The potential of *Paranosema* (*Nosema*) *locustae* (Microsporidia: Nosematidae) and its combination with *Metarhizium anisopliae* var*. acridum* (Deuteromycotina: Hyphomycetes) for the control of locusts and grasshoppers in West Africa

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Dedicated to my late grandmother
Somabey Akoehi

Abstract

The potential of *Paranosema* (*Nosema*) *locustae* (Microsporidia: Nosematidae) and its combination with *Metarhizium anisopliae* var. *acridum* (Deuteromycotina: Hyphomycetes) for the control of locusts and grasshoppers in West Africa

Agbeko Kodjo Tounou

The present research project is part of the PréLISS project (French acronym for "Programme Régional de Lutte Intégrée contre les Sauteriaux au Sahel") seeking to develop environmentally sound and sustainable integrated grasshopper control in the Sahel, and maintain biodiversity. This includes the use of pathogens such as the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum* Driver & Milner and the microsporidia *Paranosema locustae* Canning but also natural grasshopper populations regulating agents like birds and other natural enemies. In the present study which has focused on the use of *P. locustae* and *M. anisopliae* var. *acridum* to control locusts and grasshoppers our objectives were to, (i) evaluate the potential of *P. locustae* as locust and grasshopper control agent, and (ii) investigate the combined effects of *P. locustae* and *M. anisopliae* as an option to enhance the efficacy of both pathogens to control the pests. For this purpose, preliminary surveys were conducted in Senegal and Cape Verde to evaluate the presence/absence of *P. locustae* in grasshopper populations sampled from the localities where the pathogen has been previously released. Detection of *P. locustae* spores was done in frozen grasshoppers by microscopic observation followed by antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA) using polyclonal antibodies. In laboratory experiments conducted in Niger and Benin, the acute and sublethal effects of *P. locustae* on nymphal stages of the desert locust, *Schistocerca gregaria* Forskål and the Senegalese grasshopper *Oedaleus senegalensis* Krauss were investigated. To study the efficacy of the combination of *P. locustae* and *M. anisopliae*, we compared sequential and separated infections of the two pathogens in fifth instar desert locust and evaluated the production of the two agents following dual infection. In field

experiments the efficacy of mixed application of *P. locustae* and *M. anisopliae* in wheat bran formulation to control grasshoppers in southeast Niger Republic was investigated.

In the preliminary surveys several grasshopper species were collected in Senegal and Cape Verde but *Oedaleus senegalensis* was the predominant species accounting for about 80 to 90% of total grasshoppers species collected. Of the 640 and 4,481 grasshoppers sampled in Senegal and Cape Verde, respectively, 0.5% and 0.02% were found to harbour traces of *P. locustae* sporoblastes and/or spores. Due to the long gap of time between the introduction of the pathogen and the subsequent survey (i.e., 1982 and 1990 in Senegal and Cape Verde, respectively), we were not able to associate the presence of infection to the first introduction of the pathogen. However, the presence of *P. locustae* in earlier nymphal instars as reported in this survey is an indication of a possible vertical transmission from infected adults to their offspring, although horizontal infections from other sources could not be excluded.

In the laboratory bioassay testing the susceptibility of immatures stages of *S. gregaria* and *O. senegalensis* to *P. locustae*, mortality was significantly higher for inoculated nymphs after 30 days post-inoculation (except inoculated fifth instar nymphs) than in control. Infection developed in 100% of nymphs treated at first, second and third instars while between 16-27% of nymphs inoculated at fourth and fifth instars were uninfected at the end of the experiment (30 days post-inoculation). While 85-91% of control nymphs reached adulthood, none of infected first, second and third instars reached the adult stage. Between 6-30% and 55-74% of nymphs treated at fourth and fifth instars, respectively, developed to adult stage. The higher susceptibility of the younger instars compared to the older was confirmed by the Median Survival Times (MSTs) that decreased significantly and remained in general shorter in younger than older instars. In *O. senegalensis* MSTs ranged from 5 to 11 days (first instar), 9 to 18 days (third instar) and 15 to >30 days (fifth instar); and for *S. gregaria* from 6 to 14 days (first instar), 9 to 16 days (second instar), 10 to 24 days (third instar), 14 to 26 days (fourth instar) and 15 to >30 days (fifth instar). In addition

to direct mortality induced by *P. locustae*, indirect sublethal effects were observed in infected individuals. Thirty percent of infected nymphs presented abnormal development such as deformation of wings and/or hind legs. Infection status of the parents had no effect on the average number of eggs per mated-pair. However, *P. locustae* infection was 17.7%, 48.5% and 51.8% in *O. senegalensis* progeny and 20.3%, 50.4% and 58.9% in *S. gregaria* progeny, when only the adult male, female and both parents were infected, respectively. Feces, eggs and froth plug from inoculated insects had detectable level of *P. locustae* sporoblastes and spores at concentrations ranging from light (fecal pellets) to medium (froth plug and eggs). Thus, it is possible that horizontal transmission of *P. locustae* to the progeny occurred upon hatching through ingestion of remaining egg pods and/or food infected by feces from infected nymphs, although such contamination might not greatly affect the final disease prevalence recorded in the present study.

Combination of *P. locustae* and *M. anisopliae* caused additive desert locust mortalities or greater than additive, indicating synergism. No evidence of antagonistic mortality response was observed. MST decreased significantly with increasing concentration of the two pathogens. Although the combination of the two pathogens did not affect *M. anisopliae* spores' production, 3 to 20-fold and 2.5 to 8-fold fewer *P. locustae* spores yield per nymph in 3 and 10 days sequential inoculations experiments compared to single inoculation, respectively.

Based on the *P. locustae*-host instars interaction, the field trial was designed to target the younger grasshopper instars. Hence applications were conducted when the predominant grasshopper species, *O. senegalensis* was at its younger nymphal stages (first to third instars accounting for >76% of *O. senegalensis* population). Grasshopper density reduction reached 44.7%*,* 52.8%*,* 73.7% and 89.1% within 21 days in *P. locustae*, *P. locustae* in sugar solution, *M. anisopliae* and *P. locustae* + *M. anisopliae* treatments, respectively, with 48.1% *P. locustae* infection in field surviving *O. senegalensis* 28 days post-application. Moreover, among the complex of grasshopper species found in the application zone, the two most common species after *O. senegalensis* i.e., *Acrotylus blondeli* De Saussure and *Pyrgomorpha cognata*

Krauss, accounting for 7.29% and 10.72% of all grasshopper species collected respectively, were found to be highly infected by *P. locustae*. The persistence of *P. locustae* infection in living grasshopper populations suggest that the effect of *P. locustae* might continue in the next generation as demonstrated in our laboratory tests.

The present study provides an increased understanding on the potential of *P. locustae* and *M. anisopliae* to control locust and grasshopper with emphasis on the desert locust, *S. gregaria* and the Senegalese grasshopper, *O. senegalensis*. Depending on the grasshopper species and ages at the time of application, population density can be significantly reduced when applied earlier in the season. In addition, the result of this study indicated that the moderate virulence of *P. locustae* apparently appears not to be a serious problem. The sublethal effects that result from the infection of the pathogen, such as delay in host development, reduced host activity and vertical transmission might play important role in host population dynamic. Moreover, the combination *P. locustae* and *M. anisopliae* could help to significantly reduce the time required by the pathogens to kill the hosts.

Key words: additivity and synergistic, biological control, density reduction, Grasshoppers, *Metarhizium anisopliae*, *Paranosema locustae*, vertical transmission.

Zusammenfassung

Möglichkeiten von *Paranosema* (*Nosema*) *locustae* (Microsporidia: Nosematidae) allein und in Kombination mit *Metarhizium anisopliae* var. *acridum* (Deuteromycotina: Hyphomycetes) zur Kontrolle von Heuschrecken in West Afrika

Agbeko Kodjo Tounou

Das hier präsentierte Forschungsvorhaben ist Teil des PréLISS Projektes (French acronym for “Programme Régional de Lutte Intégrée contre les Sauteriaux au Sahel”), in dem versucht wird Verfahren zur integrierten Kontrolle von Heuschrecken in der Sahelzone zu entwickeln. Wesentliche Ziele sind die Verbesserung der Umweltverträglichkeit und Nachhaltigkeit von Kontrollverfahren und der Erhalt einer hohen Biodiversität. Diese Ansätze beinhalten den Einsatz des entomopathogenen Pilzes *Metarhizium anisopliae* var. *acridum* Driver & Milner sowie der Mikrosporide *Paranosema locustae* Canning aber auch die Nutzung natürlicher Gegenspieler von Heuschrecken wie beispielsweise Vögel und andere natürliche Feinde. Die vorliegende Studie beschäftigt sich insbesondere mit der Anwendung von *P. locustae* and *M. anisopliae* var. *Acridum.* Angestrebt wurde einerseits eine Bewertung des Potenzials von *P. locustae* als Gegenspieler der Heuschrecken und andererseits eine Erhöhung der Effizienz der biologischen Kontrolle durch eine Kombination von *P. locustae* und *M. anisopliae*.

Zunächst wurden erste Erhebungen im Senegal und der Republik Kap Verde (Kapverden, Kapverdische Inseln) durchgeführt, um das Vorkommen von *P. locustae* in Heuschreckenpopulationen in Gebieten zu erfassen, in denen bereits früher Freisetzungen erfolgten. *P. locustae* Sporen wurden mittels mikroskopischer Beobachtung kombiniert mit „antigen-coated plate enzyme-linked immunosorbent assay“ (ACP-ELISA) aus gefrorenen Individuen nachgewiesen. In Laborexperimenten, durchgeführt in Niger und Benin, wurden akute und subletale Effekte von *P. locustae* auf Larvenstadien von *Schistocerca gregaria* Forskål und *Oedaleus senegalensis* Krauss erfasst. Die Effizienz einer Kombination von *P.*

locustae and *M. anisopliae*, wurde mittels Infektionsstudien (einzeln, kombiniert) der beiden Pathogene an den fünften Larvenstadien (L5) von *S. gegaria* überprüft und zudem die Entwicklung (Vermehrung) der beiden Pathogene in den Zielorganismen nach kombinierten Behandlungen bestimmt. In Feldstudien in Südosten der Republik Niger wurde weiterhin die Auswirkung kombinierter Ausbringungen von *P. locustae* and *M. anisopliae* formuliert auf Weizenkleie und bei Hinzufügen von Zucker als Phagostimulant hinsichtlich ihrer Wirkung auf Heuschrecken analysiert.

In den ersten Erhebungen im Senegal and Kap Verde war *Oedaleus senegalensis* die dominierende Art mit einem Anteil von ca. 80 to 90% aller erfassten Heuschreckenarten. Nur 0.5% (Senegal) und 0.02% (Kap Verde) der Individuen enthielt geringe Mengen von *P. locustae* Sporoblasten und/oder Sporen. Aufgrund der langen zeitlichen Distanz zwischen der ersten Einführung des Pathogens und der Erhebung konnten die gefundenen Infektionen nicht der ersten Einführungen des Pathogens zugeordnet werden. Der positive Nachweis von *P. locustae* in jungen Larvenstadien der Heuschrecken läßt sich aber als ein Hinweis auf eine vertikale Übertragung von infizierten Adulten auf die Nachkommen interpretieren.

Im Labortest war die Empfindlichkeit der Larvenstadien von *S. gregaria* und *O. senegalensis* to *P. locustae* gemessen anhand der Mortalität behandelter Larven 30 Tage nach Inokulation signifikant höher als in der Kontrolle. Infektionen entwickelten sich in 100% der behandelten Larven wenn das erste, zweite und dritte Stadium (L1, L2, L3) behandelt wurden, während nach Behandlung des vierten und fünften Larvenstadiums (L4, L5) bei 16-27% der behandelten Individuen keine Infektion auftrat. Im Gegensatz zu 85-91% der unbehandelten Larven erreichte keine Larve, die als L1, L2 oder L3 behandelt wurde, das Adultstadium. Zwischen 6-30% and 55-74% der Larven behandelt als L4 oder L5 entwickelten sich zu Adulten. Die höhere Empfindlichkeit der jungen Stadien wurde auch durch die Ermittlung der mittleren Überlebensrate („Median Survival Time - MST) bestätigt, welche mit zunehmendem Alter der Larvenstadien anstieg. Bei *O. senegalensis* konnten MSTs zwischen 5 und 11 Tagen (L1), 9 bis 18 Tage (L3) und 15 bis >30 Tage (L5) und bei *S. gregaria* zwischen 6 und 14 Tagen (L1), 9 - 16 Tage (L2), 10 bis 24 Tage (L3), 14 bis 26 Tage

(L4) und 15 bis >30 Tage (L5) bestimmt werden. Zusätzlich zu der Induktion einer erhöhten Mortalität konnten nach Behandlung mit *P. locustae* subletale Effekte beobachtet werden. 30% der infizierten Larven zeigten Entwicklungsstörungen wie Deformationen der Flügel oder der Hinterbeine. Der Infektionsstatus der Eltern hatte keinen Einfluß auf die durchschnittliche Anzahl der produzierten Eier, jedoch wiesen 17.7%, 48.5% and 51.8% der Nachkommen von *O. senegalensis* und 20.3%, 50.4% and 58.9% der Nachkommen von S*. gregaria* Infektionen mit *P. locustae* auf wenn nur die adulten Männchen, Weibchen oder beide Eltern infiziert wurden. Kot, Eier und Kokons inokulierter Insekten wiesen Kontaminationen mit Sporoblasten und Sporen von *P. locustae* auf. Deshalb ist auch eine horizontale Übertragung über Aufnahme von infizierten Eischalen oder Kotpartikeln ein sehr wahrscheinlicher Infektionsweg.

Kombinationen von *P. locustae* and *M. anisopliae* ergaben additive Mortalitätsraten bei *S. gregaria* oder sogar höhere als additive Werte, was auf synergistische Effekte hindeutet. Es konnten keine Hinweise auf antagonistische Einflüsse gefunden werden. Die MSTs nahmen mit zunehmender Konzentration der Pathogene significant ab. Obwohl Kombinationen direkt die Sporenproduktion von *M. anisopliae* nicht beeinflussten, wurden 3 bis 20 und 2.5 bis 8 mal weniger Sporen von *P. locustae* bei 3 bzw. 10-tägigen Inokulationen im Vergleich zur alleinigen Behandlung mit *P. locustae* gebildet.

Der Feldversuch wurde so angelegt, dass primär junge Entwicklungsstadien der Heuschrecken getroffen werden sollten, und die Behandlungen wurden zu einem Zeitpunkt durchgeführt, zu dem von der dominanten Art *O. senegalensis* das 1. bis 3. Larvenstadium mehr als 76% der Population ausmachten. Es wurden Reduktionsraten der Heuschreckendichte von 44.7%*,* 52.8%*,* 73.7% und 89.1% innerhalb von 21 Tagen bei Behandlungen mit *P. locustae*, *P. locustae* in Zuckerlösung, *M. anisopliae* und *P. locustae* + *M. anisopliae* erreicht. Die Infektionsraten von *P. locustae* lagen 28 Tage nach der Ausbringung bei überlebenden *O. senegalensis* bei 48.1%. Innerhalb der Artengemeinschaft der Heuschrecken, die in den behandelten Arealen bestimmt wurden, waren nach *O.*

senegalensis Acrotylus blondeli De Saussure und *Pyrgomorpha cognata* Krauss mit 7.29% und 10.72% aller Heuschreckenindividuen die abundantesten Arten, die ebenfalls in hohem Masse mit *P. locustae* infiziert waren. Die Persistenz von *P. locustae* in lebenden Heuschrecken läßt vermuten, dass der Einfluß dieses Pathogens auch in folgenden Generationen die Entwicklung der Heuschrecken beeinflusst.

Die vorliegende Studie erweitert das Verständnis über das Potenzial von *P. locustae* und *M. anisopliae* als biologische Kontrollagenzien von Heusrecken im allgemeinen und S*. gregaria* and *O. senegalensis* im besonderen. Abhängig von der Heuschreckenart, der Altersstruktur der Population und der Populationsdichte können mit diesen Pathogenen signifikante Reduktionsraten bei frühen Behandlungen erreicht werden. Zudem stellt die nur moderate Virulenz von *P. locustae* keinen entscheidenden Nachteil dar. Subletale Effekte wie eine Verzögerung der Entwicklung der Zielorganismen, eine reduzierte Aktivität der Wirte sowie die intensive Verbreitung des Pathogens in der Population durch vertikale und horizontale Übertragung können die Populationsdynamik der Wirte über einen längeren Zeitraum betrachtet erheblich beeinflussen. Darüberhinaus erlaubt die Kombination von *P. locustae* mit *M. anisopliae* eine signifikante Reduktion der Zeitspanne, die *P. locustae* benötigt, um den Wirtsorganismus abzutöten.

Schlüsselworte: additive und synergistische Effekte, Biologische Kontrolle, Dichtereduktion, Heuschrecken, *Metarhizium anisopliae*, *Paranosema locustae*, vertikale Transmission.

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Abbreviations

ANOVA	Analysis Of Variance
df	Degree of freedom
e.g.	for example
F	F-value
FAO	Food and Agricultural Organization
Fig.	Figure
CI	Confidence Interval
GLM	General Linear Model
L:D	Relation of light to darkness
log	Logarithm
MST	Mean Survival Time
P	P-value (statistical significant level)
RH	Relative Humidity
SE	Standard Error of the mean
w/w	weight of sugar to weight of wheat bran/ha

1 General introduction

1.1 Origin and distribution of grasshoppers and locusts

Grasshoppers and locusts include insects in different families belonging to the super family Acridoidea and the order Orthoptera (Otte, 1981). Grasshoppers and locusts are distinguishable from other orthopterans primarily on the basis of their external morphology. The most obvious and distinctive features of grasshoppers and locusts are their enlarged hind legs and their relatively short and thick antennae. There are more than 350 grasshopper and locust species recorded from the Sahel (Mestre, 1988), of which about 30 are considered to be of regular or irregular pest status (Popov, 1988).

Grasshoppers and locusts occur in a wide variety of habitats, from low-elevation, hot, dry deserts to high-elevation, moist environment. Most species occur in arid and semi-arid environment, and it is in the warm semi-arid and arid desert grasslands that grasshopper and locust species diversity and population densities are the greatest (Otte, 1976). They are relatively large, active insects and require structurally open habitats where they are free to move, and where sunlight levels are high enough to enable them to maintain high metabolic rates. Habitat specificity varies considerably among species of grasshoppers and locusts (Joern, 1979). Some species such as *Schistocerca gregaria* Forskål and *Oedaleus senegalensis* Krauss (Orthoptera: Acrididae) are typically common in desert environments but can be found in a wide variety of habitats over wide geographic and altitude ranges when outbreaks occur. Other species are much more restricted or specific to particular types of habitats. Grasshoppers tend to feed on particular plants that occur in their preferred habitats.

1.2 Biology and ecology of locust and grasshopper

1.2.1 The Senegalese grasshopper *Oedaleus senegalensis*

The Senegalese grasshopper *O. senegalensis* is the most serious periodic grasshopper pest widely distributed throughout the Sahel but also in many other parts of the world (Cheke, 1990; Geddes, 1980). Since the 1970s, deforestation, overgrazing and droughts in Sahel have favoured the development of Kram Kram grasses (*Cenchrus biflorus* Roxb (Cyperales: Poaceae)), suitable habitats for *O. senegalensis* (Cheke et al., 1980). At high population densities, *O. senegalensis* undergoes a phase change to gregarious morph (Ahluwalia et al., 1976). *Oedaleus senegalensis* has an annual life cycle and is a pest of several subsistence crops. Early instars develop in fallow or grassland. Subsequently, with increase of population densities, late instars and adults can cause economic damage directly to pastures, and also by migrating from pasture into adjacent crops such as millet, maize, beans, sweet potato, cassava and vegetable (Boys, 1978; Popov, 1980; Lobo-Lima and Klien-Koch, 1981; Amatobi et al., 1986).

Under favourable conditions, *O. senegalensis* has a generation time of about 45 days to two months (Popov, 1980; Cheke, 1990), enabling it to complete 3 generations in the short annual rainy season. Depending on the environmental conditions, *O. senegalensis* develops through 5 to 6 nymphal stages. The pest is adapted to dry environments and when conditions are not favourable for its development, it may lay diapausing eggs that can survive underground until the rainy season (Colvin and Cooter, 1995). Hatching in such conditions occurs after 7-8 months (Gehrken and Doumbia, 1996), leading to successive serial hatchings at the beginning of the rainy season. Such a situation makes the egg diapause, an important component of the outbreaks generating mechanism (Fishpool and Cheke, 1983). Both photoperiod and temperature influence the induction of diapause in *O. Senegalensis* eggs. Diop (1993) reported that females, reared under short photoperiod (L:D 10:14 h), produced mostly diapausing eggs, while those reared under long photoperiod (L:D 14:10 h) laid essentially no diapausing eggs. According

to Colvin and Cooter (1995), high temperatures (40 °C) and long photoperiods (L:D 14:10 h) which characterize the beginning of the rainy season in the Sahel, cause non-diapausing eggs to be laid while lower temperatures (25 °C) and shorter photoperiods (L:D 12:12 h), which occur at the end of the rains, result in the production of diapausing eggs.

