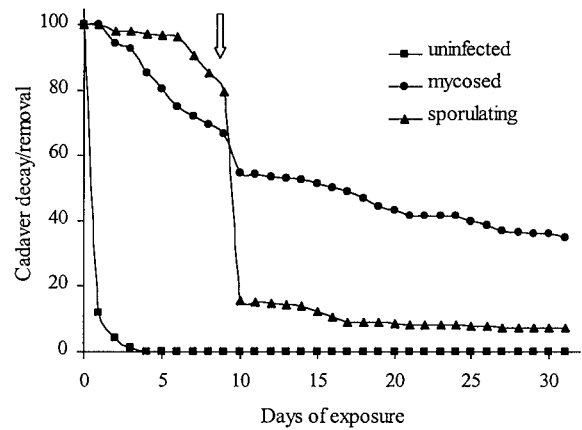


Figure 2. Cumulative measure of decay of cadavers of grasshoppers killed by *M. anisopliae* var. *acridum*, together with uninfected controls, in the Sahel. Data are expressed as a percentage of an accumulated total representing no damage/removal (see methods). Arrows indicate major rain event ($> 30 \text{ mm}$).

Table 1. Mean time to disappearance \pm SE (days) of grasshopper cadavers infected and uninfected with *M. anisopliae* var. *acridum* in the Sahel

Uninfected	Infected	
	Mycosed	Sporulating
$2.1 \pm 0.3 \text{ a}^{\text{a}}$ (0) ^b	$22.3 \pm 1.5 \text{ b}$ (38.9)	$14.3 \pm 1.6 \text{ c}$ (13.9)

^aLetters represent differences between classes of cadaver at the 1% level (log rank test).

^bPercentage of cadavers censored (not disappeared) after 30 days.

observed to avoid infected cadavers and in this case, the persistence of cadavers appeared to be regulated by climatic factors, particularly heavy rainfall (Figure 2). Thus uninfected cadavers disappeared quickly while infected cadavers persisted for several weeks at both sites with fragments still visible at the end of the observation period. Moreover, differences were observed between each category of infective cadaver. Those that had already sporulated disintegrated more rapidly compared with mycosed cadavers at both sites (Table 1).

Sporulation of infected cadavers. The percentage of infected *A. blondeli* cadavers that had visible sporulation following exposure to different microsites in the field is shown in Table 2. Both rainfall and microsite were important for sporulation (Pearson $\chi^2 = 42.2$, $P < 0.0001$; 22.6 , $P < 0.001$ respectively). Sporulation was only observed from cadavers exposed to rainfall, with increased exposure generally associated

Table 2. Percentage of *M. anisopliae* var *acidum* infected *Acrotylus* spp. cadavers sporulating within different microsites (see methods) in the Sahel during the rainy season. Sample number in parentheses. Five minor (< 10 mm) rainfall events occurred over a 22-day period, and results show observations from cadavers assessed prior to rainfall, after all rain events, and after the last three rain events. Due to destructive sampling cadavers were assessed once. All cadavers were left > eight days prior to assessment

Microsite	Prior to rainfall	After rainfall (three events)	After rainfall (five events)
– vegetation / – ground	0 a (12)	4.8 a(a) ^a (42)	41.6 b(ab)(12)
– vegetation / + ground	0 a (20)	16.9 b(a) ^b (59)	30 b(a) ^b (20)
+ vegetation / – ground	0 a (22)	7.8 a(a) (51)	31.8 b(a) (22)
+ vegetation / + ground	0 a (24)	36.8 b(b) (68)	58.3 c(b) (24)

^a Different letters directly after sample number represent differences (Pearson chi-square tests for independence on frequency data at 5% level) between microclimates due to rainfall frequency (horizontal); those in parentheses represent differences in microclimate for a given rainfall frequency (vertical).

^b Results may have been affected by high daytime ground temperatures (>60 °C).

with highest sporulation rates. There was some evidence that cadavers in shaded locations on the ground sporulated most readily (Table 2). In all cases little external sporulation was observed on the host cadaver and the majority was restricted to the host body cavity.

Measurement of infection profile from cadavers in field cages. The proportion of grasshoppers becoming infected after exposure to sporulating cadavers in field cages is shown in Figure 3. This shows transmission occurred under these field conditions, and cadavers can remain infective for a number of weeks. However the net infectivity of cadavers changed through time. Background infection levels were found to be low (<1%) throughout this period.

