<italic>Metarhizium</italic> pathogenesis of mosquito larvae.

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METARHIZIUM PATHOGENESIS OF MOSQUITO LARVAE

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ABSTRACT

Mosquitoes are arguably the most important arthropod vectors of disease, with over half the world's population at risk of mosquito-transmitted diseases. Recent studies show that *Metarhizium anisopliae*, a soil borne fungal pathogen of terrestrial insects, has potential as an environmentally friendly alternative to conventional chemical control of mosquitoes, yet the mechanism of how this terrestrial pathogen kills the aquatic larval stage is unclear. A multidisciplinary approach was taken to provide an overarching view of the host-pathogen interactions. It is demonstrated for the first time that *M. anisopliae* kills mosquito larvae via a mechanism that does not follow the traditional host-pathogen response. Although pre-formed virulence factors, mediating host mortality, are retained, unlike true fungal pathogens it does not recognise or colonise the larva host. In addition it was found, *M. anisopliae* was unable to form and consolidate firm attachment to the larval cuticle in an aqueous environment preventing the normal route of invasion and therefore pathogenesis.
Exploring protein and gene expression profiles, of two sequenced species of *Metarhizium,* elucidated candidates for transcript analysis that may be involved in pathogenesis of *Metarhizium*, providing further understanding of the genes involved in host specificity and virulence. New insights are presented on the mode of pathogenesis to both mosquito larvae and terrestrial insect models. The evidence concludes that pathogenesis in a terrestrial environment cannot be extrapolated to the aquatic environment.

DECLARATION

This work has not previously been accepted in substance for any degree and is not being concurrently submitted in candidature for any degree.

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STATEMENT 1

This thesis is the result of my own investigations, except where otherwise stated. Where correction services have been used, the extent and nature of the correction is clearly marked in a footnote(s).

Other sources are acknowledged by footnotes giving explicit references. A bibliography is appended.

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CONTENTS

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LIST OF ABBREVIATIONS

1-DE	One Dimensional Electrophoresis
ACN	Acetonitrile
ARSEF	Agricultural Research Service Collection of Entomopathogenic Fungi
AFM	Atomic Force Microscopy
ANOVA	Analysis of Variance
cDNA	Copy Deoxyribonucleic Acid
CHCA	Cyano-4-hydroxycinnamic acid
C.I.	Confidence Interval
Cryo-SEM	Cryo-Scanning Electron Microscopy
CWP	Cell Wall Protein
DNA	Deoxyribonucleic Acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
FM	Fluorescence Microscopy
FP	Faecal Pellet
GAL	*Galleria*
gDNA	Genomic Deoxyribonucleic Acid
GC-MS	Gas Chromatography couple Mass Spectroscopy
GPI	Glycosylphosphatidylinositol
GST	Glutathione-S-Transferase
HK	Heat Killed conidia
ICM	Insect Cuticle Media
ID	Infected Dead (Mosquito)
IL	Infected Living (Mosquito)
L-DOPA	3, 4-dihydroxy-L-phenyl-alanine
LOC	Locust
Ma	*Metarhizium*
MALDI-TOF MS	Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry
MDA	Malondialdehyde

MS/MS	Tandem Mass Spectrometry
nESI-LCMS/MS	Nano-Electrospray High Performance Liquid Chromatography Tandem Mass Spectrometry
PCR	Polymerase Chain Reaction
PMSF	Phenylmethanesulphonyl fluoride
PO	Phenoloxidase
qRT-PCR	Real-Time Quantitative Reverse Transcription PCR
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
SDA	Sabouraud Dextrose Agar
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
SOD	Superoxide Dismutase
SP+	*Metarhizium* conidia exposed to larvae
SP-	*Metarhizium* conidia *sans* larvae
TBA	Thiobarbituric Acid
TCA	Trichloroacetic acid
TEN	*Tenebrio*
TFA	Trifluoric acid

CHAPTER 1

GENERAL INTRODUCTION

GENERAL INTRODUCTION

This thesis concerns the mode of pathogenesis of the entomopathogenic fungi, *Metarhizium* sp. to mosquito larvae. The following chapter provides an overview of mosquitoes and their medical importance followed by a summary of biocontrol and pathogenesis of *Metarhizium* and concludes with research aims.