1.2.2 The desert locust *Schistocerca gregaria*

The desert locust, *S. gregaria* is the most commonly Orthopteran known which represents the most complex situation of all the locust pests. During periods of recession the desert locust is usually restricted to the semi-arid and arid deserts of Africa, the Near East and South-West Asia that receive less than 200 mm of rain annually. The insect generally develops from first instar nymphs to adults in about 4-6 weeks. Females lay eggs in an egg pod in sandy soils at a depth of 10-15cm below the surface. A solitary female lays about 95-158 eggs whereas a gregarious female lays usually less than 80 eggs per egg pod (Popov, 1980). Females can oviposit at least three times in their lifetime usually at intervals of about 6-11 days. Hatching occurs during rainy season in response to favourable temperature and moist conditions (Hewitt, 1979).

The most prominent feature of the desert locust is its ability to interconvert between two morphologically, physiologically and behaviourally distinct phases. As desert locusts increase in number and become more crowded, they switch from solitary to gregarious behaviour (Showler and Potter, 1991). Some adult desert locusts are pale gray or beige in the solitary phase, with the males turning pale yellow on maturation. In contrast, an adult from the gregarious (swarming) phase is bright pink when immature and bright yellow when mature. The solitarian phase prevails under endemic conditions in areas of recession, which are characterized generally by dry and warm weather. This phase lives as dispersed individuals in very low densities, feeding on a limited range of desert plants, and reproducing only when environmental conditions are sufficiently favourable. The gregarious phase is characterized by a highly cohesive behaviour, long-distance migratory aptitude,

polyphagy, synchronous and accelerated maturation of males and females and mass egg laying by gravid females at common sites.

1.2.3 Other Locusts and Grasshoppers

Other important locust and grasshopper species occur mainly in Africa, but also in the Middle East. They include among others *Zonocerus variegatus* L. and *Kraussaria anguilifera* Krauss (Orthoptera: Pyrgomorphidae); the Egyptian grasshopper *Anacridium aegyptium* L., *Cataloipus fuscocoeruleipes* Sjöstedt, *Diabolocatantops axillaris* Thunberg, *Hieroglyphus daganensis* Krauss, the brown locust *Locustana pardalina* Walk, the red locust *Nomadacris septemfasciata* Serville, *Kraussella amabile* Krauss, *Pyrgomorpha cognata* Krauss (Orthoptera: Acrididae). Some of these locust and grasshopper species may cause severe economic damage to crops while others may occur usually only in small numbers, rarely causing heavy damage and having no economic importance.

1.3 Host plants and economic importance of locusts and grasshoppers

Both grasshoppers and locusts cause direct losses to crops by voraciously devouring vegetation. They feed on several economically important crops among which rice, wheat, cotton, maize and millet are the most important. Some species are host specific to certain plants; others feed on many different species and even families of plants. Total plant loss may occur when attack coincides with vulnerable stages of the crop. Grasshoppers pose damage every year, but become very destructive during outbreak periods. In the Sahel and Sudan savannah zones of West Africa, the Senegalese grasshopper *O. senegalensis* is an important pest of *Pennisetum* (millet), the principal food crop of the region (Cheke et al*.,* 1980). In outbreak years, hopper bands can destroy millet and sorghum seedlings and farmers often have to reseed several times. In 1989, 5.7% of the farmers in northwestern Mali lost 70 to 90% of their millet crop due to grasshoppers (Cheke, 1990; Kremer and Lock, 1992). In Niger, between 10 and 82% damages measured on millet seed heads have been reported to be caused by *O. senegalensis* (Cheke et al., 1980).

1.4 Management options

1.4.1 Chemical control strategies

Control of grasshoppers and locusts has traditionally relied on synthetic insecticides and for emergency situations this is unlikely to change. Large-scale locust and grasshopper outbreaks generally demand immediate attention and significant short-term reduction of the pest populations. To prevent total crop losses following severe outbreaks, chemical controls with conventional pesticides have been the most appropriate strategy in Sahel, Northern United States and Canada, South-eastern Asia, Australia and elsewhere from the 1950s to the mid-1980s (Brader, 1988). The technique of control of locusts and grasshoppers involved the spraying of barriers of persistent organochlorine insecticides across areas infested by hopper bands. For many years, the product of choice was dieldrin, a persistent pesticide well suited for barrier treatment (Brader, 1988). However, concerns about its detrimental impact on the environment resulted in its prohibition in most countries.

Since the withdrawal of dieldrin, locust and grasshopper control has become more difficult and less efficient. In the absence of this product, other less persistent pesticides such as fenitrothion, malathion and fipronil, have been used for acridids control in Africa and in many parts around the world targeting both nymphs and adults (Prior and Street, 1997). They are sprayed or dusted directly onto hopper bands and swarms, or distributed close to them as baits. All of these techniques require much greater effort in locating and treating individual targets than the former barrier technique that had been apparently successful. Most modern pesticides such as fenitrothion that has a half-life of about 24 hours (Sekizawa et al., 1992), are much less persistent and have therefore to be applied more frequently in larger volumes. Hence, even though they are less toxic than dieldrin, their environmental impact may well be worst.

The scale and cost of pesticide application added to the concerns over the environmental and human health implications have triggered a strong interest in

international programs for the development of microbial control agents for use in integrated control of acridoid pests.

1.4.2 Biological control as alternative to conventional pesticides

Biological control of acridoid pests has been developed as an alternative to conventional chemical application. At least 200 species of insects, mites, and nematodes attack grasshoppers (Lavigne and Pfadt, 1966; Rees, 1973). Various species of flies and wasps parasitize grasshopper nymphs and eggs while other flies, beetles (including blister beetle larvae in the genus *Epicauta*), birds, and rodents are significant predators. Among diseases that occur naturally in locust and grasshopper populations, the most common are from fungal infections (Lomer et al., 2001; Wraight et al., 1998) and microsporidian, principally *Paranosema locustae* Canning (Microsporidia: Nosematidae) (Brooks, 1988; Lange, 1992, 2001, 2002).

1.5 The entomopathogenic fungi

Entomopathogenic fungi are important pathogens of many insects and other arthropods and frequently cause epizooties that can significantly reduce pest populations all over the world. Approximately 750 species of entomopathogenic fungi are known to infect a number of economically important arthropod pests and have thus received considerable attention as potential microbial control agents (Mulock and Chandler, 2000; Haraprasad et al., 2001). The majority of these entomopathogenic fungi belong to the classes of Zygomycetes, Ascomycetes and Deuteromycetes. Among entomopathogenic fungi that have been considered as alternative to synthetic chemical, species in the genera *Metarhizium* are the most widely used to control grasshoppers and locusts (Bateman, 1997; Jaronski and Goettel, 1997).

Metarhizium anisopliae Metchnikoff (Deuteromycetina: Hyphomycetes) occurs on a wide range of insects in the orders Orthoptera, Coleoptera, Lepidoptera, Hemiptera and Hymenoptera, as well in the class Arachnida. Various studies have shown the potential of *M. anisopliae* as a microbiological control agent of important pests. In Africa, since 1989, the project Lubilosa (French acronym of “Lutte Biologique Contre

les Locustes et les Sauteriaux"), a collaborative research programme involving the International Institute of Biological Control (IIBC, Ascot, UK), the International Institute of Tropical Agriculture (IITA, Cotonou, Benin) and the "Département de Formation en Protection des Végétaux" (DPFV, now part of AGRHYMET-CILSS, Niamey, Niger), has worked towards the development of an oil-based formulation of a mycopesticide containing *M. anisopliae* var. *acridum* Driver and Milner, for the control of the desert locusts and grasshoppers (Prior et al., 1992; Prior et al., 1995). Field trials demonstrating the efficacy of *M. anisopliae*, as oil formulation, have been carried out successfully (Lomer et al., 1993; Lomer et al., 2001; Douro-Kpindou *et al.,* 1995; Langewald et al., 1999; Arthurs et al., 2003). The host range of the fungus is narrow and at field application rates it is safe to non-target Hymenoptera, Coleoptera and Homoptera (Ball et al., 1994; Lubilosa unpublished results). The lack of toxicity of *M. anisopliae* to mammals and, consequently to humans (El-Kadi et al., 1983), offers a further advantage over currently used organophosphate pesticides.

1.5.1 Life cycle of entomopathogenic fungi

In general, the life cycle of entomopathogenic fungi begins with the attachment of the spore to the cuticle of the insect followed by germination and active penetration into the cuticle and a rapid proliferation of the fungal cells in the host's body. The fungus rapidly multiplies throughout the body and uses it as a nutrient source. Mortality is due to tissue destruction and occasionally to toxins produced by the fungi (Kershaw et al., 1999). The death of the infested host is followed by the outgrowth of the fungus and the production of infective spores, which can immediately repeat the cycle. The processes of germination of spore and growth on the cuticle are highly dependent on both biotic and abiotic factors. The importance of abiotic factors, such as temperature, relative humidity and light on the infection by entomopathogenic fungi has been intensively investigated (Milner et al., 1997; Estrada et al., 2000). Abiotic factors affect both fungal sporulation and the survival of the conidia (Milner et al., 1997). On the other hand, biotic factors influencing fungal infections include microbial antagonists on the host insect integument, host susceptibility and, most importantly, the varying degree of virulence of the fungal strains.

1.6 The Microsporidian, *Paranosema locustae*

Paranosema locustae is a spore-forming pathogen of the adipose tissue of orthopterans that was isolated, selected, and developed in the USA as a long-term microbial control agent of grasshoppers (Henry, 1978; Henry and Oma, 1981; Johnson, 1997; Lockwood et al., 1999). *Paranosema locustae* belongs to the Microsporidia, a group of unicellular eukaryotes that are obligate intracellular parasites of animals and some protists (Wittner and Weiss, 1999). Microsporidia were historically regarded as Protozoa or Archezoa, but recent studies at the molecular level have shown they are actually related to Fungi (Keeling and Fast 2002).

In North America, Steinhaus (1951) first noticed *P. locustae*, albeit without naming it, in 3 species of grasshoppers of the genus *Melanoplus* from Montana. Soon after, Canning (1955) described *P. locustae* as *Nosema locustae,* using diseased African migratory locusts, *Locusta migratoria migratorioides* Reiche and Fairmaire (Orthoptera: Acrididae), from a rearing colony at the Imperial College Field Station in London. Sokolova et al. (2003), while erecting the new genus *Paranosema* for another microsporidian pathogen of Orthoptera from the cricket *Gryllus bimaculatus*, transferred *N. locustae* to *Paranosema*, based on molecular and ultrastructural grounds, erecting the new combination *P. locustae*. Even more recently, Slamovits et al. (2004) proposed another new combination, *Antonospora locustae*; but Sokolova et al. (2005) provided reasons for favoring the name *P. locustae*.

One of several factors that permitted the selection of *P. locustae* for its development as a microbial control agent is its wide host range among acridomorphs. The pathogen has been extensively field-tested for long-term suppression of locusts and grasshoppers in the United States and Canada (Ewen and Mukerji, 1980; Onsager, 1988; Lockwood et al., 1988; Johnson, 1997). Although infection of *P. locustae* may occasionally result in high levels of mortality among some acridid species (Streett and Henry, 1984), realistic goals of application of this pathogen include reduced feeding by infected insects (Oma and Hewitt, 1984; Johnson and Pavlikova, 1986), delayed development (Schaalje et al., 1992), lower

reproduction (Streett et al., 1993) and potential vertical transmission of the pathogen from infected parents to offspring (Han and Watanabe, 1988; Streett et al., 1993; Raina et al., 1995). Therefore *P. locustae* appears to be a potential classical biological control agent that might simply reduce the number of grasshopper outbreaks over time.

Historical reports on the *P. locustae* have suggested that the pathogen could be of great importance in locust and grasshopper control strategies (Onsager *et al.,* 1981; Lockwood and DeBrey, 1990). According to Henry and McCleave (1963), background infection rates under field conditions were low, around 4% while Henry and Onsager (1982) reported significant reduction of grasshopper populations in the year of treatment in large-scale trials with *P. locustae*.

1.6.1 Transmission and dissemination of *Paranosema locustae*

In contrast to entomopathogenic fungi that infect through the insect's cuticle, protozoan infection occurs through ingestion of infective spores. Generally a high quantity of infective stage of the spores is required to initiate an infection that causes the premature death of the host insect. The pathogen enters the insect body via the gut wall, spreads to various tissues and organs, and multiplies, sometimes causing breakdown of tissue and septicaemia. *Paranosema locustae* infects the fat tissue, thereby disrupting the host's intermediary metabolism and competing with it for energy reserves. Infected insects may be sluggish and smaller than normal, sometimes with reduced feeding and reproduction, and difficult moulting. Death may follow if the level of infection is high. One advantage of this type of infection is that the weakened insects are more likely to be susceptible to adverse weather and other mortality factors. Spores can be released prior to death of the infected host through regurgitation or in feces and/or passed on to the next generation of host insects on or in the eggs laid by infected females.

- Vertical transmission

Vertical transmission of a pathogen is a transmission from one generation to the following one and may occur through infected females in most insects (Fine, 1975),

either via passage of the pathogen with the ovary or through contaminated eggs. It is an important mode of transmission since it can lower, even in absence of repeated applications, host population. In a laboratory study, Raina et al. (1995) reported transovarial transmission of *P. locustae* in *L. migratoria migratorioides* with the infection incidence ranging from 72 to 92% among progeny up to the F7.

- Horizontal transmission

Paranosema locustae not only can infect eggs and progeny (Bomar et al., 1993), but also be horizontally transmitted to non-infected insects. Horizontal transmission of *P. locustae* occurs when non-infected hosts acquire viable spores from infected ones either through cannibalism of dead or living insects or after ingesting diet contaminated with feces from infected insects. According to Henry and Onsager (1982), horizontal transmission in the next generation of insects infected by *P. locustae* depends probably more on transmission through feces. Reliance on horizontal transmission requires that the pathogen can persist in the infected host cadaver (Canning et al., 1982).

1.7 The PRéLISS Programme

PréLISS (French acronym for, Programme Régional de Lutte Intégrée contre les Sauteriaux au Sahel) is a collaborative programme between IITA and AGRHYMET-CILSS, Danish National Environmental Research Institute, Ornis Consult and partners from four CILSS countries (Burkina Faso, Cape Verde, Niger and Senegal). This joint programme founded initially for three years by the Danish National Environmental Research Institute, aimed to develop environmentally sound and sustainable integrated grasshopper control in the Sahel, and maintain biodiversity. To this end, the programme proposed a number of activities including three different approaches:

- Biological control approach based on strengthening indigenous natural enemies. Emphasizing the role of insects but also of birds and evaluating the impact of releasing exotic natural enemies such as *M. anisopliae* var. *acridum*, *P. locustae* and *Scelio* sp. for long-term suppression of grasshopper outbreak

- The preventative application of Green Muscle®, *M. anisopliae* var. acridum based microbial pesticide developed by the “Lubilosa” programme
- The third component is a reduced application of conventional insecticides in situations, which are unsuitable for alternatives.

The PRéLISS programme intends to study the potential negative side effects of releases of exotic natural enemies on the indigenous non-target species or natural enemies, paying special attention to the risk for bird species of high conservation value and migratory birds. The question of indirect effects of grasshopper control programs, primarily reduction or loss of basic food source for birds and other natural enemies that prey on grasshoppers, now comes up more frequently than potential toxic effects. Scientists and land managers have made a lot of progress in showing the role and benefits of wildlife, especially birds, as important contributors to regulation of grasshopper densities (Fowler et al., 1991; Bock et al., 1992). However, the overall ecology of native wild vertebrates in preventing insect pest outbreaks is virtually unexplored. Clear guidelines on how to protect these birds are needed to preserve their role as grasshopper predators and to ensure that international conventions (Bonn Convention and Ramsar) on the protection of migratory species and wetlands are not violated.

Last but not least, within the project, the Danish National Environmental Research Institute in collaboration with AGRHYMET with support from Ornis Consult in close contacts with other groups currently working on similar approaches, like CABI in UK and APLC in Australia, is developing a Geographic Information Systems (GIS) based decision-making tool. Such decision-making tool that consists of several sub models could help to determine different zones of different ecological vulnerability, natural enemy distribution, grasshopper population dynamics, crop damage and the efficacy of IPM control programme.

1.8 Research objectives

The aims of the present work were of two-fold. *Paranosema locustae* has been released in Senegal and Cape Verde Island in 1982 and 1990, respectively. The

programme was unfortunately abandoned because of lack of visible short-term impact of the pathogen on grasshoppers. Thus the first aim of the present study was to evaluate the potential of *P. locustae* to control grasshoppers in Africa. To this end, the studies included grasshopper and locust surveys to determine the presence/absence of *P. locustae* in Senegal and Cape Verde. Moreover, laboratory and field studies were conducted to evaluate the potential of *P. locustae* as control agent of grasshoppers and locusts in Africa.

The second aim was to test the interaction between *P. locustae* and *M. anisopliae* in acridid control. To address this objective we compared the susceptibility of *P. locustae*-infected and uninfected desert locust *S. gregaria* nymphs to *M. anisopliae,* and compared the production of *P. locustae* spores by desert locust nymphs infected with *P. locustae* alone and those infected with the two pathogens. In additional tests, field experiments were conducted to investigate whether the application of *P. locustae* in combination with *M. anisopliae* could be a successful strategy to control locusts and grasshoppers in West Africa and to evaluate the ecological implication of such combination in an environmental friendly IPM strategy against locusts and grasshoppers.

2 Evaluation of the persistence of *Paranosema locustae* (Microsporidia: Nosematidae) in Cape Verde and Senegal, two African ex-release countries of the pathogen

2.1 Abstract

Grasshopper surveys to detect *Paranosema locustae* Canning introduced to Senegal in 1982 and Cape Verde Island in 1990 were conducted during the year 2003. A total of 640 grasshoppers and locusts collected from two different localities in northern Senegal and 4,481 from five localities on Santiago Island were examined. Spores of *P. locustae* were found in a single *Anacridium melanorhodon* Walker (0.02%) among the grasshoppers collected in Cape Verde. Three specimens of *Oedaleus senegalensis* Krauss (0.5%) were found harbouring spores of *P. locustae* in Senegal. An additional unidentified Protozoan species was isolated from an adult desert locust, *Schistocerca gregaria* Forskål, collected at the release site of Lampsar in Senegal. Fungal infections with predominance of *Metarhizium anisopliae* Metchnikoff, were recorded in 2.4% and 6.6% of the grasshoppers collected from Cape Verde and Senegal, respectively, and bacterial infections were recorded in 0.5% of the Senegalese collection. Although few grasshoppers harbouring *P. locustae* spores were recorded from this survey, the detection of the pathogen in earlier instars nymphs is an indication that the pathogen could have been transmitted from infected adults to their offspring, although possible horizontal infections from other insects could not be excluded. These results reveal the need to continue surveying for *P. locustae* in order to accurately assess its impact on the West African grasshopper communities.

2.2 Introduction

Grasshoppers and locusts are among the most serious pests of agricultural crops in West Africa, where they cause extensive and serious damage to food crops (Hewitt and Onsager, 1983). The economic importance of the locusts and grasshoppers is reflected by the wide variety and comparatively large number of food crops they feed upon. Current control strategies for locusts and grasshoppers mainly rely on use of synthetic insecticides. Unfortunately, the reliance on chemical control might threaten the otherwise very successful biological control of other important pests in the environment. Thus, the development of alternative control methods for locusts and grasshoppers has become of paramount importance for the successful use of biological control within the context of integrated pest management (IPM) in Africa. Attempts for biological control of locusts and grasshoppers have mainly focused as well on the use of entomopathogenic fungi (Lomer et al., 2001) but also on the Microsporidia, *P. locustae* (Onsager, 1988). The Microsporidian, *P. locustae* is after entomopathogenic fungi, the most promising biological agent for reducing grasshopper and locust populations. Releases of *P. locustae* were conducted in several countries, USA, Canada, Argentina but also in China and Australia for locusts and grasshoppers control (Lange and De Wysiecki, 1996; Yan et al., 1996). Studies of the impact of applications of *P. locustae* conducted in Argentina and China indicate possible establishment and long-term impacts of the pathogen (Johnson, 1997; Lange, 2001, 2003).

Preliminary field trials using *P. locustae* against grasshoppers in Africa were conducted from 1982 to 1990 in countries like Mali, Senegal, Cape Verde, Niger and South Africa (Fishpool, 1982; Henry, 1992; Nasseh et al., 1992). The great mobility of African grasshopper species made experimental results difficult to interpret. However in the regions where *P. locustae* were released, no major grasshopper outbreaks were recorded following introduction of the pathogen (Langewald, pers. commun.). Given the lack of information about the long-term impact and establishment of *P. locustae* in African introduced localities and in the light of the pathogen situation in Argentina, there is a need for further studies in Africa especially to gather information

on the persistence of *P. locustae* in locust and grasshopper populations. Hence, in this study, our objective was to find out whether *P. locustae* could be re-isolated in Cape Verde and Senegal, two former release countries of the pathogen.

2.3 Materials and methods

2.3.1 Site description

In Cape Verde grasshoppers were sampled in Calabaceira (014º 55' N 023º 35' W), San Martinho (014º 56' N 013º 33' W), Vale Da Custa (014º 55' N 023º 29' W), Achada Barnelo (014º 55' N, 023º 38' W) and Tarrafal (015º 16' N 023º 44' W) all located on Santiago Island. In Senegal, samples were collected from Lampsar (016º 06' N 016º 22' W). Additional samples were taken from Mpal, 30km South-East Saint Louis. The Mpal area was selected because (1) grasshopper density was very low in Lampsar at the time of the survey and (2) this area was the closest locality with notable grasshopper populations. Grasshopper collections were undertaken from July to August 2004 in Senegal and in September on Santiago Island in Cape Verde. In each of the seven localities, criteria for site selection included high and/or diverse grasshopper population densities, and slightly green and/or humid areas.