Estimating the transmission coefficient in the field. Following incubation in plastic bottles, $19.1 \pm 4.6\%$ of grasshoppers collected from the infective plot died of *Metarhizium* infection. No fungal infections occurred from grasshoppers collected prior to placement of sporulating cadavers. No rainfall occurred during the period grasshoppers were exposed to these sporulating cadavers. Based on this information, the transmission coefficient can be estimated. The density of healthy hosts at time t can be expressed as a function of initial density and risk of infection (Equation(1)). Thus the transmission coefficient can be derived (Equation(2)).

$$Ht = H_0 \exp(-\beta t I), \quad (1)$$

$$\beta = \frac{1}{tI} \ln \left(\frac{H_0}{Ht} \right), \quad (2)$$

H_0 is the initial density of healthy hosts, Ht is the density of healthy hosts at time t , t is the period of exposure, I is the density of infectious cadavers and β is the transmission coefficient from infectious cadavers to healthy hosts. Ht was not assessed, but was assumed to reflect the decrease in initial density of healthy hosts resulting from infection within the infective plot. This yields a transmission coefficient for this set of conditions of $0.45 \text{ m}^2 \text{ day}^{-1}$. However, this may be a conservative estimate, as it does not account for movement of grasshoppers in and out of the infective plot, and the actual exposure period for a subset of the grasshoppers sampled may have been less than 48 h.

Discussion

Following the large-scale field trial described in Langewald et al. (1999), the populations of live grasshoppers were reduced by $\approx 90\%$ in the 2–3 weeks following treatment. However, the density of grasshopper cadavers remained consistently low throughout the mycopesticide plot throughout this time (Arthurs, 2000). This inconsistency implies that predation contributed significantly to the fate of diseased grasshoppers (and hence also to the fate of the pathogen) before they were killed by the infection. An important aspect for the secondary transmission of *M. anisopliae* var. *acidum* is, therefore, likely to be its persistence within infected cadavers coupled with its ability to sporulate and reinfect hosts even at a low density.

In the present studies, and in marked contrast to the patterns of predation of infected grasshoppers mentioned above, field observations indicate that grasshopper cadavers infected with *M. anisopliae* var. *acidum* are unattractive to scavengers in this environment. Consequently the pathogen is likely to persist for several weeks, or even months, following death of the host. Like many, this pathogen produces anti-bacterial metabolites during necrophilic growth stages to prevent competition from common saprophytes (Hajek & St. Leger, 1994). Few studies have compared scavenging rates between uninfected and fungus-infected prey, although predator avoidance of *Metarhizium*-infected locust cadavers has also been observed in the Karoo, South Africa while uninfected cadavers were rapidly scavenged (Arthurs, 2000). In laboratory studies Roy et al. (1998) showed

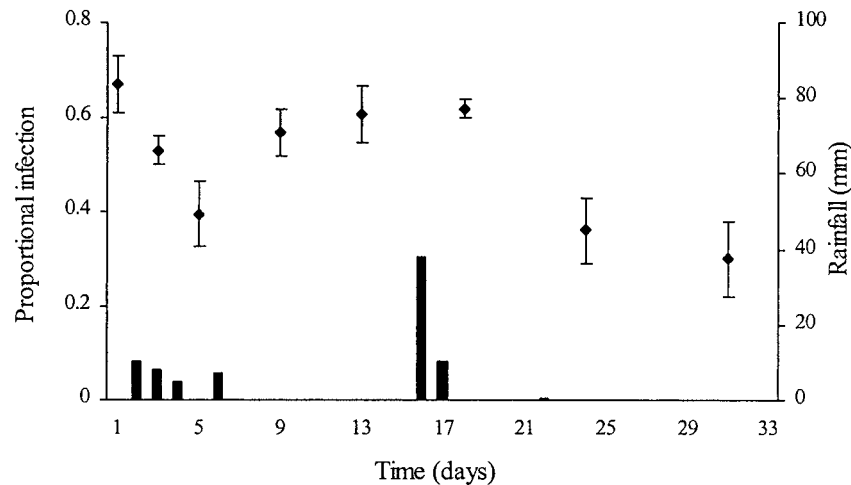


Figure 3. The infectivity profile for cadavers of *Acrotylus* spp. infected with *M. anisopliae* var. *acridum* in the Sahel as determined by the sequential exposure of uninfected hosts to sporulating cadavers in field cages. Rainfall occurring during the study is shown by the histogram.

that cadavers of aphids *Acyrtosiphon pisum* Harris infected with the entomopathogenic fungus *Erynia neoaphidis* Remaudiere & Hennebert were seldom consumed by starved foraging Coccinellid beetles, although uninfected cadavers were readily taken. In the present study, the factor most strongly associated with the disintegration and disappearance of cadavers was rainfall. Pre-sporulated cadavers were significantly less persistent in the environment compared with non-sporulating ones, probably because sporulation reduced the cuticular integrity of the host cadaver (Gabriel, 1968), facilitating its more rapid break up. The amount and frequency of rainfall has also been highlighted as the most important environmental factor associated with the persistence of cadavers of the clearwing grasshopper, *Camnula pellucida* Scudder, infected with *Entomophaga grylli* Fresenius (Sawyer et al., 1997) and cadavers of gypsy moth larvae *Lymantria dispar* L. infected with *E. maimaiga* Humber (Hajek et al., 1998).