1.1 BIOLOGY OF MOSQUITOES

Due to their importance as vectors of a wide range of debilitating viral and parasitic disease affecting both humans and animals, mosquitoes are at the centre of worldwide entomological research. As a consequence of their unique ability to colonise temporary as well as permanent water sites both clean and polluted, means that almost all aquatic habitats are a potential breeding site. *Anopheles* larvae can thrive in many environments, as a result they are often found in association with other species of mosquito in both fresh and salt water, grassy marshes, mangroves, ditches, edges of streams as well as small water collections. Arboreal species such as *Aedes cretinus*, *An. plumbeus*, *Ochlerotatus geniculatus* and *Orthopodomyia pulcripalpis*, preferentially reside in tree-holes, in contrast to species like *Cx. p. pipiens, Ae. aegypti*, *Ae. albopictus* and *Oc. j. japonicas*, which prefer small water fills and many manmade containers such as tyres, water drums and clays pots. This opportunistic habit has contributed to their spread globally, due to increased international trade of tyres and "Lucky bamboo" (McMichael & Lindgren 2011; Medlock *et al.* 2012).

Like all diptera mosquitoes exhibit complete metamorphosis, but require an aquatic habitat for development. After hatching they pass through four larval stages, a pupal stage and finally emerge as an adult mosquito (Figure 1.1). The larval development is temperature dependent, and depending on species there can be great differences in the optimum temperature for development (Table 1.1).

Table 1.1: Development time of larvae maintained at 25°C and 30°C (±2°C).

Species	Days	
	25°C	30°C
***Aedes aegypti* (Strain AeAe)**	7-10	5-6
***Anopheles stephensi* (Strain Beech)**	11-12	11-12
***Culex quinquefasciatus* (Strain Muheza)**	7-8	6-7