2.3.2 Sampling methods

The grasshopper survey in Senegal was done along transects in a total of 48 natural grasslands and fallows (28 in Lampsar and 20 in Mpal). On the Santiago Island, Cape Verde, 9 representative plots in natural grasslands and improved pastures were surveyed in each of the 5 localities in the centre of each treatment plots; four diagonal transects were laid out, starting 10 m from the central point and extending towards the four corner points of each plot. The grasshopper population density was monitored by counting every 5 m the number of grasshoppers within 25 imaginary one-square-meter-quadrates along these lines (Kooyman et al., 1997).

Sampling was performed over 3 days with 3 surveyors alone transect in natural grasslands and fallows using a 30 cm diameter, 65 cm depth sweep net. Collected grasshoppers were placed in cages and brought to the laboratory where they were

counted and classified by species and age. These insects were, then, frozen at -4 °C and later shipped to IITA Benin station where they were examined for presence or absence *P. locustae* spores.

2.3.3 Diseases diagnosis

Disease diagnosis was conducted in two steps. In the first step, microscopic observation was done to identify any pathogen that might infect the collected grasshoppers. The second step that used ACP-ELISA test was set up for *P. locustae* detection and was used for samples that showed signs of Microsporidian infection from the first step observation.

Microscopic observation

For the microscopic observation, each frozen nymph was dried for 48 hours at room temperature and transferred onto sterile microscope slides placed inside sterile Petri dishes, containing 85 mm diameter moistened sterile filter paper. Cadavers were monitored daily under a microscope for fungal outgrowth and sporulation on the insect cuticle. When entomopathogenic fungi were detected, they were isolated, cultured on PDA (Potato Dextrose Agar) medium and identified by the morphological characteristics to particular fungal species at IITA Benin station. For *P. locustae* detection, insects were crushed and suspended in 10 ml distilled water. One drop of each suspension was observed with a phase contrast microscope at 400x magnification to search for the characteristic spores of protozoan and/or those of any other pathogens (Kleespies et al., 2000). Once a suspension was suspected to harbour Protozoan spores, a spore suspension was mixed with wheat bran. Healthy fifth instar *S. gregaria* nymphs reared at IITA-Benin were supplied with the treated wheat bran to initiate infection. A total of fifteen insects were infected with each suspension suspected to harbour Protozoa spores. Two weeks after insects have been fed with the treated wheat bran, 10 individuals were randomly selected from each treatment and crushed individually and the extract used for *P. locustae* spores detection.

Protocol for detection of P. locustae using ACP-ELISA test

Grasshoppers that were infected with extract from grasshoppers suspected to harbour protozoan spores were tested for *P. locustae* by antigen-coated plate enzyme-linked immunosorbent assay (ACP-ELISA) (Mih et al., 1995) using polyclonal antibodies produced at IITA Ibadan. Samples were extracted (1:10 w/v) in coating buffer pH 9.6, containing 2% (w/v) polyvinylpyrrolidone (PVP), 0.1% (w/v) Na_2SO_3 and 2.93 g $NaHCO_3$ in 1 litre of distilled water. Blocking was set for 60 min at 37 °C with freshly prepared 2% (w/v) skimmed milk in phosphate-buffered saline (PBS) pH 7.4 (8.0g NaCl, 1.1g KH_2PO_4, 0.2g KCl) containing 0.05% v/v Tween 20 in 1 litre of distilled water. Samples were tested in duplicate wells along with 4 negative (healthy) and 2 positive (pure *P. locustae* pure culture, stored at –20 °C) controls. *Paranosema locustae* bound antibodies were detected with alkaline phosphate conjugate of goat anti-rabbit diluted 1/30000 in conjugate buffer (1/2 PBS, 0.05% Tween 20, 0.02% egg albumin and 0.2% PVP). The ρ-nitrophenylphosphate substrate (1mg/ml 10% diethanolamine, pH 9.8) was incubated overnight at 37 °C before plates were read at 405 nm wavelength (A_{405}) with Bio-RAD model 2550 EIA reader. Each plate was blanked with the substrate buffer pH 9.8 (97 ml Diethanolamine in 800ml distilled water. pH was adjusted with concentrated HCl and made up to 1000ml with distilled water). A sample was considered diseased if the A_{405} value was at least twice the mean of the healthy controls.

2.4 Results

2.4.1 Grasshopper collection

A total of 640 and 4,481 frozen grasshoppers belonging to various acridid species were collected from Senegal and Cape Verde, respectively (Table 2.1). *Oedaleus senegalensis* was, by far, the most common species on the sites, representing 82% and 89% of the populations recorded from Senegal and Cape Verde, respectively.

Table 2.1. Acridid species sampled in Senegal and Cape Verde 2003 surveys.

Acridid species	Senegal		Cape Verde Island	
	Number	%	Number	%
Oedaleus senegalensis Krauss	525	82.0	4002	89.3
O. nigeriensis Uvarov	30	4.7	-	-
Trilophidia conturbata Walker	7	1.1	-	_
Humbe tenuicornis Schaum	5	0.8	-	-
Duronia chloronata Stål	10	1.6	27	0.7
Acrotylus longipes Charpentier	8	1.3	362	8.1
Pyrgomorpha cognata Krauss	17	2.7	-	-
P. vignaudii Guérin-Méneville	15	2.3	-	-
Diabolocatantops axillaris Thunberg	2	0.31	-	-
Acrida bicolor Thunberg	12	1.9	14	0.3
Chrotogonus senegalensis Krauss	5	0.8	-	-
Schistocerca gregaria Forskål	4	0.6	-	-
Anacridium melanorhodon Walker	-	-	76	1.7

The age structure of grasshoppers at the time of collection in Senegal was dominated by adults (e.g., up to 87% of *O. senegalensis* collected were adults) (Table 2.2). In Cape Verde however, grasshoppers collected were mainly young (e.g., up to 96% of *O. senegalensis* being between first and fourth instar nymphs (Table 2.3).

Table 2.2. Age structure of the common grasshopper species collected in Senegal.

Species[a]	Instars (%)					
	first	second	third	fourth	fifth	Adult
Oedaleus senegalensis	2.1	0.8	3.1	2.9	2.5	88.6
O. nigeriensis	-	-	26.7	10.0	13.3	50.0
Acrida bicolor	16.7	33.3	-	-	8.3	41.7
Pyrgomorpha vignaudii	-	6.7	-	13.3	33.3	46.7
P. cognata	-	-	11.7	17.8	11.7	58.8

[a]The percentage in each age class is calculated for each of the common species; [b]The prevalence of each species, based on a total sample of 640 individuals.

Table 2.3. Age structure of the three common grasshopper species collected in Cape-Verde.

Species[a]	Instars (%)					
	first	second	third	fourth	fifth	Adult
Oedaleus senegalensis	3.0	2.6	60.0	30.9	2.1	1.4
Acrotylus blondeli	3.3	7.7	34.5	43.4	6.4	4.7
Anacridium melanorhodon	-	-	6.6	22. 4	29.0	42.0

[a]The percentage in each age class is calculated for each of the common species; [b]The prevalence of each species, based on a total sample of 4481 individuals.

Grasshopper densities at the time of the survey are listed in Table 2.4. In Cape Verde the survey was conducted when rainfall densities were very high, ranging from 23 to 55 grasshoppers/m^2. In Senegal however the grasshopper density was low and never reached 3 individuals/m^2 (Table 2.4).

Table 2.4. Locust and grasshopper densities in localities surveyed in 2003.

Localities surveyed	Mean densities (individuals/m^2)
Cape Verde Island	
Achada Barnelo	46.7
Calabaceira	23.3
Sao Martinho	47.8
Tarrafal	4.9
Vale Da Custa	55.1
Senegal	
Lampsar	1.5
Mpal	2.2

2.4.2 Pathogens detected by microscopic observation

Examination of grasshoppers collected in Senegal and Cape Verde showed that 0.6% (four insects) of the 640 locusts and grasshoppers collected in Senegal and 0.02% (one insect) of the 4481 insects collected in Cape Verde harboured Microsporidian spores. Microsporidian spores were detected in the two localities surveyed in Senegal and in one locality (Calabaceira) in Cape Verde. In addition to Microsporidian spores, fungal infections were recorded in 2.40% (107 insects) and 6.56% (42 insects) of the grasshoppers collected from Cape Verde and from Senegal respectively, and bacterial infections were recorded in 0.5% (three insects) of the Senegalese collection. Apart from traces of mixed fungal infections like *Penicillium* spp. and *Fusarium* spp., the fungus recorded in this survey was mainly *M. anisopliae* 75% (80 insects) and 64% (27 insects) in Cape Verde and in Senegal, respectively. Fungal infections were recorded from grasshoppers collected at the Lampsar site on 16th August after the slight rainfall (1.5 mm) of 15th August 2003.

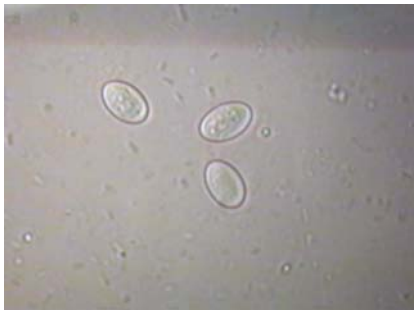
2.4.3 Occurrence of *P. locustae* in collected grasshoppers

Presence of *P. locustae* spores was noted in one *A. melanorhodon* nymph (third instar) from Calabaceira (0.02% of Cape Verde collection) and three *O. senegalensis* (0.5% of the Senegalese collection): one adult and two nymphs (third and fifth instar nymphs) in Senegal (Table 2.5). The three *P. locustae*-infected grasshoppers recorded in Senegal were collected at the site of Lampsar (fifth instar of *O. senegalensis*) and from Mpal (adult and third instar of *O. senegalensis*). In addition to the presence of *P. locustae* spores that were detected by the ACP-ELISA test, some unknown protozoan spores were found in an adult *S. gregaria* from Lampsar (Table 2.5 and Figure 2.1A).

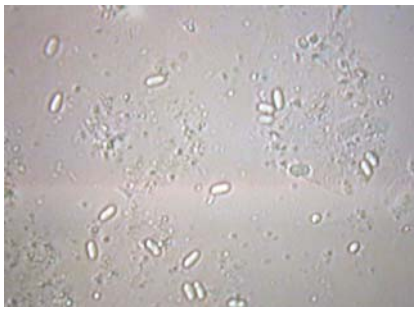
Table 2.5. Detection of *P. locustae* in grasshoppers collected from Senegal and Cape Verde.

Samples tested	A_{405}[a]	Infection status[b]
Negative control	0.149	-
Positive control	3.222	+++
Senegal	0.320	+
Senegal	0.335	+
Senegal	0.361	+
Cape Verde	0.390	+
Senegal[c]	0.142	-

[a]Wavelength for detection of *P. locustae*; [b]+ = *Paranosema locustae* detected in the extract; - = *Paranosema locustae* not detected in the extract; [c]Unknown protozoa isolated from *Schistocerca gregaria* in Senegal.



A



B

Figure 2.1. *Paranosema locustae* spores isolated from *Oedaleus senegalensis* (A) and unknown protozoan spores isolated from *Schistocerca gregaria* in Senegal.

2.5 Discussion

The results from the present study revealed low number of insects harbouring entomopathogenic fungus and *P. locustae* spores. The grasshopper surveys were conducted during raining season particularly in Cape Verde that might favour the increase of fungal infection through increasing relative humidity. At the time of collection in Lampsar, grasshopper (mainly adult hoppers) densities ranged between 0 and 2 per square meter. However, an increase in adult grasshopper densities up to 5 grasshoppers per square meter was observed from 15 to 16th August after the rainfall recorded in the morning of 15th August. Indeed, almost 85% of the adult grasshoppers from Lampsar were collected within the 2 days. It is therefore possible that most adult grasshoppers, collected from the survey sites in Lampsar were not from the area but rather have come from some other areas as a consequence of the rain. The restriction of the surveys to the pathogen introduction areas in Cape Verde, the low impact of grasshoppers over the pathogen introduction area in Senegal and the very long time-lag between introduction of the pathogen and the survey period (21 and 13 years in Senegal and Cape Verde, respectively), may be the principal reasons for the low prevalence of *P. locustae* recorded during the surveys. Moreover, the continuous use of synthetic chemicals for control of grasshoppers in Sahel could have greatly contributed to the display of many natural enemies, including *P. locustae*. The fact that most of the few grasshoppers that were infected by *P. locustae* were young nymphal instars could be an indication of vertical transmission from infected parents. However there is a possibility that *P. locustae* infections recorded from this survey being attributed to horizontal transfer or natural occurrence at the time of the survey. Spores of *P. locustae* are released into the environment when the infected insect dies and the body disintegrates, allowing the spores to escape from the infected tissue. Infective spores may overwinter in the soil, in insect feces, in egg pod of the hosts or in dispersing hosts that can be important sources of inoculums for inducing diseases. In a three-year study, Germida (1984) and Germida et al. (1987) reported that large numbers of *P. locustae* spores could still be detected in soil, depending on the extent of infection in the resident grasshopper populations.

The ability of the pathogen to persist in a given environment depends on the efficacy with which it is transmitted from one individual to another (Anderson and May, 1981). Among factors that determine transmission and dissemination of *Paranosema* spp., environmental factors such as solar radiation, ambient temperature, and relative humidity are of great importance as they influence the infectivity of the pathogen (Frixione et al., 1992; Maddox and Solter, 1996; Maddox, 1977).

The present study is the first of the kind conducted after the release of *P. locustae* in Africa from 1982 to 1990 to evaluate its persistence in the released localities. The results reveal that *P. locustae* could still be reisolated from the African former released countries. However, further studies are still needed to evaluate the real impact of *P. locustae* in grasshopper communities in Africa. In that respect and due to the continuous annual movement of grasshoppers, we recommend that surveys for *Paranosema* species associated with the locust and grasshopper be continued. In addition, efforts should be made to recover African isolates of *P. locustae* from field populations of locust and grasshopper by undertaking large-scale surveys covering different grasshopper biotopes in Africa. This could help to gather data on prevalence, host range, and geographical distribution of *P. locustae* for management of grasshoppers and others pests in Africa.

3 Susceptibility of immature stages of *Schistocerca gregaria* and *Oedaleus senegalensis* (Orthoptera: Acrididae) to *Paranosema locustae* (Microsporidia: Nosematidae) and its vertical transmission in the infected Adults[a]

3.1 Abstract

The present study reports the relative susceptibility of immature stages of the desert locust, *Schistocerca gregaria* Forskål and the Senegalese grasshopper, *Oedaleus senegalensis* Krauss to *Paranosema locustae* Canning. Both development and mortality of infected nymphs were affected by *P. locustae*. Median Survival times were significantly lower for younger instar nymphs than for older instar nymphs. Infection developed in all nymphs inoculated at first, second and third instars whereas 16 to 27% of nymphs inoculated at fourth and fifth instars were uninfected at the end of the experiment (30 days post-inoculation). While 85 to 91%, 6 to 30% and 55 to 74% of control nymphs, nymphs treated at fourth and fifth instars, respectively, reached adulthood, none of the inoculated first, second and third instars developed to adult stage. *Paranosema locustae* infection in *S. gregaria* and *O. senegalensis* had no effect on the average number of eggs per mated pair. However, depending on the infection status of the parents, 20 to 60% and 17 to 52% of *S. gregaria* and *O. senegalensis* progenies, respectively, had detectable levels of *P. locustae* spores. *Paranosema locustae* spores were detected in fecal pellets, eggs and froth plug from inoculated insects at concentrations ranging from light (feces) to medium (froth plug and eggs), but might not affect the disease prevalence in the progeny.

[a] This chapter has been partly submitted to BioControl as Agbeko Kodjo TOUNOU, Christiann KOOYMAN, Orou Kobi DOURO-KPINDOU and Hans Michael POEHLING (2007): Susceptibility of immature stages of *Schistocerca gregaria* and *Oedaleus senegalensis* to *Paranosema locustae* and its vertical transmission in infected adults

3.2 Introduction

The desert locust, *Schistocerca gregaria* Forskål and the Senegalese grasshopper, *Oedaleus senegalensis* Krauss (Orthoptera: Acrididae), are the most damaging locust and grasshopper species of Sahelian cereal crops (Geddes, 1980; COPR, 1982; Popov, 1988; Krall, 1994; Kogo and Krall, 1997). Efforts to reduce crop losses during outbreaks often involve extensive use of synthetic pesticides. In some Sahelian countries, plant protection agencies are still spraying large areas with pesticides against grasshoppers. For instance, between 1997 and 1999, the "Direction de Protection des Végétaux" of Niger treated 587,440 ha out of 1,455769 ha infested with grasshoppers (DPV, 1998-2000). During the recent major desert locust outbreak in 2004, a total of 12 million ha have been sprayed with synthetic pesticides, exceeding US$60 million and harvest losses were valued up to US$2.5 billion, with disastrous effects on the food security situation in West Africa (FAO, 2005).

Concerns over the environmental and human health impact of chemical control of locusts and grasshoppers have led to considerable interest in the development of alternative control methods using mycopesticides (Bateman et al., 1997; Prior and Streett, 1997; Lomer et al., 2001). *Paranosema locustae* was selected in the early 1980's for long-term suppression of grasshoppers. Lange (2005) revised the host and geographical range of *P. locustae* and reported 121 species of Orthoptera, including *S. gregaria* and *O. senegalensis*, from North and South America, Africa, Australia, China and India to be susceptible to *P. locustae*. However, no detailed data are available on the susceptibility of *S. gregaria* and *O. senegalensis* to *P. locustae*. A recent investigation reported second and third instar nymphs of *Melanoplus sanguinipes* Fab. (Orthoptera: Acrididae) to be more susceptible to *Paranosema cuneatum* Henry than fifth instar nymphs (Erlandson et al., 1986). However, to the best of our knowledge, there has been no systematical study on intergenerational transmission of *P. locustae* in both species. The main objectives of our study were, thus, to evaluate the susceptibility of different developmental stages of *S. gregaria*

and *O. senegalensis* to *P. locustae* and to evaluate the potential for *P. locustae* to be transmitted from infected adults to the offspring.

3.3 Material and methods

3.3.1 Study organisms

Insects. *Schistocerca gregaria* and *O. senegalensis* were obtained from the mass rearing unit of the "Centre Regional AGRHYMET" station in Niamey. Insects were reared from non-diapause egg. In order to provide adequate numbers of nymphs of the same age for the bioassays, the insects were continuously reared in synchrony from egg at 32 °C (temperature in cages), 85-90% RH and 14:10 (L:D) photoperiod (Diop, 1995). The ages of nymphs used in the study were as follows: first instar (<24 hours-old), second instar (4 day-old), third instar (11 day-old), fourth instar (16 day-old) and fifth instar (21 day-old). The five developmental stages of *S. gregaria* were tested and due to the limited number of *O. senegalensis*, only first, third and fifth instar nymphs were used for the bioassays.

Pathogen. *Paranosema locustae* spores (originally isolated from *Melanoplus differentialis* Thomas (Orthoptera: Acrididae) from Dr. John Henry's laboratory at Montana State University in Bozeman) were provided by Dr. Lee Anne Merrill (M and R Durango, Inc. P. O. Box 886 Bayfield, CO 81122 USA) in water suspension. Spores were stored frozen at –20 °C in a freezer for one week before use in bioassays. Experiments were replicated three times over a period of three weeks with 30 insects per replicate. For each replicate, fresh suspensions were prepared. Following the "Lubilosa" guidelines, spore concentrations were quantified using a Thomas counting chamber and serial dilutions were made with distilled water, to determine the appropriate concentration for the bioassay (Bateman et al., 1997).

3.3.2 Susceptibility of immature stages of *S. gregaria* and *O. senegalensis* to *P. locustae*

Nymphs were transferred to new portable wooden cages measuring 30 cm x 30 cm x 30 cm with metal wire mesh (2 cm dia.) sides where they were starved for 24

hours before use in bioassays. Groups of 30 starved nymphs were inoculated with 3.16 x 10^4, 5.62 x 10^6 and 1.0 x 10^7 spores per os infused in 10g wheat bran which they were fed on. The nymphs were allowed to feed for three days and only the groups of nymphs that consumed the entire diet were used in the experiment. Nymphs fed with untreated wheat bran were used as control. Following inoculation, insects were reared in a group (Price et al., 1999) in the wooden cages. A canvas sleeve was fitted on one side of the cages in order to handle the insects and provide them with fresh millet leaves. Host mortality was assessed daily over 30 days and dead insects were continuously removed from the cages to avoid scavenging, and cadavers were transferred to labelled vials and dried for 48 hours. Any morphological abnormal development in inoculated insects was noted.