In the current study field observations also showed that rainfall was essential for sporulation to occur on infected cadavers. The importance of environmental moisture for spore formation of entomopathogenic fungi in host cadavers is well documented (see Fargues & Luz, 1998; Hajek et al., 1990; Luz & Fargues, 1998; MacLeod et al., 1980; Milner & Lutton, 1986; Newman & Carner, 1975; Steinkraus & Slaymaker, 1994; Wilding, 1969) although relationships between sporulation and humidity vary considerably between different species of entomopathogenic fungi. Climatic data indicates that heavily overcast conditions associ-

ated with prolonged or intermittent rainfall are similarly associated with increased and more prolonged periods of high relative humidity during the night (Figure 1). Cooler night-time temperatures (20–30 °C) are also conducive to sporulation of *M. anisopliae* var. *acridum* (Thomas & Jenkins, 1997).

Sporulation also varied between microsites, being most likely within cadavers in sheltered locations on the ground. Such locations maintained slightly higher ambient humidity compared with exposed ones following rainfall and also provided additional moisture from a damp substrate. Such contact has been shown to favour sporulation of other entomopathogenic fungi compared with saturated humidity alone (Sprenkel & Brooks, 1977; Wilding, 1969). Thus, it is probable that in this environment sporulation is only likely to proceed during the night under wet conditions when high moisture availability and suitable temperatures typically occur.

While sporulation is necessary for reinfection, another important parameter in the epizootiology of host-entomopathogen dynamics is the lifespan of the infectious stage in the environment. Abiotic factors are known to be critical for the survival of spores of entomopathogenic fungi. In particular, the detrimental effects of UV radiation are well documented, with measured half-lives of *M. anisopliae* conidia being typically only a few hours under direct exposure to simulated sunlight in the laboratory (Moore et al., 1996; Roberts & Campbell, 1977; Zimmermann, 1982). In tropical areas conidial survival of *M. anisopliae* and *Beauveria bassiana* is proportional to the

degree of protection from UV (Daoust & Pereira, 1986). High temperatures are also detrimental, although thermal tolerances of conidia are influenced by their moisture content (Morley-Davies et al., 1995; Zimmermann, 1982) and conidial desiccation occurring during hot dry weather will greatly increase the thermal resilience of conidia.

Despite exposure to high UV and temperature, the field cage study showed that sporulating cadavers remained infective to grasshoppers throughout the study period, showing that at least some spores survived for extended periods within protected fragments of cadaver. The mechanisms governing the risk of infection are complex and in this case were thought to reflect both changes in the net infectivity of cadavers coupled with changes in host behaviour, both largely in response to climatic conditions (see below).

Many fungal entomopathogens are known to be able to undergo interruptions in spore production and release as cadavers dehydrate and rehydrate in response to fluctuating moisture conditions (Hajek & Soper, 1992; Millstein et al., 1983; Tyrrell, 1988). Therefore the production and dispersal of conidia and hence the net infectivity of cadavers may change as they break up and decay. The influence of climate on secondary infection is unlikely, however, to be solely dependent on the pathogen. Grasshopper activity is also related to climate, this time through their ability to thermoregulate and maintain a preferred body temperature (Chappel & Whitam, 1990). In the present study, grasshoppers were markedly inactive when thermoregulation opportunity was restricted during periods of cooler wet weather. Under these circumstances the risk of infection may have been low independent of cadaver infectivity. This latter explanation would account for the initial decline in infection rate in Figure 3. That reinfection was possible during dry conditions, however, demonstrates that unlike sporulation, high humidity is unnecessary for the infection process, possibly because water loss through parts of the living insect's cuticle creates a humid microclimate for germinating conidia (Chapman, 1971). Drawing these arguments together, it appears that infection profiles associated with cadavers may vary between season and ecological zone, as well as between host/pathogen systems. They may also differ from those associated with mycoinsecticide sprays.

Relatively few measurements of the transmission coefficient parameter, β , exist for entomopathogenic fungi due to difficulties with both manipulating and monitoring insect and pathogen populations. However,

the present estimate of $0.45 \text{ m}^2 \text{ day}^{-1}$ is comparable to estimates obtained from field cage studies using grasshopper cadavers infected with *Metarhizium* spp. in more humid areas, i.e., 0.4 for *Hieroglyphus daganensis* Krauss and 1.8 for *Zonocerus variegates* L. (Thomas et al., 1995). However, our estimate is high compared with those from other entomopathogens, such as viruses, which are typically at least an order of magnitude lower (see Anderson & May, 1981). Our estimate may, however, reflect the high mobility of hosts and the large number of conidia that were produced on the infective cadavers used in this study (i.e., incubated under optimum conditions in the laboratory).

In summary, these studies indicate that *M. anisopliae* var. *acidum* can be persistent in host cadavers in the environment and go on to sporulate and reinfect new hosts at a low field density, thus contributing to secondary control following application of a mycoinsecticide. However, at least in arid or semi-arid areas, rainfall may be critical for this process, by influencing the infectivity and persistence of cadavers. In addition, the cooler and overcast conditions associated with rain may limit host thermoregulation and promote faster host mortality rates (Blanford & Thomas, 1999) thus encouraging fungal transmission within a defined control season.

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