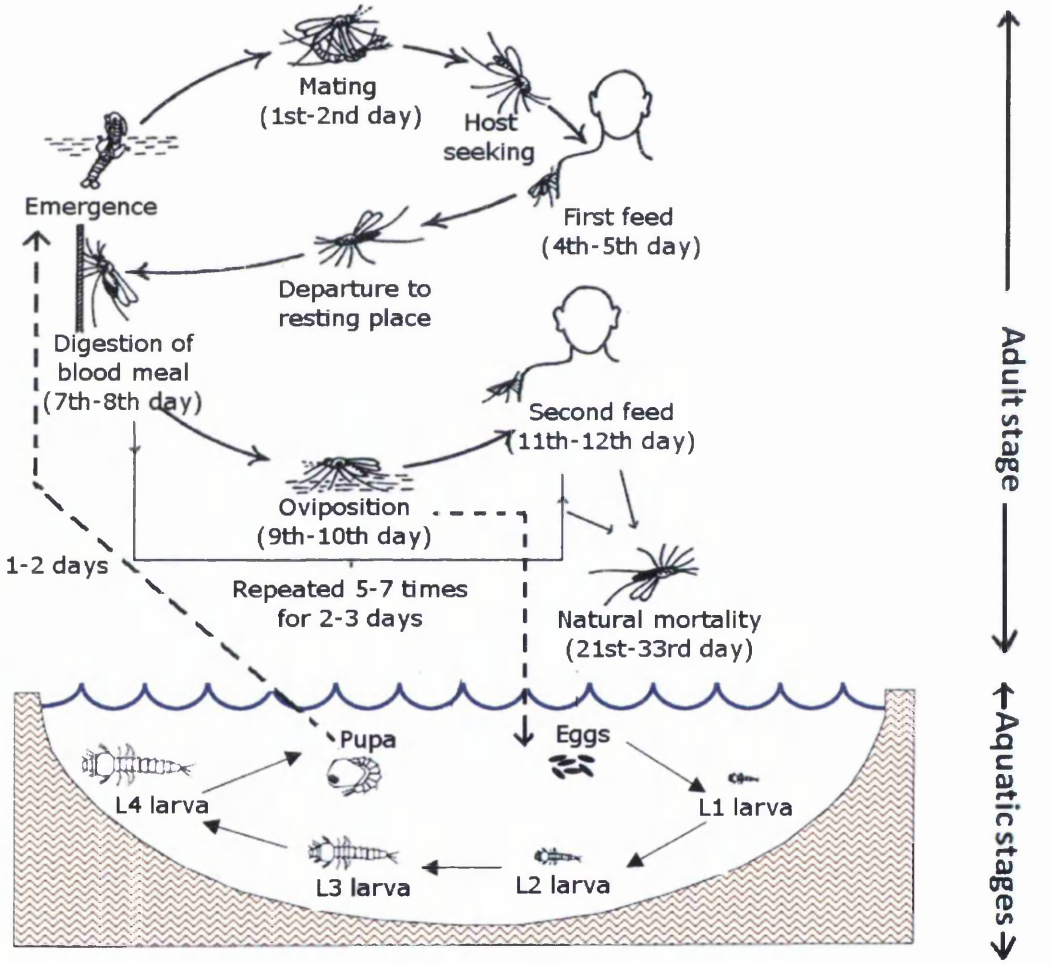

Figure 1.1: Mosquito life cycle. Mosquitoes pass through four larval stages before pupation and finally emerging as adults. Typical development from egg to emergence is 5-7 days (adapted from http://eltahir.mit.edu/tags/dengue-mosquito-climate-change).

1.1.2 Larval anatomy and behaviour

The larval body can be divided into three distinct sections 1) the head with eyes, mouth-parts and antennae; 2) a broader, sclerotized thorax and 3) the abdomen, which is further divided into seven segments with three modified posterior segments (Figure 1.2). The posterior segments bear four anal papillae modified for the regulation of electrolyte levels. Along the posterior segments, where tracheal trunks open at spiracles, the segment is modified with either spiracular lobes (in anophelines) or a siphon (in culicines), facilitating the intake of oxygen. Typically, anopheline larvae lie parallel to the water surface. The body is covered in palmate setae, allowing the larvae to maintain this position, whilst keeping spiracular lobes in direct contact with the air. In contrast, culicine larvae hang horizontally, using the siphon to break the water surface and access air.

Figure 1.2: Cryo-SEM image of *Aedes aegypti* larvae, a typical body plan for culicine larvae.

Mosquito larvae feed upon particulate matter within the water body, this largely consists of; microorganisms, protozoa, algae and detritus. Feeding behaviour can be broadly separated into three categories; 1) filter or suspension, 2) browsers or 3) predators. *Anopheles* and *Culex* species are categorised as 'active' suspension-feeders, by beating the mouth brushes (Figure 1.3) they feed on particulate matter within the water column, as well as grazing from the microbial film at the surface (Dahl, Widahl & Nilsson 1988; Merritt, Dadd & Walker 1992; Merritt *et al.* 1996; Workman & Walton 2003). In contrast, *Aedes* preferentially feed through browsing, harvesting particulates from surrounding substrates. *Aedes* species, however, do not feed exclusively in this manner, they will also filter feed (Eisenberg, Washburn & Schreiber 2000). Although larvae are not discriminate in what they ingest, particular

matter is usually less than 50 µm (Merritt *et al.* 1992, 1996). The behaviour and feeding habits are important to consider in the development of a larval control strategy.