3.3.3 Effect of *P. locustae* on host fecundity and its vertical transmission in F1 generation

Nymphs inoculated in the previous section as fifth instar and that developed to adults were used in the vertical transmission experiment. Adult insects were paired randomly and kept in rearing cage (25 cm x 30 cm x 25 cm) with metal wire mesh (2 cm dia.) for three weeks. The cage bottom was designed to hold four oviposition trays, measuring 10 cm high and 3 cm wide. Each tray was filled with 10% humid sterilized sand. Egg pods were recorded weekly over the three weeks observation period and incubated at 32 ± 1 °C and 70% RH. Newly hatched nymphs, both dead and alive, from individual egg pods were recorded daily. The living nymphs were transferred to another cage for a further eight days before examination for infection. Each pair was examined for the infectivity of individuals at the end of the experiment and assigned to the following combinations (H ♀ x H ♂; H ♀ x I ♂; I ♀ x H ♂; I ♀ x I ♂, with ♂ (male) and ♀ (female), H (Healthy) and I (Infected)).

3.3.4 Effect of *P. locustae* on the growth of *O. senegalensis* and *S. gregaria*

Insects were inoculated with *P. locustae* at 0 (control), 3.16 x 10^4 and 3.16 x 10^5 spores as described above. Inoculated nymphs were caged as described above. Insects that survived the host growth experimental period were individually weighed.

In order to avoid bias in the fresh mass measure, nymphs of the same age (<24 hours-old) were used in the host growth experiment.

3.3.5 Examination for *P. locustae* infection

In all experiments, the pathogen infection, the cause of death as well as the degree of infection in fecal pellets (collected from the rearing cages during the last two weeks of the mating experiment) and egg pods (both eggs and froth plug that surrounds the eggs) were confirmed by microscopic observations. Nymphs, both inoculated and hatched from vertical transmission experiments, were individually examined. Representative samples of fecal pellets and egg pods were also examined. For detailed examination, samples were homogenized in 10 ml of distilled water. Four to six microscopic preparations from each homogenized sample were examined as hanging drops using a phase contrast microscope (400x). *Paranosema locustae* spores, recognized by their distinctive shape and size were counted, and the degree of infection in fecal pellets and egg pods was classified, using a standard rating scale of 0-5. The spore concentrations were estimated as none (0 spore/microscopic field), trace (0-1 spore/microscopic field), light (1-5 spores/field), medium (5-100 spores/field) and heavy (more than 100 spores/field) (Johnson and Dolinski, 1997).

3.3.6 Analysis of data

Data were arcsine or log transformed where necessary before analyses. Median survival times (MST) were calculated using Kaplan Meier Survivorship analysis (SPSS, 1999). The efficacy of the pathogen was compared using the final mortalities (i.e., 31 days cumulative mortalities). All statistical analyses were performed using the general linear model (GLM). Means were compared using Tukey range test and the probability level was set at $p = 0.05$.

3.4 Results

3.4.1 Susceptibility of immature stages of *S. gregaria* and *O. senegalensis* to *P. locustae*

Infection developed in all nymphs inoculated as first, second and third instars. In *O. senegalensis*, 30 to 74.2% of nymphs inoculated at fifth instar reached adulthood with an average infection of 57.9%. Similarly 6.3 to 55.3% and 33.7 to 82.5% of *S. gregaria* nymphs inoculated at fourth and fifth instar reached the adult stage with an average infection of 74.8% and 48.6% respectively. Insects infected at first, second and third nymphal stages died as fourth instar or remained as moribund fifth instar at the end of the experiment. Infected nymphs developed morphological abnormalities such as reduced size (Figure 3.1), abnormal wings and/or hind legs development. Wings were deformed, reduced or appeared scorched and curled at the edges (Figure 3.2).

Survival of both species was affected by *P. locustae* infection. The overall Median survival time (MST) values are summarized in Table 3.1. For each developmental stage, MSTs significantly decreased with increasing pathogen concentrations and remained shorter in younger nymphs compared to the older nymphs ($p< 0.0001$). In both species fifth instar nymphs survived the longest after *P. locustae* infection, resulting in MSTs exceeding the 30 days of the bioassay as obtained with the lowest concentration tested (Table 3.1). Results on nymphal mortality are presented in Table 3.2. Mortality was significantly affected by both host developmental stage ($F = 191.52$; $df = 4, 60$; $p < 0.0001$ and $F = 80.22$; $df = 2, 24$; $p < 0.0001$) and pathogen concentrations ($F = 325.78$; $df = 3, 60$; $p < 0.0001$ and $F = 150.04$; $df = 3, 24$; $p < 0.0001$) for *S. gregaria* and *O. senegalensis*, respectively.

Figure 3.1. Reduced size in *Schistocerca gregaria*, 21 days after being infected with *Paranosema locustae* as first instar; uninfected (bottom) and infected (top).

Figure 3.2. Malformation of wings and posterior legs as sublethal effect in of *Schistocerca gregaria* nymph infected with *Paranosema locustae*.

Table 3.1. Median survival time (MST) in nymphal stages of *Oedaleus senegalensis* and *Schistocerca gregaria* inoculated with different concentrations of *Paranosema locustae*.

Nymphal Stages	Median Survival Time (days ± SE)[ab]			
	Paranosema locustae concentrations[c]			
	1.0×10^7	5.62×10^6	3.16×10^4	Control
	Oedaleus senegalensis			
1	5.0 ± 0.4 Cc	7.0 ± 0.3 Cb	11.0 ± 0.4 Ba	-[d]
3	9.0 ± 0.5 Bc	14.0 ± 0.9 Bb	18.0 ± 0.8 Aa	-[d]
5	15.0 ± 1.3 Ab	18.0 ± 1.7 Aa	-[d]	-[d]
	Schistocerca gregaria			
1	6.0 ± 0.3 Cc	9.0 ± 0.7 Cb	14.0 ± 0.8 Ca	-[d]
2	9.0 ± 0.7 Bc	14.0 ± 0.7 Bb	16.0 ± 2.2 Ca	-[d]
3	10.0 ± 0.6 Bc	14.0 ± 0.5 Bb	24.0 ± 1.2 Ba	-[d]
4	14.0 ± 1.5 Ac	16.0 ± 1.1 Ab	26.0 ± 1.5 Aa	-[d]
5	15.0 ± 1.1 Ab	17.0 ± 2.3 Aa	-[d]	-[d]

[a]MST values were calculated using Kaplan Meier Survivorship analysis; [b]Means in the same columns followed by the same upper case letters and in the same lines followed by the same lower case letter are not significantly different (p = 0.05; Tukey-test); [c]Spores were provided to a group of thirty nymphs; [d]MST exceeded the observation period.

Table 3.2. Thirty days cumulative mortality of nymphal stages of *Oedaleus senegalensis* and *Schistocerca gregaria* inoculated with *Paranosema locustae* in caged bioassay.

	Mortality (% ± SE)[a]			
Nymphal	*Oedaleus senegalensis*			
stages	1.0×10^7	5.62×10^6	3.16×10^4	Control
1	100.0 ± 0.0 Aa	98.4 ± 1.1 Aa	76.8 ± 3.7 Ab	11.6 ± 1.1 Ac
3	88.5 ± 3.1 Ba	71.1 ± 2.6 Bb	57.9 ± 2.1 Bc	12.4 ± 2.4 Ad
5	70.0 ± 1.9 Ca	52.8 ± 2.9 Cb	25.8 ± 2.2 Cc	13.5 ± 1.9 Ad
	Schistocerca gregaria			
1	100.0 ± 0.0 Aa	100.0 ± 0.0 Aa	62.5 ± 2.1 Ab	8.9 ± 1.1 Ace
2	100.0 ± 0.0 Aa	91.1 ± 4.8 Ba	57.5 ± 2.2 Ab	11.1 ± 2.9 Ac
3	94.6 ± 4.0 Ba	76.9 ± 3.1 Cb	55.8 ± 2.9 Bc	14.6 ± 2.2 Ad
4	93.7 ± 1.9 Ba	77.5 ± 1.1 Cb	44.7 ± 4.0 Cc	13.1 ± 1.2 Ad
5	66.3 ± 3.8 Ca	53.5 ± 3.8 Db	17.5 ± 1.1 Dc	13.3 ±1.9 Ac

[a]For each species means in the same columns followed by the same upper case letters and in the same lines followed by the same lower case letter are not significantly different (p = 0.05; Tukey-test).

3.4.2 Effect of *P. locustae* on the host growth

Infected insects showed slower development compared to non-infected insects. Marked effects of *P. locustae* on the host fresh mass was noted (Figures 3.3 and 3.4). Host body mass in infected nymphs was lowered 6 to 7-fold in *O. senegalensis* and 7 to 8-fold in *S. gregaria*. Regardless of the host species, no differences were observed between the two concentrations tested (p> 0.05). However, nymphs treated

with *P. locustae* showed significantly lower fresh mass compared to untreated nymphs (F = 192.66; df = 2, 6; p< 0.0001 and; F = 105.15; df = 2, 6 p< 0.0001) for *S. gregaria* and *O. senegalensis*, respectively.

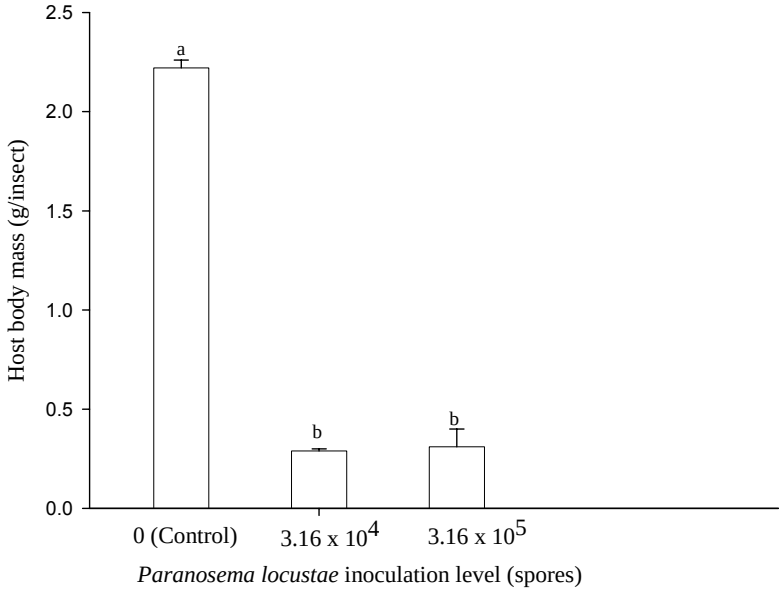


Figure 3.3. Host fresh mass of *Schistocerca gregaria*, 21 days after being infected with *Paranosema locustae* at first instar nymphs. Spores were provided to a group of thirty nymphs. Different lower case letters indicate significantly different values (*p*< 0.05).

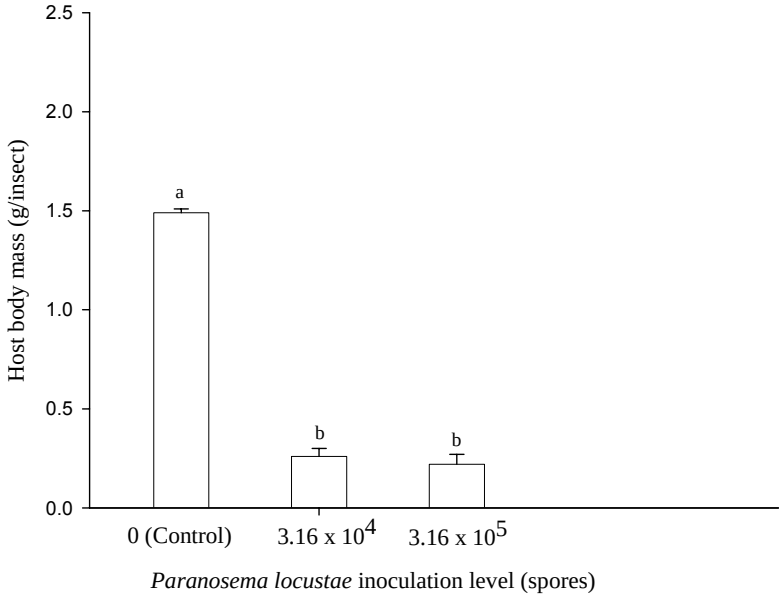


Figure 3.4. Host fresh mass of *Oedaleus senegalensis*, 21 days after being infected with *Paranosema locustae* at first instar nymphs. Spores were provided to a group of thirty nymphs. Different lower case letters indicate significantly different values ($p < 0.05$).

3.4.3 Effects of *P. locustae* infection on host fecundity and its vertical transmission

The average fecundity and percentage of infection among progeny are presented in Table 3.3. Infection status of the parents had no effect on fecundity of either *S. gregaria* (F = 4.38; df = 3,129; p = 0.09) or *O. senegalensis* (F = 6.07; df = 3, 69; p = 0.29). The results show evidence of *P. locustae* transmission to the progeny when either one or both parents are infected (Table 3). No differences in disease prevalence were observed between (I ♀ x I ♂) and (I ♀ x H ♂) mated-pairs. However, progeny infection was higher in (I ♀ x I ♂) and (I ♀ x H ♂) than in (H ♀ x I ♂). *Paranosema locustae* infection was particularly high in nymphs that died at hatching; e.g., 83.5 ± 2.1%, 78.9 ± 1.9% and 72.6 ± 1.7% (*O. senegalensis*); 75.8 ± 1.8% 70.3 ± 0.5% and 69.8 ± 0.9% (*S. gregaria*), from (H ♀ x I ♂), (I ♀ x I ♂), (I ♀ x H ♂) and (H ♀ x I ♂) mated-pair, respectively.

Results from the examination of feces, eggs and froth plug for presence of *P. locustae* spores are presented in Table 3.4. No spores were observed in any of the examined samples from uninfected insects. However samples from inoculated insects showed evidence of *P. locustae* spores at concentrations ranging from ranging from light (feces) to medium (froth plug and eggs).

Table 3.3. Vertical transmission and effect on fecundity of *Paranosema locustae* in *Oedaleus senegalensis* and *Schistocerca gregaria*.

	Oedaleus senegalensis[e]					*Schistocerca gregaria*[e]				
Infection status[a]	No. pairs	Avg fecundity[b]	No. nymphs examined alive	No. nymphs examined dead[c]	Prevalence of infection[d]	No. pairs	Avg fecundity[b]	No. nymphs examined alive	No. nymphs examined dead[c]	Prevalence of infection[d]
H ♀ x H ♂	30	26.6 ± 4.3 a	583	17	0.2 ± 0.2 c	45	31.3 ± 5.6 a	871	29	0.0 ± 0.0 c
H ♀ x I ♂	15	21.1 ± 3.4 a	210	90	17.7 ± 4.9 b	22	28.2 ± 4.9 a	352	88	20.3 ± 6.4 b
I ♀ x H ♂	14	20.8 ± 6.3 a	202	78	48.5 ± 2.8 a	31	26.9 ± 3.9 a	537	117	50.4 ± 3.4 a
I ♀ x I ♂	14	23.6 ± 5.6 a	182	98	51.8 ± 2.8 a	34	26.2 ± 4.2a	488	132	58.9 ± 3.2 a

[a]H , Healthy adult; I infected adults; [b]Number of eggs per pair; [c]Dead nymphs recorded at hatching; [d]Percentage of nymphs infected eight days after hatch was determined with phase microscopy; [e]Means within a column followed by the same letter are not significantly different (p = 0.05; Tukey-test).

Table 3.4. Presence of *Paranosema locustae* spores in eggs, egg pod and feces of infected *Oedaleus senegalensis* and *Schistocerca gregaria*.

Infection Status[b]	*Oedaleus senegalensis*[a]			*Schistocerca gregaria*[a]		
	Eggs	Froth plug	Feces	Eggs	Froth plug	Feces
H ♀ x I ♂	10 - 15	9 - 11	1 - 3	9 - 11	8 -13	1 - 3
I ♀ x H ♂	12 - 21	15 -17	2 - 3	14 -18	20 - 24	1 - 3
I ♀ x I ♂	16 - 20	13 -19	2 - 4	11 - 20	12 - 26	2 - 5
H ♀ x H ♂	-	-	-	-	-	-

[a]Number of spores/microscopic field; [b]H , Healthy adult; I, infected adults

3.5 Discussion

Host developmental stage at inoculation affected both survival time and mortality rates. All nymphs inoculated at first, second and third instars showed evidence of infection whereas between 15 and 27% of the fourth and fifth instars were uninfected at the end of the observation period. The difference in susceptibility is not well understood. Nevertheless, based on the prior investigations that reported locusts and grasshoppers to cease feeding after *P. locustae* application (Johnson and Pavlicova, 1986), we can speculate that the lower sensitivity to *P. locustae* in older instar nymphs compared to younger nymphs may, in part, be explained by their ability to starve over longer periods. This may occur because the older nymphs have greater energy reserves and/or may also be more tolerant to infection. Our results corroborate findings from previous studies that reported younger nymphs to be more susceptible to microsporidian infection and to succumb more easily than older nymphs even at low level of infection (Henry, 1973; Tanada, 1976; Erlandson et al., 1986; Lockwood and DeBrey, 1990; Johnson, 1997; Lockwood et al., 1999; Lange et al., 2000).

The greatest effects of *P. locustae* are of a sublethal nature although direct host mortality is commonly seen to be the first objective in pest control. These include

reduced food consumption (Oma and Hewitt, 1884; Johnson and Pavlicova, 1986), delayed development (Henry, 1969), decreased activity and migratory capability (Canning, 1962; Bomar et al., 1993) and reduced fecundity (Ewen and Mukerji, 1980) as well as alterations in development (Henry and Oma, 1981; Lange et al., 2000). Sublethal effects observed in this study might be directly related to host-pathogen interactions. *Paranosema locustae* primary infects host fat body, which performs a dynamic role in both intermediate metabolism and energy reserve, thus, compromising energy reserve for growth of the host. Infected insects therefore exhibit morphological deformities (Henry, 1969; Henry and Oma, 1981; Lange et al., 2000). Moreover, uninfected nymphs would develop faster and weigh more than infected nymphs. Delayed development and reduced host fresh mass in insect infected by *P. locustae* had been reported by Schaalje et al. (1992) and associated with reduction in food intake following infection (Oma and Hewitt, 1984 Johnson and Pavlikova, 1986).

Our results show that *P. locustae* infection has no effect on fecundity of inoculated late stages of *S. gregaria* and *O. senegalensis*. Similar observations have been made by Streett et al. (1993) and Erlandson et al. (1986) who reported *Paranosema* spp. infection in fifth instar grasshoppers *Chorthippus cutipennis* Harris and *Melanoplus sanguinipes* (Fab.) (Orthoptera: Acrididae) to have no effect on the host fecundity. This contradicts the results of Henry (1972), Ewen and Mukerji (1980) who reported in field studies *P. locustae* infection to reduce fecundity in *M. bivittatus* and *M. sanguinipes*. However, these authors did not deal specifically with fifth instar nymphs.

Transmission of microsporidia between hosts occurs when spores are ingested. However, vertical transmission from parent to progeny via eggs is known to be an important route for many of the insect-infecting microsporidia (Canning, 1982; Raina et al., 1995). Most reports on the vertical transmission of *P. locustae* have associated the pathogen transmission from parents to the offspring to be maternal-mediated (Ewen and Mukerji, 1980; Wilson, 1982; Canning et al., 1985; Han and Watanabe, 1988; Solter et al., 1990; MacVean and Capinera, 1991; Streett et al., 1993; Raina et al., 1995). Streett et al. (1993) reported maternally mediated transmission of

Paranosema sp. in grasshopper *C. curtipennis* with more than 70% of the progeny being infected within 5 days after hatching. Raina et al. (1995) described the loci of *P. locustae* to include the ovaries and developing embryo, and confirmed the transmission of the pathogen to occur up to the F14 generation in *L. migratoria migratorioides*. They reported the occurrence of vegetative stages of *P. locustae* in the germ band and spores in the yolk and concluded that some stages of *P. locustae* might directly have invaded the embryonic tissues and many other stages by using lipoproteins of the yolk, developed into spores and remained suspended in the yolk. Therefore, progeny infection recorded in this study might be a result of embryo being infected inside the eggs, given the medium spore concentrations recorded in the eggs and the high disease prevalence in nymphs that died upon hatching.

Twenty percent of offspring from paired set-ups where only males were infected showed evidence of infections, suggesting the possibility of paternally mediated vertical transmission in this experiment. Paternal-mediated vertical transmission of microsporidia-infected insects occurred less frequently than maternal-mediated transmission (Thomson, 1958; Kellen and Lindegren, 1971). Brooks (1968) reported stages of *Paranosema heliothidis* Lutz and Splendore in the spermatogonia and spermatozoa of *Heliothis zea* Boddie (Lepidoptera: Noctuidae) and concluded on the possibility of the paternal-mediated vertical transmission in this pest. In Indian-meal moth, *Plodia interpunctella* Hübner (Lepidoptera: Pyralidae), Kellen and Lindegren (1971) reported evidence of paternal transmission of *Vairimorpha plodiae* Kellen and Lindegren (Microsporidia: Burenellidae).

Although we cannot discount the possibility of horizontal transmission of *P. locustae* in progeny upon hatching, such transmission might not greatly affect the outcome of this study given the high level of infections recorded in nymphs that died at hatching. It had been determined that spores from vertical transmission were detected in first instar nymphs between 3 and 5 days after hatch, but spores were not detected in inoculated nymphs until 7 to 9 days (D.A.S., unpublished data). Henry (1972) reported that *P. locustae* required at least 13 days for the initial generation to develop in the laboratory at 35 °C, and Raina et al. (1995) confirmed infection to occur within 13 to 15 days in the haemocyte and fat-body tissue of inoculated

insects. It is therefore unlikely that cross contamination of progeny affected prevalence of infection in our experiment. Any possible horizontal infection that may occur during the eight caging days through ingestion of food contaminated by feces from infected nymphs could not be detected in the current experiment.

Results from this study suggest that *P. locusta*e could play an important role as long-term biocontrol agent of *S. gregaria* and *O. senegalensis* in Sahel. Further studies are however needed to investigate different strategies of for reducing the time to death when using *P. locustae* as biological control agent of acridid pests. One possibility in such directions could be the combination of *P. locustae* with other pathogens such as *Metarhizium anisopliae* var. *acridum,* the most common pathogen used as Green Muscle® for grasshopper control in Africa.

4 Interaction between *Paranosema locustae* (Microsporidia: Nosematidae) and Green Muscle®, *Metarhizium anisopliae* var. *acridum* (Deuteromycotina: Hyphomycetes) infecting the desert locust *Schistocerca gregaria* (Orthoptera: Acrididae)[b]

4.1 Abstract

We studied the interaction between two pathogens namely; *Paranosema locustae* Canning and *Metarhizium anisopliae* var. *acridum* Driver and Milner on the desert locust, *Schistocerca gregaria* Forskål. Fifth instar nymphs reared in the laboratory were inoculated either separately or sequentially at various concentrations of the two pathogens. Host mortality was assessed at daily intervals and pathogen spore production evaluated. Combination of *P. locustae* and *M. anisopliae* resulted in additive or more than additive locust mortality indicating synergism. No antagonistic effects on host mortality were observed between the two pathogens. Survival time decreased for nymphs infected with both pathogens compared to nymphs infected with the pathogens independently. *Paranosema locustae* spore production was significantly reduced by additional *M. anisopliae* infection. For example, nymphs with 3 days and 10 days existing *P. locustae* infection, infected with *M. anisopliae,* produced 3 to 20-fold and 2.5 to 8-fold fewer spores than nymphs infected with *P. locustae* alone, respectively. Reduction of time to death and *P. locustae* spore production in mixed infection suggest that *M. anisopliae* will negatively affect the survival and persistence of *P. locustae* as a regulating factor of locust populations.

[b] This chapter was partly submitted to Journal of Invertebrate Pathology as Agbeko Kodjo TOUNOU, Christiann KOOYMAN, Orou Kobi DOURO-KPINDOU and Hans Michael POEHLING (2007): Interaction between *Paranosema locustae* and *Metarhizium anisopliae* var. *acridum,* two pathogens of the desert locust, *Schistocerca gregaria.*

4.2 Introduction

The desert locust, *Schistocerca gregaria* Forskål (Orthoptera: Acrididae) is a serious pest that causes extensive damage to food crops in Africa and western Asia (Steedman, 1988; Showler, 1993) particularly during outbreaks. The prevailing method for locust control involves the application of synthetic insecticide. Nevertheless, the development of pesticide tolerance and concerns about environmental contamination and safety has increased the increase in alternative strategies using biopesticides based on fungal and microsporidian spores (Johnson et al., 1992; Johnson and Goettel, 1993; Inglis et al., 1995; Lomer et al., 1999; Mulock and Chandler, 2000; Haraprasad et al., 2001; Lange, 2005). The Lubilosa programme has developed an oil formulation containing the fungal pathogen *Metarhizium anisopliae* var. *acridum* Driver and Milner (Deuteromycotina: Hyphomycetes) for control of locusts and grasshoppers (Bateman et al., 1997; Johnson, 1997; Lomer et al., 1999; Lomer et al., 2001). The process of mycopathogenesis begins with attachment of the conidia to the cuticle of the insect, followed by germination and penetration of the cuticle by the germ tube (Charnley, 1984). Consequently, death of the insect results from toxaemia or nutrient depletion.

Paranosema locustae Canning (Microsporidia: Nosematidae) is known to be infective to more than 100 species of Orthopteran from North and South America, Africa, Australia, China and Indian (Lange, 2005). It has been efficient against the earlier instars of acridid species and showed promising results for long-term management of grasshopper and locust populations (Bomar et al., 1993; Streett et al., 1993). Its limitations however, lie in its inefficiency against late instars (Lockwood and DeBrey, 1990; Lange et al., 2000). In the previous laboratory experiments (Chapter 3), we observed *P. locustae* to be highly virulent to earlier instars *S. gregaria* whereas late instars (e.g., fifth instar) showed more tolerance to the pathogen infection.

Insects treated with *M. anisopliae* or *P. locustae* die more slowly compared to those treated with synthetic insecticides. Using the fungal and microsporidian components in combination could help to improving their efficacy for pest control. An

enhancement in the speed and level of kill is desirable particularly for late instar nymphs. An advantage of such formulation is the recycling potential of the pathogens to new generation of acridids through vertical and/or horizontal transmission (Raina et al., 1995; Langewald et al., 1999). This recycling potential however, might be, hampered by a number of factors including interactions with other control agents (Solter et al., 2002; Pilarska et al., 2006). Recently, there has been a growing recognition in the importance of higher-level interaction between natural enemies. Most frequently, studies on host-pathogen interaction have focused on the direct interaction between one disease causing agent and one host. Under natural conditions however, evidence of mixed infections involving two or more parasites are common (Hochberg and Holt, 1990; Koppenhöfer and Kaya, 1997; Ishii et al., 2002; Solter et al., 2002; Pilarska et al., 2006). The concern then is to understand how the combined pathogens interact with each other and with the host pest. In mixed infections, complex interactions between pathogens on one hand and between pathogens and host on the other hand will arise. In such a case, the efficacy of one or both pathogens is improved or suppressed, or one may be suppressed and the other one enhanced (Cox, 2001; Solter et al., 2002; Pilarska et al., 2006), leading to dramatic changes in population dynamics. The objective of the present study was to examine the mixed infection of *P. locustae* and *M. anisopliae* in desert locust *S. gregaria* as an alternative to chemical control. To address this objective we determined, in laboratory experiments (1) the compatibility of *P. locustae* and *M. anisopliae* var. *acridum* and the kinds of interactions that occur when both agents are combined for the control of *S. gregaria*, (2) the production of *P. locustae* and *M. anisopliae* spores in both single and mixed infections.

4.3 Materials and Methods

4.3.1 Study organisms

Insects. *Schistocerca gregaria* was obtained from the laboratory culture at IITA-Benin as fifth instar nymphs (21 day-old). Insects were reared continuously from egg to adult at 31 ± 1 °C, 12:12 (L: D) photoperiod and 85-90% RH following the protocol

developed by "Lubilosa" unit at the IITA Benin station (Bateman et al., 1997). Adults were fed with cassava leaves (*Manihot esculenta* Crantz (Euphorbiales: Euphorbiaceae)).

Pathogens. The spores of *P. locustae* were those provided by Dr. Lee Anne Merrill (M and R Durango, Inc. P. O. Box 886 Bayfield, CO 81122 USA) (Chapter 3). The isolate of *M. anisopliae* var. *acridum* (strain IMI 330189, isolated from *Ornithacris cavroisi* Finot (Orthoptera: Acrididae) in Niger) was the standard isolate used in locust biocontrol in Africa (Lomer et al., 2001). Conidia were produced in vitro by the production unit at IITA-Benin, West Africa (Cherry et al., 1999). *Metarhizium anisopliae* spore viability was determined prior to the application. Samples of conidia formulated in a 50:50 mixture of ondina oil (Shell Pty, Ltd) and pure paraffin (Jet A1 grade) were streaking out onto agar plates and geminating conidia were counted. Percentage germination was > 90% after 24 hours at 25 °C.

The experiments were replicated three times over a period of three weeks with 30 nymphs per replicate. In each replicate, fresh suspensions were prepared. Following the "Lubilosa" guidelines, spore concentrations were quantified using Thomas counting chamber and serial dilutions were made using 50:50 mixture of ondina oil and pure paraffin for *M. anisopliae* and distilled water for *P. locustae,* to determine appropriate concentration for the bioassay (Bateman et al., 1997).

4.3.2 Inoculation of hosts

Fifth instar nymphs were transferred to new portable wooden cages measuring 30 cm x 30 cm x 30 cm with metal wire mesh (2 cm dia.) sides, where they were starved for 48 hours before their use in treatments. Test insects were inoculated either separately (single inoculation) or in combination (mixed inoculations). Given the relatively slow action of *P. locustae* that requires longer time to invade host tissue and reproduce (Henry, 1982), no simultaneous inoculations were tested in this bioassay.

For the single inoculations with *P. locustae*, groups of 30 starved nymphs were inoculated with 1×10^4, 1×10^5 and 1×10^6 spores per os infused in 10 g wheat bran

which they were fed on. The nymphs were allowed to feed for three days and only groups of nymphs that consumed the entire diet were considered for the experiment. Inoculations with *M. anisopliae* were done at dosages of 1 x 10^2, 1 x 10^3 and 1 x 10^4 spores/nymph following the standard technique in which individuals were inoculated with a 1-*µ*l drop of spores suspension, placed at the base of the pronotal shield (Arthurs and Thomas, 2001; Elliot et al., 2002).

The mixed inoculations reflect the biological control situation where *M. anisopliae* is introduced into locust population carring *P. locustae*. Thus, the two pathogens were examined as sequential infections with *P. locustae* spores administrated 3 days or 10 days before *M. anisopliae*. A total of nine treatments were carried out following the inoculation procedure described above with the nymphs being inoculated with the three concentrations of the two pathogens in all combinations.

Nymphs fed with untreated wheat bran and/or inoculated with 1-*µ*l drop blank ondina oil formulation were used as control. Experiments were conducted under 31 ± 1 °C temperature regimen and 85-90% RH and treated insects were fed fresh cassava leaves.

4.3.3 Data collection

Mortality was assessed daily for 21 days and dead insects were continuously removed from the cages to avoid scavenging. Cadavers were transferred individually onto filter paper in aerated labelled vials where they were dried for 48 hours. The filter paper was subsequently moistened with sterile water and the cadavers observed and incubated for a further five days to encourage fungal growth (Elliot et al., 2002).

Insects, both dead and alive, were examined at the end of the experiment to confirm infection. For the pathogen spore production assays, only dead insects were examined. Insects were homogenized individually in 10 ml of distilled water. For a detailed examination samples of the suspension were examined using a phase contrast microscope (400x) with hemacytometer (Bateman, 1997) and each type of spores was counted. Pure *P. locustae* and *M. anisopliae* spore suspensions were

used for comparison and the distinction between the two pathogens was made based on spore morphology.

4.3.4 Analysis of data

Data were arcsine or log transformed where necessary before analyses. Median survival times (MST) were calculated using Kaplan Meier Survivorship analysis (SPSS, 1999) and the criteria of significance was the failure of the 95% confidence limits to overlap. Comparisons of mean percentage mortality (i.e., 21 days cumulative mortalities) and significant difference between separate and sequential treatments as well as time effect of inoculation of *M. anisopliae* following *P. locustae* on production of spores per nymphs were conducted with GLM and Tukey studentized range test, p = 0.05.

Analysis for additive, antagonistic, and synergistic interaction was based on binominal test and comparison of the expected and observed percentage mortality as adapted from Robertson and Preisler (1982). Expected mortality at a set of concentrations of *P. locustae* and *M*. *anisopliae* was based on the formula $P_E = P_0 + (1-P_0)(P_1) + (1-P_0)(1-P_1)(P_2)$, where P_E is the expected mortality on combination of both pathogens, P_0 is the natural (control) mortality, P_1 is the mortality after treatment with *P. locustae* alone and P_2 is the mortality after treatment with *M. anisopliae* alone. $X^2 = (L_o-L_E)^2/L_E + (D_o-D_E)^2/D_E$ with L_o the number of living nymphs observed, L_E is the number of living nymphs expected, D_o is the number of dead nymphs observed and D_E is the number of dead nymphs expected was used to test the hypothesis of independence with 1 df and p = 0.05. Additivity was indicated if $X^2 < 3.84$. Antagonism was indicated if $X^2 > 3.84$ and $P_C < P_E$ where *Pc* is the observed mortality of the combination of the two pathogens and P_E the expected mortality of the combination. Synergism was indicated if $X^2 > 3.84$ and $P_c > P_E$.

4.4 Results

4.4.1 Host mortality

Infection was observed in all inoculated nymphs as single infection (separate treatments) or mixed infection (sequential treatments). Survival of *S. gregaria* was affected by both pathogens and mortality was higher in mixed than in single infections and increased significantly with increasing of either concentrations/dosages of both pathogens or time period between the two successive inoculations ($p < 0.0001$). In single infection host mortality did not exceed 50% over the experiment period of 21 days; subsequently the Median Survival Time (MSTs) for these treatments were not estimated, with the exception of *M. anisopliae* applied at 1 x 10^3 and 1 x 10^4 spores/nymph that result in MSTs of 18.8 ± 0.6 and 12.3 ± 0.9 days, respectively. Overall MSTs in mixed infection declined significantly with increasing concentrations of both pathogens (Tables 4.1 and 4.2). Nymphs infected sequentially with *P. locustae* followed by *M. anisopliae* succumbed more easily than nymphs infected with each pathogen alone, even at low level of pathogens combination (Tables 4.1 and 4.2).

Table 4.1. Median Survival Time (MST) for fifth instar desert locust *Schistocerca gregaria* inoculated with *Metarhizium anisopliae* after sequential oral inoculation with *Paranosema locustae* with a 3-day time interval between inoculations.

	MST (days ± SE) and 95%CI[a]		
	M. anisopliae[b]		
P. locustae[c]	1 x 10^2	1 x 10^3	1 x 10^4
1 x 10^4	-[c]	15 ± 1.5 (12.1 – 19.9)	11 ± 0.8 (10.3 – 13.7)
1 x 10^5	12 ± 1.5 (9.1 – 14.9)	9 ± 0.9 (7.3 – 10.7)	7 ± 0.5 (9.5 – 12.6)
1 x 10^6	10 ± 1.2 (7.7 – 13.3)	8 ± 1.2 (5.7 – 10.3)	5 ± 0.6 (3.8 – 6.2)

[a]Kaplan Meier Survivorship analysis was used to calculate the MST and the criteria oft significance was the failure of the 95% confidence limits to overlap; [b]Spores per nymph; [c]Nymphs were inoculated in groups of thirty individuals feeding them with 10g wheat bran treated with each spore concentration.

Table 4.2. Median Survival Time (MST) for fifth instar desert locust *Schistocerca gregaria* inoculated with *Metarhizium anisopliae* after sequential oral inoculation with *Paranosema locusta*e with a 10-day time interval between inoculations.

MST (days ± SE) and 95%CI[a]			
	M. anisopliae[b]		
P. locustae[c]	1 x 10^2	1 x 10^3	1 x 10^4
1 x 10^4	9.0 ± 0.6 (7.8-10.2)	8 ± 0.5 (6.9-9.1)	6 ± 0.6 (4.8-7.3)
1 x 10^5	9.0 ± 0.5 (8.1-9.9)	5 ± 0.5 (4.0-6.0)	4 ± 0.3 (3.5-4.5)
1 x 10^6	8 ± 0.7 (6.6-9.4)	5 ± 0.3 (4.5-5.5)	3.0 ± 0.3 (2.4-3.6)

[a]Kaplan Meier Survivorship analysis was used to calculate the MST and the criteria oft significance was the failure of the 95% confidence limits to overlap; [b]Spores per nymph; [c]Nymphs were inoculated in groups of thirty individuals feeding them with 10g wheat bran treated with each spore concentration.

The mortality response while combining the two pathogens in *S. gregaria* as adapted from Robertson and Preisler (1982) are summarized in Table 4.3. The sequential inoculation experiment showed an increase in *S. gregaria* mortality with increasing of either concentrations of both pathogens or time period between the two successive inoculations. There were no antagonistic interactions between the two pathogens. However, five and six of the nine combinations in the two pathogens caused synergism of *S. gregaria* mortalities in 3-day and 10-day sequential inoculation experiment, respectively, the remaining combinations indicating additive interactions (Table 4.3).

Table 4.3. Mortality response of fifth instar desert locust, *Schistocerca gregaria* to *Paranosema locustae* and *Metarhizium anisopliae*.

Paranosema concentrations[c]	*Metarhizium* Spores/nymph	% mortality Observed 3 days[a]	% mortality Observed 10 days[a]	Expected[b]	X^2 3 days[a]	X^2 10 days[a]	Response 3 days[a]	Response 10 days[a]
0	0	12,2	12,2		-	-	-	-
1×10^4	0	25.6	25.6		-	-	-	-
1×10^5	0	40	40		-	-	-	-
1×10^6	0	45.6	45.6		-	-		
0	1×10^2	31.9	31.9		-	-	-	-
0	1×10^3	60.6	60.6		-	-	-	-
0	1×10^4	74.0	74.0		-	-	-	-
1×10^4	1×10^2	45.6	53.2	55.5	3.58	0.18	Additive	Additive
1×10^4	1×10^3	74.4	76.7	74.3	0.00	0.25	Additive	Additive
1×10^4	1×10^4	77.8	87.8	83.0	1.74	1.33	Additive	Additive
1×10^5	1×10^2	67.8	82.3	64.1	0.53	11.34	Additive	Synergistic
1×10^5	1×10^3	90.0	100.0	79.2	6.33	20.17	Synergistic	Synergistic
1×10^5	1×10^4	97.8	100.0	86.3	10.06	10.79	Synergistic	Synergistic
1×10^6	1×10^2	84.5	85.9	67.5	11.89	11.76	Synergistic	Synergistic
1×10^6	1×10^3	100.0	100.0	81.2	20.86	15.76	Synergistic	Synergistic
1×10^6	1×10^4	100.0	100.0	87.6	12.76	9.64	Synergistic	Synergistic

[a]Time interval between inoculation of the nymphs with *P. locustae* and *M. anisopliae*; [b]$P_E = P_0 + (1-P_0)(P_1) + (1-P_0)(1-P_1)(P_2)$, where P_E is the expected mortality on combination of the two pathogens, P_0 is the natural (control) mortality, P_1 is the mortality after treatment with *P. locustae* alone and P_2 is the mortality after treatment with *M. anisopliae* alone; [c]Nymphs were inoculated in groups of thirty individuals feeding them with 10g wheat bran treated with each spore concentration.

4.4.2 Pathogen spore production

Results on spore production in either single infection or mixed infection are presented in Tables 4.4, 4.5 and 4.6. In single infection (Table 4.4), spores yield per nymph increased significantly with increasing concentrations of *P. locustae* ($F = 11.1$; $df = 2$; $p < 0.0001$) or dosages of *M. anisopliae* ($F = 21.5$; $df = 2$; $p < 0.0001$).

Mixed infection occurred in all inoculated nymphs with always a predominance of *M. anisopliae* over *P. locustae*. *Metarhizium anisopliae* spore production did not differ significantly between single infection and mixed infection treatments ($P > 0.05$). However, both the time and *M. anisopliae* dosages significantly affected *P. locustae* spore production ($p < 0.0001$), leading to 3 to 20-fold and 2.5 to 8-fold fewer *P. locustae* spores yield per nymph in 3 and 10-day sequential inoculations experiments compared to single inoculations, respectively (Tables 4.5 and 4.6).

Table 4.4. *Paranosema locustae* and *Metarhizium anisopliae* spore production in fifth instar desert locustae *Schistocerca gregaria* inoculated separately with each pathogen.

Pathogen spore production (mean ± SE)[ab]							
Paranosema locustae concentrations[c]				*Metarhizium anisopliae* dosages (spores/nymph)			
Control	1 x 10^4	1 x 10^5	1 x 10^6	Control	1 x 10^2	1 x 10^3	1 x 10^4
0.0 ± 0.0 d	4.9 ± 0.7 c	7.8 ± 0.5 b	14.4 ± 0.6 a	0.0 ± 0.0 d	9.94 ± 0.3 c	13.4 ± 0.7 b	15.1 ± 0.9 a

[a]Mean x 10^8 spores: *Paranosema locustae*, mean x 10^9 spores: *Metarhizium anisopliae*; [b]For each pathogen means followed by the same letter are not significantly different (p = 0.05; Tukey-test); [c]Nymphs were inoculated in groups of thirty individuals feeding them with 10g wheat bran treated with each spore concentration.

Table 4.5. *Metarhizium anisopliae* spore production in fifth instar desert locust *Schistocerca gregaria* inoculated with one of a series of dosages of *Metarhizium anisopliae* 3 or 10 days after exposure to *Paranosema locustae*.

	Metarhizium anisopliae spores/nymph (mean ± SE)[a] x 10^9					
	Metarhizium dosages (spores/nymph)					
Paranosema	1 x 10^2		1 x 10^3		1 x 10^4	
Concentrations[c]	3 days[b]	10 days[b]	3 days	10 days	3 days	10 days
1 x 10^6	10.0 ± 1.1 Aa	10.5 ± 0.5 Aa	13.2 ± 0.6 Aa	12.8 ± 0.3 Aa	14.3 ± 0.7 Aa	14.9 ± 0.8 Aa
1 x 10^5	10.5 ± 0.6 Aa	9.37 ± 0.3 Aa	12.9 ± 0.3 Aa	12.5 ± 0.2 Aa	14.8 ± 0.4 Aa	15.0 ± 0.7 Aa
1 x 10^4	9.76 ± 0.5 Aa	9.52 ± 0.1 Aa	13.3 ± 0.7 Aa	12.8 ± 0.5 Aa	14.6 ± 0.4 Aa	14.5 ± 0.7 Aa

[a]Means in the same columns followed by the same upper letters and in the same lines followed by the same lower letter are not significantly different (p = 0.05; Tukey-test); [b]Time interval between inoculation of the nymphs with *P. locustae* and *M. anisopliae;* [c]Nymphs were inoculated in groups of thirty individuals feeding them with 10g wheat bran treated with each spore concentration.