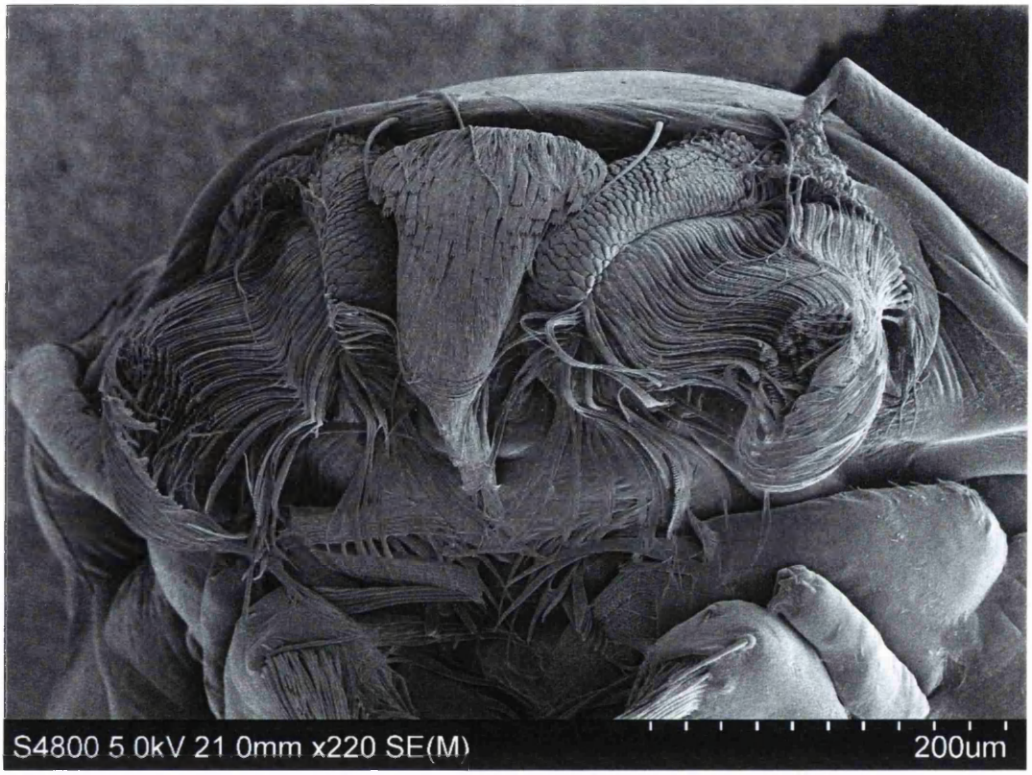

Figure 1.3: *Aedes aegypti* mouth brushes, adapted for filter feeding and browsing.

1.2 MEDICAL IMPORTANCE OF MOSQUITOES

Mosquitoes are arguably the most important vector of human disease, threatening more than three billion people in the tropical and subtropical regions, thus representing a greater health threat in these regions than any other arthropod vector of disease. Due to their blood-sucking habit, mosquitoes are able to vector and transmit parasites and pathogens from one vertebrate host, to another and are responsible for the transmission of the causative agents of several medically important diseases including; malaria, dengue, yellow and Chikungunya fever, encephalitis and filariasis (Table 1.2) (Mackenzie, Gubler & Petersen 2004; Brailsford & Berchi 2007; Becker *et al.* 2010a; Medlock *et al.* 2012).

Table 1.2: Examples of Mosquito Bourne Diseases

Disease	Type of infection	Country	Vector	Infects
Chikungunya	Arbovirus: Togaviridae (Alphavirus)	Africa and Asia	*Ae. aegypti*, *Ae. albopictus*	Humans
Dengue Fever (DF)	Flavivirus	Africa, Asia, Western Pacific region, Carribean, Central and South America and the Eastern Mediterranean	*Ae. aegypti*, *Ae. albopictus*	Humans
Eastern Equine Encephalomyelitis (EEE) virus	Arbovirus: Togaviridae (Alphavirus)	North, South and Central America and the Caribbean	*Cs. melanura,Cs. morsitans, Cq. pertubans, Ae. vexans, Oc. sollicitans,*	Humans, wide range of mammals, birds, reptiles and amphibians
Lymphatic Filariasis	Nematode	Tropics and subtropics	*Cx. quinquefasciatus*, *Anopheles* and *Aedes*	Humans, wide range of mammals and birds
Malaria	Protozoan	Subtropics, Sub-Saharan Africa	Anopheles Gambiae Complex *An. gambiae s.s, An. arabiensis*	Humans, wide range of mammals, birds, reptiles and amphibians
Rift Valley Fever (RVF)	Bunyaviridae (Phlebovirus)	Sub-Saharan and Northen Africa	*Cx. pipiens*, *Ae. africanus*, *Anopheles* spp.	Humans domesticated animals: cattle, sheep, camels and goats.
Ross River Virus (RRV)	Arbovirus: Togaviridae (Alphavirus)	Australia, Papua New Guinea	*Ae. vigilax*, *Ae. aegypti*, *Ae. polynesiensis*and *Ma. Uniformis*	Humans
West Nile Virus (WNV)	Flavivirus	Africa. Europe, Middle East, Asia and North America	*Cx. pipiens, Cx. modestus, An. maculipennis, Oc. Cantans, Cx. quinquefasciatus*	Humans, birds
Yellow Fever (YF)	Flavivirus	North, South and Central America, Africa, Europe and the Caribbean	*Ae. aegypti*	Humans, Monkeys and numerous other vertebrates