Table 4.6. *Paranosema locustae* spore production in fifth instar desert locust *Schistocerca gregaria* inoculated with one of a series of dosages of *Metarhizium anisopliae* 3 or 10 days after exposure to *Paranosema locustae*.

	Paranosema locustae spores/nymph (means ± SE)[a] x 10^8					
	Metarhizium dosages (spores/nymph)					
Paranosema	1 x 10^2		1 x 10^3		1 x 10^4	
Concentrations[c]	3 days[b]	10 days[b]	3 days	10 days	3 days	10 days
1 x 10^6	3.20 ± 0.4 Aa	3.61 ± 0.3 Aa	1.91 ± 0.3 Ab	2.88 ± 0.7 Aa	1.35 ± 0.1 Ab	1.83 ± 0.5 Aa
1 x 10^5	2.27 ± 0.2 Ab	3.23 ± 0.2 Aa	1.39 ± 0.2 Bb	1.93 ± 0.3 Ba	1.21 ± 0.1 Ab	1.68 ± 0.2 Ba
1 x 10^4	0.98 ± 0.2 Ba	1.98 ± 0.4 Ba	0.49 ± 0.1 Ba	1.60 ± 0.1 Ca	0.25 ± 0.1 Aa	1.10 ± 0.1 Ca

[a]Means in the same columns followed by the same upper letters and in the same lines followed by the same lower letter are not significantly different (p = 0.05; Tukey-test); [b]Time interval between inoculation of the nymphs with *P. locustae* and *M. anisopliae;* [c]Nymphs were inoculated in groups of thirty individuals feeding them with 10g wheat bran treated with each spore concentration.

4.5 Discussion

The microsporidian, *P. locustae* and the entomopathogenic fungus *M. anisopliae* offer great advantages over organophosphate pesticides used for the control of locusts. Throughout our experiments, combination of *P. locustae* and *M. anisopliae* var. *acridum* resulted in a higher mortality of fifth instar *S. gregaria* than either pathogen alone. In our experiments, no evidence of antagonistic interaction between the two biopesticides on host mortality was noted.

The increase in *S. gregaria* mortality indicates that the performance of the relative virulence of *M. anisopliae* may be altered by the presence of less virulent *P. locustae* and vice versa. The two pathogens act either independently or synergistically depending on the concentrations of the less virulent pathogen (i.e., *P. locustae*). The synergistic combinations of *P. locustae* and *M. anisoplia*e in this study showed an increase of 13 and 21% in host mortality over the expected for an additive response. This represents a substantial improvement over the single use of either *P. locustae* or *M. anisopliae* for *S. gregaria* control. Such a synergistic response is a result of interspecific interactions, such as a host debilitating effect of *P. locustae* even at sublethal dosages. Hence, *P. locustae* acts to reduce the host cellular defenses, indirectly enhancing the performance of *M. anisopliae*. *Paranosema locustae* infects the fat tissue of the host, disrupting the host intermediary metabolism and competing for energy reserves. Infected insects are therefore stressed and become more sensitive to *M. anisopliae* infections. This was evidenced by the increased mortality rates of *S. gregaria* in 10-day sequential inoculation treatments.

There have been numerous studies on the effect of multiple pathogens infecting the same host. These include among other interactions virus vs. virus (Ishii et al., 2002), virus vs. microsporidia (Fuxa, 1979; Cossentine and Lewis, 1984; Moawed et al., 1987; Bauer et al., 1998), virus vs. fungus (Malakar et al., 1999), fungus vs. fungus (Thomas et al., 2003), microsporidia vs. microsporidia (Solter et al., 2002; Pilarska et al., 2006), bacteria vs. nematode (Koppenhöfer and Kaya, 1997) and bacteria vs. microsporidia (Pierce et al., 2001). Double infections by different types of pathogens usually led to an increase in insect mortality, especially when infections

were sequential rather than simultaneous (Jacques and Morris, 1981). In a laboratory study, Bauer et al. (1998) reported pre-existing microsporidian infection to increase nuclear polyhedrosis virus mortality in gypsy moth larvae, particularly at high microsporidian concentrations, and Pierce et al. (2001) reported that European corn borers preinfected with *Nosema pyrausta* Paillot were killed more easily by *Bacillus thuringiensis* Berliner than were uninfected corn borers. Koppenhöfer and Kaya (1997) studied the effect of time lag between successive inoculations of entomopathogenic nematodes and *B. thuringiensis* for scarab grub control and concluded that grub has to be exposed to *B. thuringiensis* at least 7 days before the addition of nematodes to induce additive and synergistic effects.

Results from pathogen spore production indicated that overall, fifth instar *S. gregaria* infected with *P. locustae* produced particularly high numbers of spores, although late instars of locust and grasshopper are commonly cited not be highly sensitive to microspiridian infections. These data agree, however, with the study of Canning (1962) who reported spore production to be negatively correlated to host mortality rates. High sporulation of *M. anisopliae* was recorded in our study. This may have occurred due to the high virulence of the pathogen on one hand, and relative humidity (>90%) and temperature (30 °C) in the experimental room on the other hand. These conditions correspond well with the pathogen's optimum growth conditions (Milner et al., 1997).

The interaction between the two pathogens showed that *M. anisopliae* is dominant over *P. locustae* in desert locust *S. gregaria*. This can be explained by the differences in pathogen development after infection. *Metarhizium anisopliae* is a saprophytic pathogen that produces infective spores upon host death. In contrast *P. locustae*, an obligate parasite, can only develop and replicate within a living host. Hence, the reduction in *P. locustae* replication under a co-infection regime is a direct consequence of the reduction of host survival following additional infection with *M. anisopliae. Paranosema locustae*-infected nymphs are more sensitive to *M. anisopliae* than uninfected nymphs; consequently they are less likely than uninfected nymphs to survive even reduced concentrations of *M. anisopliae*. Therefore, infected nymphs that might have matured and produced infected adults that could

subsequently have contributed to further transmission of the pathogen are unlikely to do so. Our data suggest that additional use of *M. anisopliae* may not exclude *P. locustae*; the two pathogens rather coexist and develop within the same host, but the virulent *M. anisopliae* affected negatively the rate of multiplication of the less virulent *P. locustae*. When *M. anisopliae* was added to an existing *P. locustae* infection, spores, normally present by day 3 post-inoculation might not develop. It had been determined that spores from inoculated nymphs were not detected until 7-9 days (D.A.S., unpublished data). Raina et al. (1995) reported infection to occur within 13-15 days in the haemocyte and fat body tissue of inoculated insects, and Henry (1972) reported that *P. locustae* required at least 13 days for the initial generation to develop in the laboratory at 35 °C. Moreover, when *M. anisopliae* was added to an existing *P. locustae* infection present by day 10, nymphs produced more spores of *P. locustae* than those with an existing *P. locustae* infection present by day 3. Such result suggests that time to death of the host and production of *P. locustae* spores are related to the pathogen virulence. According to Ebert (1994) and Wells et al. (1995), virulence is the consequence of balancing host mortality and propagule production. Both *P. locustae* and *M. anisopliae* required host nutrients and energy resources for their growth and development following infections. It is therefore not surprising that competition between the two pathogens for common host resources are detrimental to the replication of the relatively less virulent *P. locustae* without strong effects on mortality rates. Our finding agrees with Wilson and Burke (1978) who reported *Pleistophora* (*Endoreticulatus*) *schubergi* to be dominant over *Nosema fumiferanae* in the spruce budworm, *Choristoneura fumiferana* (Clem.). Similarly, Ishii et al. (2001) reported that direct competition of mixed virus infections in Lepidoptera for common resources limit pathogen replication. In contrast, Solter et al. (2002) reported mixed infection of three species of microsporidian to have no dominant effect of a single species, and Knell et al. (1998) studied competition between *B. thuringiensis* and a granulosis virus of the Indian meal moth and concluded that there was no evidence that any of the species could out-compete the others.

Mixed applications of *P. locustae* and *M. anisopliae* may offer a powerful and reliable tool for locust control, and this approach will be more desirable than using

each pathogen alone. However, the additive and synergistic effect observed in the present study need to be confirmed under field conditions. Moreover, because *M. anisopliae*, when added to an existing *P. locustae* infection, could induce adverse effects on its replication, the use of the two pathogens in a classical biocontrol will be detrimental for the latter. Therefore, detailed descriptions of abundance and distribution of *P. locustae* in Africa and its movement between infected host areas where *M. anisopliae* is applied are required. These would assist in the determination of the appropriate conditions in the use of the two pathogens in a way that could reduce adverse effects on the survival of *P. locustae* in locust populations.

5 Field efficacy of *Paranosema locustae* (Microsporidia: Nosematidae) and Green Muscle®, *Metarhizium anisopliae* var. *acridum* (Deuteromycotina: Hyphomycetes) in wheat bran application for the control of locusts and grasshoppers in Sahel[c]

5.1 Abstract

Field trials were conducted to evaluate the efficacy of *Paranosema locustae* and *Metarhizium anisopliae* in wheat baits application for controlling grasshoppers in southeast Niger. Treatments consisted of wheat bran mixed with *M. anisopliae*, *P. locustae* + *M. anisopliae* or with *P. locustae* spores and *P. locustae* + sugar. *Oedaleus senegalensis*, *Pyrgomorpha cognata* and *Acrotylus blondeli* were the predominant species at the time of application representing ca. 94% of the total population. Bran application was done when *O. senegalensis* (ca. 75% of the population), was at its early instar nymphs, with first, second and third instars totalling 64 to 85%. Grasshopper population reduction, *P. locustae* prevalence and level of infections in the predominant species were monitored. Manual application of *P. locustae* and *M. anisopliae* formulated in wheat bran has proven to induce consistent pathogen infection in grasshopper populations. Population density over the three weeks monitoring, typically decreased by 44.7 ± 6.9%, 52.8 ± 8.4%, 73.7 ± 5.5% and 89.1 ± 1.8% in *P. locustae*, *P. locustae* + sugar, *M. anisopliae* and *P. locustae* + *M. anisopliae* treated plots, respectively. *Paranosema locustae* prevalence in surviving adult grasshoppers after 28 days post-application was 48.1 ± 2.3%, 28.9 ± 4.8% and 27.4 ± 3.7%, with infection level of $6.2 \pm 0.8 \times 10^6$, $2.3 \pm 0.3 \times 10^4$ and $2.1 \pm 0.3 \times 10^3$ spores/mg host weight in *O. senegalensis*, *A blondeli* and *P. cognata*, respectively. Other species that each accounted for <2% of the community, namely *Aiolopus thalassinus*, *A. simulatrix*, *Acorypha glaucopsis*, *Acrotylus patruelis*, *Anacridium melanorhodon*, *Diabolocatantops axillaris*, *Kraussaria angulifera* and *Schistocerca gregaria* were found to show sign of infection.

[c] This chapter was partly submitted to BioControl as Agbeko Kodjo TOUNOU, Christiann KOOYMAN, Orou Kobi DOURO-PINDOU and Hans Michael POEHLING (2007): Combined field efficacy of *Nosema locustae* and *Metarhizium anisopliae* var acridum for the control of sahelian grasshoppers

5.2 Introduction

The control of Sahelian grasshoppers by entomopathogenic agents is receiving intensified attention from ecologists. Among pathogens that are known to cause disease in locusts and grasshoppers, *Paranosema locustae* Canning (Microsporidia: Nosematidae) and *Metarhizium anisopliae* var. *acridum* Driver and Milner (Deuteromycotina: Hyphomycetes) have been widely tested (Johnson and Henry, 1987; Langewald et al., 1997; Johnson, 1997). Field trials conducted in Cape Verde and Mauritania established that the predominant Sahelian species, particularly *Oedaleus senegalensis* Krauss (Orthoptera: Acrididae), could be infected by field application of *P. locustae* spores, and as revealed in Cape Verde trials, density reduction could result from the application of the pathogen (GTZ, 1992; Henry, 1992).

The way *P. locustae* is offered to the target host in the field has been suggested to be one of the important factors that determine the potential of the pathogen to control grasshoppers (Onsager et al., 1980; Onsager et al., 1981). Formulation of *P. locustae* as a wheat bran bait has resulted in numerous successful introductions of the pathogen into field populations (Johnson, 1997; Lockwood et al., 1999). An advantage in such applications is that the infective spores are concentrated on the bait particles and the target host becomes thus infected by ingesting small quantities of bait.

The Lubilosa programme has carried out number of field trials for grasshopper control using *M. anisopliae* var. *acridum* (Bateman, 1997; Milner et al., 1997). The successful development of methods for mass-producing infective spores of *M. anisopliae* var. *acridum* has led to the commercial development of this fungus as a microbial insecticide under the name Green Muscle® (Lomer et al., 2001). The pathophysiology of *M. anisopliae* var. *acridum* makes this product an ideal candidate for augmentative biological control of locusts and grasshoppers. Unlike microsporidia, which must be ingested to initiate infection, *M. anisopliae* is able to invade actively through the cuticle (Charnley, 1992). In previous studies the efficacy of oil-based *M. anisopliae* var. *acridum* formulations in controlling acridid populations was demonstrated (Kooyman and Godonou, 1997; Langewald et al., 1999).

Application of *M. anisopliae* var. *acridum* using ULV spray appears efficient since it increases the coverage applied surface and consequently reducing cost. The infective spores are thinly dispersed over the treated area and are likely to come into contact with the target host. Although application of *M. anisopliae* var. *acridum* as spray has proven to be the most efficient, there are cases where wheat bran bait has been successfully used for controlling grasshoppers with entomopathogenic fungi (Caudwell and Gatehouse*,* 1996a, b; Caudwell, 1993; Inglis et al., 1996; Bextine and Thorvilson, 2002).

In the previous laboratory studies we reported younger grasshopper instars to be more susceptible to *P. locustae* than older instars (Chapter 3). Moreover additive and synergistic mortality responses were observed when combining *P. locustae* and *M. anisopliae* var. *acridum* (Chapter 4). Our interest in the present study was two-fold. The first objective was to evaluate the within-season impact of *P. locustae* on the population density of grasshoppers targeting particularly early developmental stages. The second objective was to test different possibilities of enhancing the efficacy of wheat bran formulation of *P. locustae* by mixing the pathogen with *M. anisopliae* or formulating the *P. locustae* in a sugar formulation.

5.3 Materials and Methods

5.3.1 Field Site

The studies described below were conducted throughout the 2004 rainy season (August-September) in Maine Soroa district (13°13'N, 12°08'E) in southeast of Niger, West Africa. Three sites were chosen in a dry savanna grassland and for the purpose of this paper, these sites will be referred to as Site A, Site B and Site C. The experimental site consisted of an open savannah, dominated by *Cenchrus biflorus* Roxb, *Tragus berteronianus* Schultes and *Chloris prieuri* Kunth (Cyperales: Poaceae) that ranged from 5-15 cm height at the time of applications. The field site were scattered with trees of *Acacia* spp., *Balanites* spp. and *Pergularia tomentosa* bushes.

5.3.2 Bait formulation and application

Spores of *P. locustae* were provided by Dr. Lee Anne Merrill (M and R Durango, Inc. P. O. Box 886 Bayfield, CO 81122 USA) (see chapters 3 and 4 for details). Spores were stored in aqueous suspension frozen at –20 °C in a freezer for three weeks before use in trials. The isolate of *M. anisopliae* var. *acridum* (strain IMI 330189) was the standard isolate used in locust biocontrol in Africa (Lomer et al., 2001) (see chapter 4 for details).

Dried spores sealed in aluminium sachets, were transported from IITA-Benin to Niger in a cool box. Fresh suspensions were prepared few hours before applications. A hemacytometer was used for counting the spores, and desired concentrations were made up as water-based suspensions. Each concentration was homogenized in 2 litres distilled water or sugar solution and applied to 50 kg wheat bran in plastic containers and mixed by hand.

Fifteen 25-ha plots were marked out in the three selected sites (five plots/site). Within each site, one randomly chosen plot was treated with each of the dosages listed below. The quantity of the infective spores ingested has been reported to be the most important factor in controlling grasshoppers (Henry et al., 1973). Therefore, in the present study wheat bran was mixed with sugar formulation in one *P. locustae* treatment assuming that such formulation could phagostimulate grasshoppers to consume more treated bran. Inoculum was spread with hand by 21 persons walking at a speed of 5km/h and a track interval of 5 m. Hand-held global positioning system was used to help the operators to maintain the 5 m apart during treatments. The treatments are:

(1) *P. locustae*: 5 x 10^9 spores mixed with 2 kg wheat bran/ha;

(2) *M. anisopliae*: 5 x 10^{12} spores mixed with 2 kg wheat bran/ha;

(3) *P. locustae* in sugar formulation: 5 x 10^9 spores in 2 kg wheat bran/ha, mixed with sugar (4% w/w);

(4) *P. locustae* (2.5 x 10^9 spores) + *M. anisopliae* (2.5 x 10^{12} spores) mixed with 2 kg wheat bran/ha;

(5) Untreated plots used as control

The bait applications were carried out on three consecutive days, always in the morning when the hoppers were still roosting on bush. The average wind speed at the time of application was 1.5-2.3 m/s. The application dates are as followed:

- Site A: 21 August 2004
- Site B: 22 August 2004
- Site C: 23 August 2004.

5.3.3 Population structure and cage incubation samples

Population structure was determined by collecting samples of a maximum of 100 insects each per plot, sweeping sweepnets fewer than 10 times to minimize dispersal of tracer and cross-contamination between the hopers (Langewald et al., 1997). The collections were made in proximity to, but not within the density transects (Onsager et al., 1980), shortly before application (day 0) and 3 days post-application. Collected grasshoppers were counted and classified by species and age structure.

From the 100 hoppers collected, 50 individuals, consisting mainly of second and third instars *O. senegalensis*, were selected for mortality assessment. To avoid injury from handling as a mortality factor, only outwardly healthy and active hoppers were selected. Hopper samples were reared in group (Price et al., 1999; Douro-Kpindou, 2001) in portable wooden cages (see chapter 3 and 4 for details). The caged samples were stored under a shade roof and mortality was assessed daily over 21 days. Samples collected at day 0 were used to assess pathogen infection status of the sites before application. Cadavers from the cage samples were transferred onto Petri dishes containing wet filter paper. The number of cadavers with external sign of *M. anisopliae* spore (sporulation) was noted. For confirmation of *P. locustae* infection samples of cadaver homogenates were examined under microscope (Johnson and Dolinski, 1997). Pathogen incidence was calculated as percentage of dead grasshoppers either presenting external sign of *M. anisopliae* infection or harbouring *P. locustae* spores.

5.3.4 Population density monitoring

Grasshopper densities, determined on a density per-unit basis were evaluated, at day 0, and subsequently at 3-day intervals until the 21st day post-application. In the center of each plot, four diagonal transects were laid out, starting 10 m from the central point and extending toward the four corners of the plot. The grasshopper population density was monitored by counting every 5 m the number of grasshoppers within 25 imaginary one-square-meter-quadrates along each cross line (Kooyman et al., 1997). The same observer always counted the same line to decrease variability.

5.3.5 Prevalence of *P. locustae* and its level of infection in field surviving adult *O. senegalensis*, *A. blondeli* and *P. cognata*

At day 28 post-application, grasshoppers were sampled from plots that received *P. locustae* spores (i.e., *P. locustae*, *P. locustae* + sugar and *P. locustae* + *M. anisopliae*). Collected grasshoppers were transported frozen to the Lubilosa unit of the "Centre Regional AGRHYMET" station in Niamey where insects were classified by species and analyzed for infection.

Representative samples of each species were selected from the frozen samples and examined for presence of spores. The prevalence of infection was calculated as percentage of grasshoppers showing *P. locustae* spores and the degree of infection was determined separately for the three predominant species (*O. senegalensis*, *A. blondeli*, *P. cognata*) by microscopic observation. A total of 225 adults per species (75 per treatment per plot) were randomly selected from grasshoppers that showed evidence of infection. For detailed examination, the selected hoppers were weighed individually and homogenized in 10 ml of distilled water. Four to six microscopic preparations from each homogenized sample were examined as hanging drops using a phase contrast microscope (400x) and *P. locustae* spores recognized by their distinctive shape and size were counted (Johnson and Dolinski, 1997). Spore concentrations were calculated by direct counting (Bateman et al., 1997) and expressed as the number of spores per milligram of grasshopper weight.

5.3.6 Analysis of data

Data were arcsine or log transformed where necessary before analyses. Since the density of treated populations depends on initial population size within plots and progressing fluctuations not only on treatments but on natural occurring changes too, we calculated corrected percent efficacy according to the Henderson-Tilton formula:

$$\%Efficacy = \left[1 - \left(\frac{T_a}{T_b}\right) x \left(\frac{C_b}{C_a}\right)\right] x100$$

where T_b et C_b represent densities before treatments in treated and control plots and T_a and C_a represent densities after treatment in treated and control plots, respectively (Henderson-Tilton, 1955). Densities after treatment were pooled from day 15 to day 21. Means comparisons were performed using one-way-ANOVA and Tukey test for mean separation ($P < 0.05$) (SPSS, 1999).

5.4 Results

5.4.1 Meteorological conditions

The meteorological conditions (a typical summer conditions in Maine Soroa) such as air temperature, relative humidity and rainfall recorded in the treatment zone (Meteorological station of Maine Soroa district) during the application and data record are shown in Figures 5.1, 5.2 and 5.3. Air temperature reached a maximum of 31 °C during the early afternoon and a minimum of 24 °C at dawn (Figure 5.1). Relative humidity generally measured 100% at night and declined to 22-73% during the heat of the day (Figure 5.2). Prior to and during the course of the trials, a total of 165.7 mm rainfall were recorded in Maine Soroa, with 70.6 mm before the beginning of the applications. The last day of treatment was followed in the night by 47.4 mm of rainfall (Figure 5.3).

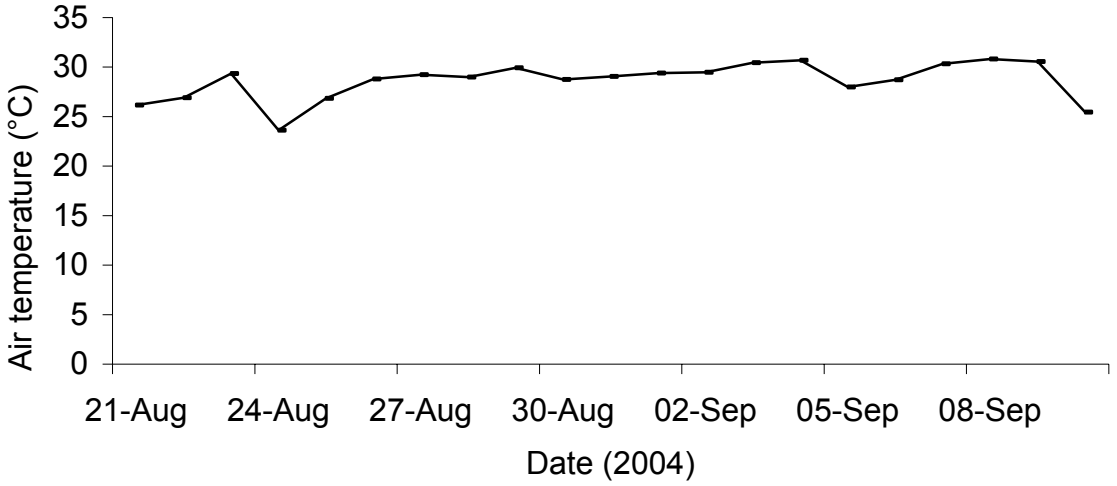


Figure 5.1. Meteorological conditions of air temperature recorded at Maine-Soroa station during the field trial of *Paranosema locustae* and *Metarhizium anisopliae* var. *acridum* against grasshopper in southeast Niger.

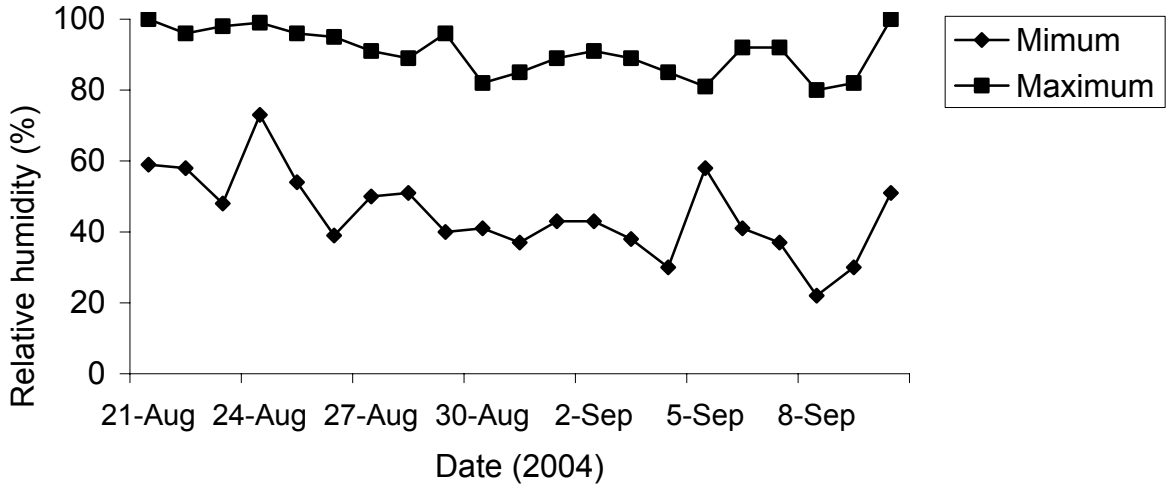


Figure 5.2. Meteorological condition of relative humidity recorded at Maine-Soroa station during the field trial of *Paranosema locustae* and *Metarhizium anisopliae* var. *acridum* against grasshopper in southeast Niger.

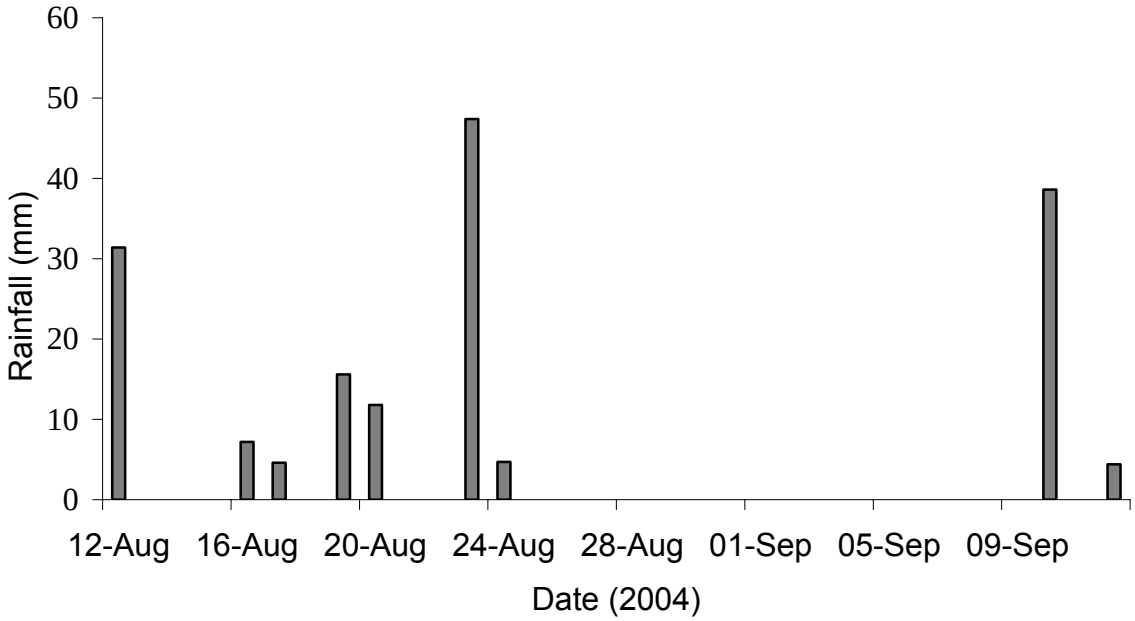


Figure 5.3. Rainfall recorded at Maine-Soroa station during the field trial of *Paranosema locustae* and *Metarhizium anisopliae* var. *acridum* against grasshopper in southeast Niger.

5.4.2 Population structure

A total of 25,376 grasshoppers were collected during the experiment (Table 5.1). The grasshopper community in this area can be described as Sahel- to semi-desert type consisting mainly of the following species: *O. senegalensis*, *Acrotylus blondeli* De Saussure, *Pyrgomorpha cognata* Krauss, *Diabolocatantops axillaris* Thunberg, *Chrotogonus senegalensis* Krauss (Orthoptera: Acididae), *Poekilocerus bufonius* Klug (Orthoptera: Pyrgomorphidae). The first three represented ca. 94% of the total population at the time of the experiment. On all three sites, *O. senegalensis*, which accounted for 75% of all grasshopper species, was dominated by first, second and third instars totalling 64 to 85% (Table 5.2).

Table 5.1. Species present (as number and percentage of total grasshoppers collected at the three sites, using sweepnets) during the 2004 rainy seasons in Maine Soroa district, southeast Niger.

	Site A	Site B	Site C	Total
Schistocerca gregaria Forsakål	0 (0.00)	4 (0.05)	1 (0.01)	5 (0.02)
Aiolopus thalassinus Fabricius	10 (0.11)	8 (0.10)	7 (0.09)	25 (0.1)
Anacridium melanorhodon Walker	5 (0.06)	16 (0.19)	4 (0.05)	25 (0.1)
Cryptocatantops haemorrhoidalis Krauss	7 (0.08)	11 (013)	7 (0.09)	25 (0.1)
Ornithacris cavroisi Finot	12 (0.13)	5 (0.06)	8 (0.1)	25 (0.1)
Aiolopus simulatrix Walker	22 (0.24)	10 (0.12)	18 (0.22)	50 (0.2)
Ochrilidia spp. Fieber	10 (0.11)	32 (0.39)	8 (0.1)	50 (0.2)
Tenuitarsus sudanicus Kevan	19 (0.21)	21 (0.26)	10 (0.12)	50 (0.2)
Truxalis spp. L.	17 (0.19)	15 (0.18)	18 (0.22)	50 (0.2)
Stenohippus spp. Uvarov	22 (0.24)	10 (0.12)	19 (0.24)	51 (0.2)
Morphacris fasciata Thunberg	14 (0.16)	19 (0.23)	23 (0.29)	56 (0.26)
Acorypha glaucopsis Walker	30 (0.33)	17 (0.21)	29 (0.36)	76 (0.30)
Acrotylus patruelis Herrich-Schaeffer	24 (0.27)	41 (0.50	11 (0.14)	76 (0.30)
Poekilocerus bufonius Klug	5 (0.06)	28 (0.34)	43 (0.53)	76 (0.30)
Kraussaria angulifera Krauss	39 (0.43)	85 (1.03)	33 (0.41)	157 (0.62)
Acrida bicolor Thunberg	69 (0.77)	53 (0.65)	37 (0.46)	159 (0.63)
Chrotogonus senegalensis Krauss	85 (0.95)	115 (1.40)	78 (0.97)	278 (1.10)
Diabolocatantops axillaris Thunberg	110 (1.22)	127 (1.55)	93 (1.16)	330 (1.30)
Pyrgomorpha cognata Krauss	668 (7.43)	648 (7.89)	533 (6.63)	1849 (7.29)
Acrotylus blondeli De Saussure	1025 (11.40)	829 (10.09)	867 (10.78)	2721 (10.72)
Oedaleus senegalensis Krauss	6796 (75.60)	6119 (75.50)	6197 (77.04)	19217 (75.73)

Table 5.2. Age structure of the three predominant grasshopper species.

Species	Instar[a] first	second	third	fourth	fifth	adult	% of total[b]
			Site A (n = 433)[c]				
O. senegalensis	31.4	22.8	30.6	2.9	3.5	8.8	86.1
A. blondeli	0.0	4.9	46.3	28.8	12.2	9.8	9.5
P. cognata	0.0	0.0	0.0	18.2	18.2	63.6	2.5
			Site B (n = 401)				
O. senegalensis	29.9	14.2	28.5	7.6	11.0	8.7	85.8
A. blondeli	0.0	15.4	41.0	30.8	7.7	5.1	9.7
P. cognata	0.0	0.0	0.0	7.7	15.4	76.9	3.2
			Site C (n = 312)				
O. senegalensis	30.0	16.3	20.7	5.4	10.8	16.7	65.1
A. blondeli	3.6	15.5	32.1	20.2	19.0	9.5	26.9
P. cognata	0.0	5.9	11.8	23.5	35.3	23.5	5.4

[a]The percentage in each age class is shown for each of the dominant species; [b]The relative abundance of each species, based on grasshopper sampled shortly before pathogen applications; [c]Number of grasshoppers sampled per site.

5.4.3 Population development

Results on population counts in the untreated and treated plots over the 21 days observation period, are presented in Figure 5.4. In the untreated plots grasshopper counts increased from 10.44 ± 1.24 to 20.02 ± 2.25 grasshoppers/m^2. The counts in the treated plots however, declined with time and ranged from 11.12 ± 2.09 to 9.72 ± 1.01, from 11.52 ± 2.10 to 8.86 ± 1.05, from 11.00 ± 2.50 to 4.09 ± 1.35 and from 13.36 ± 3.78 to 2.20 ± 1.09, in *P. locustae*, *P. locustae* + sugar, *M. anisopliae* and *M. anisopliae* + *P. locustae*, respectively.

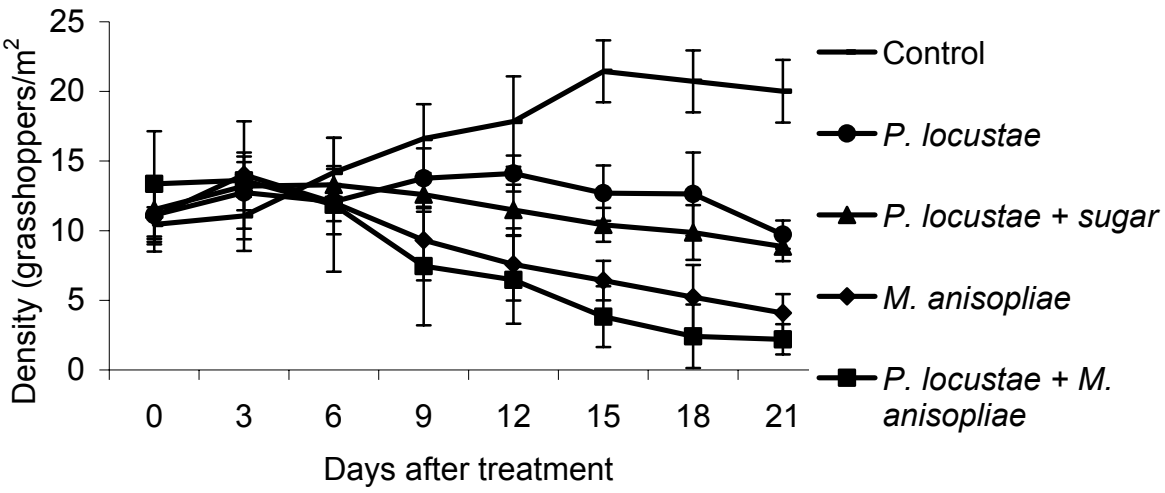


Figure 5.4. Mean count (± SE) of grasshoppers per square metre over a period of 21 days in untreated plots, plots treated with a wheat bran formulation of *Paranosema locustae*, *Paranosema locustae* + sugar, *Metarhizium anisopliae* var. *acridum* and *P. locustae* + *M. anisopliae* in southeast Niger.

Percents efficacy of the treatments are summarized in Table 5.3. Treatment efficacy differed significantly ($F = 14.16$; $df = 3, 8$; $p < 0.0001$), with *M. anisopliae* + *P. locustae* inducing the greatest population reduction followed by *M. anisopliae*. No differences were observed between *P. locustae* + sugar and *P. locustae* treatments (Table 5.3).

Table 5.3. Corrected densities of grasshoppers based on counts on the day of application (day 0) shortly before treatment, and subsequently at 3-day intervals over 21 days using Henderson and Tilton (1955) formula.

Treatments	Host density reduction (% ± SE)[ab]
P. locustae	44.67 ± 6.92 c
P. locustae + sugar	52.8 ± 8.44 c
M. anisopliae	73.71 ± 5.47 b
M. anisopliae + *P. locustae*	89.05 ± 1.79 a

[a]Means followed by the different letters are significantly different (*p = 0.05*; Tukey test); [b]Grasshopper counts were pooled from day 15 to day 21.

5.4.4 Cage incubation

The cumulative mortality data of the samples collected from treated and untreated plots, and pathogen incidence in the dead hoppers are presented in Table 5.4. A mortality rate of 18.67% ± 3.27 was recorded in the control over the 21-day assessment period and remained significantly lower than mortality from the treated samples (df = 139.81; df = 4, 10; p< 0.0001). No sporulations were observed on the cadavers from control samples. A total of 12 hoppers died during the first 2 days in all cages including control. None of these hoppers showed either sign of *M. anisopliae* mycosis or presence of *P. locustae* spores. Four specimens of *O. senegalensis* presented however, typical red/black coloration and putrefaction, which characterized bacterial infection caused by *Serratia marcescens*. Insects that died later showed evidence of pathogen infections with cadavers presenting characteristic red body coloration typical for *M. anisopliae* or harbouring *P. locustae* spores. Mixed infection of the two pathogens with predominance of *M. anisopliae* spores was noted in about 5% of the insects that died from *M. anisopliae* + *P. locustae* caged samples.

Table 5.4. Cumulative total mortality and pathogen incidence of caged grasshoppers sampled from untreated (control) and treated (*Paranosema locustae*, *P. locustae* + sugar, *Metarhizium anisopliae* var. *acridum* and *P. locustae* + *M. anisopliae* var. *acridum,* mixed with wheat bran) plots 3 days after applications and incubated in the shade over 21 days.

Treatments	Mortality (% ± SE)[a]	Pathogen infection (% ± SE)[a] in dead grasshoppers		
		Numb. examined	*P. locustae*	*M. anisopliae*
Control[a]	18.67 ± 2.33 d	28	0.00 ± 0.00 c	0.00 ± 0.00 b
P. locustae	55.67 ± 1.86 c	84	69.79 ± 2.14 a	0.00 ± 0.00 b
P. locustae + Sugar	57.33 ± 2.19 c	86	65.22 ± 1.03 a	0.00 ± 0.00 b
M. anisopliae	72.67 ± 2.03 b	109	0.00 ± 0.00 c	98.45 ± 0.13 a
P. locustae + *M. anisopliae*	93. 67 ± 1.87 a	141	21.23 ± 1.41 b	87.18 ± 0.91a

[a]Mean followed by the same letter in the same columns are not significantly different ($p = 0.05$; Tukey test); [b]Three untreated plots (25 ha each) were used as control.

5.4.5 Prevalence of *P. locustae* and its level of infection in field surviving adult *O. senegalensis*, *A. blondeli* and *P. cognata*

Disease prevalence and the levels of infection (number of spores) in infected insects are presented in Table 5.5. No background infections occurred in the untreated plots. In treated plots however, infections of grasshoppers with *P. locustae* were common, with consistence prevalence of infection in the three predominant species after 28 days post-application. Disease prevalence varied significantly among grasshopper species, with population of *O. senegalensis* showing higher proportion of infection ($F = 10.22$; $df = 2, 6$; $p = 0.012$) and level of infection ($F = 666.58$; $df = 2, 6$; $p < 0.0001$) than *A. blondeli* and *P. cognata*.

Table 5.5. Prevalence of *Paranosema locustae* and its level of infection (number of spores) in *Oedaleus senegalensis*, *Acrotylus blondeli* and *Pyrgomorpha cognata,* the three predominant grasshopper species, 28 days post-application during the 2004 rainy seasons in Maine Soroa district, southeast Niger.

Species[a]	Prevalence of *P. locustae*[b]		Infection level[b]	
	No. examined	Mean (% ± SE)	No. examined	Avg no. spores/mg grasshopper wt
O. senegalensis	1233	48.08 ± 2.26 a	225	$6.22 \pm 0.77 \times 10^6$ a
A. blondeli	500	28.93 ± 4.74 b	225	$2.25 \pm 0.32 \times 10^4$ b
P. cognata	378	27.36 ± 3.71 b	225	$2.05 \pm 0.30 \times 10^3$ c

Mean followed by the same letter in the same columns are not significantly different ($p = 0.05$; Tukey test); [a]*Paranosema locustae* infection level was quantified only for the three dominant species. However, other species that each accounted for <2% of the community (e.i., *Aiolopus thalassinus* Fabricius, *A. simulatrix* Walker, *Acorypha glaucopsis* Walker, *Acrotylus patruelis* Herrich-Schaeffer, *Anacridium melanorhodon* Walker, *Diabolocatantops axillaris* Thunberg, *Kraussaria angulifera* Krauss and *Schistocerca gregaria* Forsakål were found to show sign of infection; [b]The data were pooled from the three sites.

5.5 Discussion

The incorporation of water-based suspension of *M. anisopliae* var. *acridum* and *P. locustae* either alone or in combination, and sugar solution-based formulation of *P. locustae* into wheat bran bait resulted in significant decrease in grasshopper densities in all treated plots compared to untreated plots. In previous studies the efficacy of *M. anisopliae* var. *acridum* in controlling grasshopper populations was demonstrated against several species (e.g., Lomer et al., 2001; Arthurs et al., 2003). Both nymphs and adults are reported to be susceptible to oil-based formulations of *M. anisopliae* var. *acridum* and field treatments with plot sizes between 1 and 50 ha resulted in population reductions of up to 90% in two to three weeks (Langewald et al*.,* 1999).

Bait formulation protect spores from sunlight and attract grasshoppers, thereby, increasing contact with the spores. The high mortality and density reductions recorded in bait formulations of *M. anisopliae* var. *acridum* result from grasshoppers becoming surface contaminated while ingesting the bait substrate. Caudwell and Gatehouse (1996ab) suggested that insect intestinal canals are inhospitable to fungi and, despite the fact that the foregut and hindgut are lined with unsclerotized cuticle, invasion rarely occurs there. According to Charnley (1992), digestive enzymes, adverse pH, speed of food throughput, protection by the peritrophic membrane and antifungal phenols induced loss of fungal viability after ingestion. Therefore, fungal infections may have occurred mainly by contact with mouthparts and other areas of the body during feeding resulting in greater mortality than the edible baits.

First evidence of mycosis in the treated plots appeared from day 6 post-application when few grasshoppers showing characteristic red coloration were commonly observed lying dead on the soil, other being directly scavenged by coleopteran species and ants. Nearly all cadavers from the cage samples (samples from *M. anisopliae* plots) developed clearly signs of mycosis upon death. The high relative humidity and temperature recorded over the first 3 days in the treatment zone would have favored infection development in grasshoppers, the majority of which presumably became infected during this period.

Species in the subfamily Oedipodinea (e.g., *O. senegalensis*) are generally susceptible to *P. locustae* (Lange, 2005). Given the high proportion of *O. senegalensis* in the selected sites, it is reasonable to expect relatively high rate of *P. locustae* infection in this species and subsequently significant population density reduction in the present study. Another factor that may have contributed to the density reduction in the *P. locustae* treatment was the timing of application during the peak of first, second and third instars. Up to 85% of *O. senegalensis* populations was between first and third instar nymphs during the treatment. In general, targeting earlier instars grasshopper is considered to be the most suitable for *P. locustae* application (Erlandson et al., 1986, Lange et al., 2000). Field studies indicated that young grasshoppers are more susceptible to *P. locustae* and are killed at lower levels of infection than are older nymphs (Henry, 1973). Bomar and Lockwood (1991) showed that the third instar (compared to earlier and later stages) consumes the greatest amount of bran relative to its body weight, and Streett (1995) recommended that *P. locustae* treatment should be applied to hatchlings if significant population reduction is to be expected. Most commonly, *P. locustae* is considered as a long-term control agent and that infection continue into the year after treatment (Henry and Onsager, 1982; Lockwood and DeBrey, 1990; Bomar et al., 1993). However, some field results suggested that with proper timing, grasshopper reduction of 50-60% could be obtained within 4 to 6 weeks, with 35-40% infection in survivors (Henry et al., 1973; Henry and Oma, 1974). One important factor that may contribute to the effectiveness of *P. locustae* treatment was the rate of application. Dosage of 2.5 x 10^9 spores applied at a rate of 1.68 kg wheat bran per hectare has been recommended for an optimal application (Bomar and Lockwood, 1991). Although hand spreading method used in the present study may not ensure even coverage of the treated plot, it appears that there was probably sufficient bran for most grasshoppers given that twice of the recommended dosage was applied at a rate of 2kg bran.

Baits used for the formulation of insect control agents may be made up of several components including attractant and phagostimulants (Caudwell, 1993; Caudwell and Gatehouse 1996ab). Certain sugar derivatives such as fructose, fucose, glucose, and

particularly sucrose and raffinose act as feeding stimulants. Insects fed bait carrier with sugar derivatives as added phagostimulants induce sustained proboscis extension response as well as an extended gustatory period, leading to the increase in the rate of infective spores ingested (Lopez et al., 2000). The *P. locustae* and sugar bait developed in this study was not effective at causing higher mortality and population reduction compared to *P. locustae* bait without sugar. Detailed comparisons of research findings are difficult because of lack of data the use of sugar as phagostimulant in bait formulation of *P. locustae*. We can only speculate that grasshoppers might not be able to consume over certain quantity of bait. Moreover, it is possible that consumption of as little as a single *P. locustae*-treated bran could induce mortality, although individuals varied greatly in their susceptibility.

Population density was significantly lowered in combined application compared to either pathogen applied alone, suggesting that the performance of the relative virulent *M. anisopliae* is enhanced by the presence of less virulent *P. locustae* and vice versa. The two pathogens act either independently or synergistically to increase host mortality and subsequently reduce population density. It is appeared that insects infected with both pathogens succumb to mycosis rather than microsporidiosis, however impact of the two pathogens acting together became more obvious later in the course of the experiment. This was evidenced by the significantly higher decline in grasshopper densities in the plots treated with combination of the two pathogens compared to plots treated with each pathogen alone. There have been numerous studies on the effect of multiple pathogens infecting the same host (Koppenhöfer and Kaya, 1997; Bauer et al., 1998; Malakar et al., 1999; Pierce et al., 2001; Ishii et al., 2002; Thomas et al., 2003; Pilarska et al., 2006). Double infections by different types of pathogens usually led to an increase in insect mortality. In the previous laboratory bioassay, we observed desert locust *S. gregaria* nymphs infected with combination of *P. locustae* and *M. anisopliae* var. *acridum* to be more likely to die than nymphs infected by each pathogen alone and speculated that the slow acting *P. locustae* stresses its hosts in a way that makes them less able to trigger the physiological and behavioural defense mechanism they have against *M. anisopliae*.

The most significant observation concerning the environment in the present study was the consistent high incidence of infection by *P. locustae* at the three treated sites. This is highly relevant for the transmission of the pathogen to the second generation of grasshopper and the long-term management concerns. Although the transmission of microsporidia between hosts occurs horizontally when the spores are ingested, the vertical transmission from parent to progeny via eggs is known to be an important route for many of the insect-infecting microsporidia (e.g., Ewen and Mukerji, 1980; Streett et al., 1993; Raina et al., 1995). In preliminary laboratory examinations we observed that 18-52% of the offspring from infected *O. senegalensis* were infected when the parents were infected at late instars, which suggests survival and continual impact of *P. locustae* as a regulating factor of grasshopper populations in this study. The results obtained from this study confirm that *P. locustae* used as microbial bioinsecticide alone or in combination with *M. anisopliae* has some potential as within-season biological agent of the main West African grasshopper species. This field application also provided evidence that integration of *P. locustae* with *M. anisopliae* on bait-carrier could be developed as a viable option to (1) total reliance on chemicals for grasshopper control, (2) to overcome the delayed action of *P. locustae*. However additional studies are required when applying the two pathogens simultaneously as microbial insecticide, to determine their abundance and distribution and their movement to ensure their survival in locust and grasshopper communities.

6 General Discussion

Locusts and grasshoppers are the most treating agricultural pests that represent with drought the first reason of famine in Sahelian regions of Africa. Concern about environmental and toxicological issues resulting from wide spread application of synthetic pesticide has stimulated studies on the development of microbiological insecticides based on Microsporidian *Paranosema locustae* Canning and the entomopathogenic fungus *Metarhizium anisopliae* var. *acridum* Driver and Milner. The main objectives of this study were to: (i) investigate the potential of *P. locustae* to control locusts and grasshoppers (Chapters 2 and 3) and (ii) the possibility of its combination with *M. anisopliae* to increase the efficacy of the two pathogens in regulation of locust and grasshopper populations in West Africa (chapters 4 and 5).

The evaluation of the relative susceptibility of developmental stages of *O. senegalensis* and *S. gregaria* to different dosages of *P. locustae* indicated both lethal and sublethal effects of the pathogen against the hosts. Considering the lethal effects all tested nymphal stages suffered from significantly higher mortality compared to the control with always the younger nymphal instars being more susceptible compared to the older. Sublethal effects of *P. locustae* in the tested hosts included among other, delayed and abnormal development of the infected hosts, reduced host weight and vertical transmission of the pathogen from infected adults to the offspring. Laboratory investigation of the interaction of *P. locustae* and *M. anisopliae* indicated that additional inoculation with *M. anisopliae* following infection with *P. locustae* cause additive and synergistic mortality response of the two pathogens in fifth instar *S. gregaria*, resulting in both a significant reduction of Median Survival Time (MSTs) and a higher mortality in mixed infections compared to the single infection. Significantly fewer *P. locustae* spores were yielded in mixed infections compared to nymphs infected with *P. locustae* alone. However, *M. anisopliae* production was not affected by the mixed infections. Last but not least, based on the laboratory bioassay testing the relative susceptibility of grasshopper and locust developmental stages to *P. locustae*, a field trial was designed to target the younger instar of grasshopper. The results showed that within season grasshopper population reduction might be feasible when the pathogen is applied earlier after hatching. Moreover combined

application of *P. locustae* and *M. anisopliae* under field condition confirmed that mixed application of the two pathogens could help to increase host population reduction in short time periods.

One of the main characteristics of the African grasshoppers and locusts is their ability to undertake long distance dispersal. Since *P. locustae* does not act quickly in killing its host, infected grasshoppers have the possibility to move to other localities, carrying the pathogen in their body. Normally, the transmission microsporidia between hosts occurs when the spores are ingested. Vertical transmission from parent to progeny is known to be an important route for many of the insect-infecting microsporidia. The pathogen overwinters in living grasshopper and is transmitted to the next generation via eggs. Such transmission route appears as the most appropriated in the sahelian zone of Africa given the high temperatures that could compromise the survival of dry spores released in the environment. Most terrestrial host microsporidia are to some degree tolerant to desiccation but they do not usually survive as long as they do in refrigerated aqueous suspensions or frozen (Rai et al., 2000).

In addition to the long time lag between introduction of *P. locustae* in Sahel and the high temperatures recorded in Sahel, the continuous use of synthetic chemical for control of locusts and grasshoppers may have contributed indirectly to the display of many other natural enemies among which *P. locustae* through host population reduction. van der Valk and Niassy (1997) summarized the impact of insecticide treatments on natural enemies of locusts and grasshoppers, and discussed the possibility that insecticide application may have increased the impact of acridids in the Sahel (van der Valk et al., 1999). During the outbreak of the desert locust *S. gregaria* in the Sahel between 1986 and 1989, over US$ 200 million were spent on the application of 15 million litres of chemical pesticides to control grasshoppers and locusts (Symmons, 1992).

Synthetic pesticide applications should not be considered as the only factor that contributes to the display of biological control agents in pest populations. Indeed the present study showed that continuous application of *M. anisopliae* in the areas where

the relatively less virulent *P. locustae* is introduced could contribute to the reduction in the prevalence of the latter over time. Following the first introduction of *P. locustae* in Africa conducted from 1982 to 1990 in, among others, Senegal, Mali and Cap Verde, the use of *P. locustae* in Africa has been abandoned in the favour of the relatively more virulent entomopathogenic fungus *M. anisopliae*, because these first field tests did not yield expected results. In contrast however, repeated and wide applications of *M. anisopliae* were conducted in Sahelian grasshopper ecological zones as an alternative to the intensive use of synthetic pesticides (Lomer et al., 1993; Douro-Kpindou et al., 1995; Langewald et al., 1999; Lomer et al., 2001; Arthurs et al., 2003).

In the light of the results from the laboratory bioassays testing different concentrations of *P. locustae* spores against different nymphal instars of *O. senegalensis* and *S. gregaria*, the effects of *P. locustae* on the two species can be classified as direct and indirect effects. The direct host mortality effect of *P. locustae* is greatly pathogen concentration dependent with younger instars being more susceptible than older instars. The longer Median Survival Time (MST) recorded in older nymphal developmental stage compared to the younger, confirms the high susceptibility of the latter. Almost 100% mortality can be reached in first and second instars in a maximum of 10 days while most fourth and fifth instar nymphs can survive *P. locustae* infection up to adulthood. Mortalities recorded when targeting the host at older stages remain low irrespective of the pathogen dosages. Although some of the results show a low susceptibility of the tested host to the pathogen (e.g., low concentrations against fifth instars, the indirect sublethal effect of the pathogen can be considered as an additional important factor that can contribute to overcome its low virulence. Younger instars infected at low *P. locustae* spore concentrations that did not die from the pathogen infection in most cases did not reach adulthood and remained small in their size with low weight. Moreover common malformations of wings and hind legs observed in infected nymphs that developed to adults could affect the population dynamics of grasshoppers and locusts since morphologically affected hosts are unlikely to move and fly as normal healthy ones. Only infected adults that have survived the pathogen infection were able to mate and produce eggs

with very high potential to transmit *P. locustae* to the hatching nymphs. However the slow development of *P. locustae* infection remains one important limitation in the use of this pathogen since for the grower the first and probably the only objective is to kill the pest fast and with high efficacy to avoid resurgence of the pest and need for repeated treatments.

Our laboratory study testing the host mortality response to the combination of *P. locustae* and *M. anisopliae* indicated that a combination of the two pathogens increased significantly host acute mortality with consistent reduction in host survival time. This is further supported by the fact that after inoculation of *Paranosema*-infested fifth instar nymphs, total mortality rates were recorded within 4 to 7 days in host infected at the highest concentrations of the two pathogens. However, although the two pathogens showed ability to develop in the same host, the production of *P. locustae* spores was significantly lowered in dual infection. A successful integration of the two control agents, i.e. *P. locustae* and *M. anisopliae* var. *acridum*, depends on the possibility to minimize direct and indirect detrimental effects on the relatively less virulent *P. locustae*.

Application of *P. locustae* alone or in mixture with sugar or *M. anisopliae* resulted in significant grasshopper density reduction in three weeks, with up to 40% *P. locustae* infection in surviving grasshopper in plots that have received *P. locustae* spores, suggesting the possible persistence of the pathogens in the following generations. The general ability of *M. anisopliae* var. *acridum* and *P. locustae* to infect locusts and grasshoppers (Brooks, 1988; Henry, 1990; Langewald et al., 1999), the high relative humidity (leading to a >90%) and temperature (24-31 °C) at the time of pathogen applications, which correspond with the optimum growth conditions for both pathogens (Ouedraogo et al., 1997; Hallsworth and Magan, 1999; Lowman et al., 2000) might have contributed to the high infection and density reduction recorded in the present experiment. Moreover, the relatively high predominance of younger instars of the Senegalese grasshopper, *O. senegalensis* (>76% were represented by first, second and third instar nymphs) expands the previous laboratory observations on the high virulence of *P. locustae* against young

grasshopper and locust instars. The field trial confirmed the efficacy of combination of *P. locustae* and *M. anisopliae* for control of locust and grasshopper populations.

Results obtained from this study suggest that *P. locustae* is of a value as long-term biological control agent of *S. gregaria* and *O. senegalensis* in West Africa. Although *P. locustae* does not cause conspicuous mortality as do the chemicals currently used, its increased developmental time, the decreased survival of the infected host, and the fact that infected nymphs are apparently unable to reach adulthood, would have an impact on the intrinsic rate of population increase, leading to lower population density levels in the long term. In our study we have observed that both *O. senegalensis* and *S. gregaria* readily consumed wheat bran the way in which *P. locustae* is delivered (Henry and Oma, 1981). In large sector of the locust and grasshopper outbreak area, ground cover is relatively poor, meaning that nymphs would easily locate baits.

Grasshopper control in the Sahel has always been difficult to justify in purely economic terms. Most commonly, the value of the millet crop is so low that farmers are not ready to invest in any inputs, and in case of emergencies due to grasshopper outbreaks, simple food aid is considered to be cheaper than plant protection operations. Cost–benefit calculations are difficult to apply in such marginal systems where only a minor proportion of the crop is reaching the markets. Apart from logistic and socio-economic problems such as disruption of social structures, distortion of local food markets, and unemployment linked to food aid, there are other externalities, connected with grasshopper damage justifying control operations, which should be evaluated in term of costs arising from negative impact on general environmental and health problems. The currently applied curative treatments rely on synthetic insecticides covering much larger areas compared with preventative approaches using biological control agents, putting under risk human health and the environment. One reason for this approach is that it is rather easy for plant protection agencies to find emergency financial support from donor agencies, while finding sustainable support for a more preventative control strategy is difficult. The plant protection services in Sahelian countries have financed the greater part of their operations since 1985 from external aid. For instances, foreign aid covered over 75%

of the costs of Senegal's plant protection agency, over 96% of the costs in Niger and 87% of the costs in Mali (Kremer and Lock, 1992). In such situations, farmers benefit from free governmental services, which has attributed to their attitude, that grasshopper control is a public pest and the government's responsibility. This is reflected by the fact that in these countries, governmental spray operations are commonly used as political strategies.

Another field for economic studies is the comparison of the direct costs of different control options, and the quantification of the benefit of integrated control approaches compared to the old fashion curative control strategy. Given the cost and general environmental and health problems associated with large-scale applications of insecticides, number of projects in Africa, namely LUBILOSA and PRéLISS programs proposed that integrated pest management techniques be developed and used to keep grasshoppers below economically damaging levels as an alternative to standard grasshopper control programs. This approach could help to reduce the amount of pesticides applied, which in turn will reduce not only application costs, but also the negative effects on non-target organisms. Compared to synthetic pesticides, the host range of *P. locustae* and *M. anisopliae* var. *acridum* is generally narrow (Lomer et al., 1999, Lomer et al., 2001; Lange, 2005; Solter et al., 2005) and at field application rates they are safe to non-target Hymenoptera, Coleoptera and Homoptera (Ball et al., 1993; Prior, 1997). The lack of toxicity of *P. locustae* and *M. anisopliae* to mammals, birds and other beneficials (El-Kadi et al., 1983, Zimmermann, 1993), offers an advantage over currently organophosphate pesticides in the Sahel, where knowledge on the safe handling and application of pesticides is often lacking.

Large numbers of microsporidian spores can sometimes be obtained from field-collected hosts. However, because of the greater certainty that the spores are all of the same species, production in the laboratory is preferred as a source for experimental work. Unlike *M. anisopliae* that are produced on readily available media and applied with conventional spray equipment (Cherry et al., 1999), *P. locustae* is commonly produced in vivo although some cases of successful in vitro propagation have been reported (Khurad et al., 1991). In general, mycopesticides are estimated

to cost in the range of $10 to $20 per ha including production and application costs, which is somewhat higher than most chemicals (Quinn et al., 2000). Nevertheless, an advantage of the *M. anisopliae* and *P. locustae* formulations is their long persistence in the field. Thomas et al. (1997) reported a half-life of the spray residue of 7.7 days under Sahelian conditions. Healthy grasshoppers migrating into treated plots can still be infected by the fungus up to three weeks after application. Similarly, infection of *P. locustae* occurs vertically via eggs (Henry and Omar, 1981; Canning, 1982; Raina et al., 1995) and most importantly, horizontally through ingestion of propagules (Henry and Oma, 1981; Johnson, 1997; Lockwood et al., 1999; Lange, 2002). Infected grasshoppers are weakened and become attractive as a food source to the healthy grasshoppers, thereby spreading the disease. Grasshoppers are extremely migratory and can move over great distances. Therefore, frequent application of *P. locustae* throughout the season will help to spread the infection further and aid in long term control.

Pathogens used in the present study were derived from commercial products (*M. anisopliae* var. *acridum* from Green Muscle® IITA-Benin and *P. locustae* from M and R Durango, Inc. P. O. Box 886 Bayfield, CO 81122 USA). This suggests that microbial control of locusts and grasshoppers combining the two pathogens in bait could be readily implemented. Innovative products such as *P. locustae* and *M. anisopliae* formulations, however, are unlikely to be accepted unless they are easy to handle. The oil-based *M. anisopliae* formulation has proved to be easy to handle, involving a minimal amount of preparation before spraying, and thus helped to achieve an acceptably high work rate (Douro-Kpindou et al., 1995; Bateman et al., 1997; Johnson, 1997; Langewald et al., 1999; Lomer et al., 1999; Lomer et al., 2001). Interestingly, manual application of wheat bran formulation of *P. locustae* and *M. anisopliae* in the present study has proven to induce consistent pathogen infections in grasshopper populations in Sahel. Such method could be more appropriated for the Sahelian growers given the small handled quantity of bran (2kg) applied per hectare. Compared to the use of toxic pesticide that requires or need more protection or risk avoiding technique for application, the hand spreading method required little work and less experiment from the operator.

However, further surveys are needed to evaluate the real impact of *P. locustae* in grasshopper communities in Africa. To this end, efforts should be made to recover African isolates of *P. locustae* from field populations of grasshopper. This could help to gather data on prevalence, host range, and geographical distribution of *P. locustae* for management of grasshoppers and others pests in Africa. Moreover, additional experiments are required to evaluate the survival of *P. locustae* in egg pods over the dry season, to determine the chance of vertical transmission of the pathogen in diapausing grasshopper species. Other modes of transmission of *P. locustae* (e.g., through feces, cannibalism) that contribute to the persistence of the pathogen should be investigated. Different techniques of applications and formulations should be tested to fully evaluate the impact of the Microsporidian *P. locustae* on locust and grasshopper populations. Moreover, studies on potential negative effects resulting from combining *P. locustae* with other biological control agent of locusts and grasshoppers like the entomopathogenic fungus *M. anisopliae* need to be carried out in order to achieve a suitable combination of existing biocontrol agents against locusts and grasshoppers. The facility and safety in the application of wheat bran containing *P. locustae* and *M. anisopliae* used as bait for effective control of locusts and grasshoppers in Sahel (as revealed by our field experiment conducted in Niger), where knowledge of the safe handling and application of pesticide is often lacking, is a prerequisite for taking into consideration such option in grasshopper management in Africa directly at the growers level.

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I am solely responsible for the views expressed in this thesis and hope that the assembled information will provide a useful tool in integrated pest managements of Grasshoppers and Locusts using the Microsporidia, *Paranosema locustae* and the entomopathogenic fungi, *Metarhizium anisopliae*.

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