As a consequence of their ability to colonise temporary as well as permanent water sites, there are few aquatic habitats that do not lend themselves as a breeding site, often resulting in overlapping habitat range of the three major genera of vectors of disease: *Aedes*, *Anopheles* and *Culex* (Yasuoka & Levins 2007; Becker *et al.* 2010b).

1.2.1 *Aedes* sp.

Aedes aegypti is the primary vector of dengue virus, the causative agent of dengue fever, a severe flu-like illness infecting 50-100 million children and adults every year (WHO 2014a). Although the disease itself seldom ensues death, severe infections have the potential to develop into the more severe dengue haemorrhagic fever, causing pneumonia and respiratory distress, internal bleeding and organ failure (Petersen & Marfin 2005; WHO 2014a). *Aedes* is predominately found in tropical and sub-tropical regions; however, recently disease transmission via this vector has increased in many urban and sub-urban areas causing a major international public health concern. The spread of dengue is linked with both expanding geographic distribution of the four dengue serotypes (DEN1, DEN2, DEN3 and DEN4) as well as the increasing habitat range of both *Ae. aegypti* and *Ae. albopictus* (secondary vector) (Becker *et al.* 2010a; Medlock *et al.* 2012). In addition to dengue fever, *Ae. aegypti* is also a highly effective vector of yellow fever, historically the most important and dangerous mosquito borne disease (Becker *et al.* 2010a), a causative agent of haemorrhagic fever, resulting in 29,000 – 60,000 deaths in Africa in 2013 (WHO 2014b).

1.2.2 *Anopheles* sp.

Malaria is amongst the world's most prevalent infectious diseases, affecting 135 – 287 million people a year resulting in an estimated 627,000 deaths annually (WHO 2014c). Affecting more than 100 tropical countries, approximately 40% of the world's population is at risk of malaria, however, it is most prevalent in sub-Saharan Africa (Becker *et al.* 2010a; WHO 2014c). Malaria is transmitted exclusively via *Anopheles* mosquitoes, of which there are currently 20 species implicated (WHO 2014c). Although they are not a primary vector, Anopheline mosquitoes are also effective vectors of lymphatic filariasis (WHO 2014d).

1.2.3 *Culex* sp.

In addition to Anopheline vectors, *Cx. quinquefasciatus* is one of the primary vectors of lymphatic filariasis, infecting 120 million people across 73 countries, it is the fastest spreading vector borne disease of humans within the tropics (Scholte *et al.* 2003a; WHO 2014d). *Cx. quinquefasciatus* belongs to the *Cx. pipiens* complex and is the third most commonly distributed mosquito in the world (Scholte 2004). Although filariasis is not of medical importance in Europe, it does pose a serious threat to animals, causing cardiovascular dirofilariasis in dogs and other canids (Becker *et al.* 2010a).

1.3 BIOLOGICAL CONTROL OF INSECT PEST SPECIES

Increasing reports of resistance in mosquito populations to insecticides, and emergent resistance of pathogens to available drugs (Otranto & Wall, 2008), as well as increasing concern for the safety of chemical pesticides on human and environmental health, all present a serious threat to conventional chemical control efforts (Hemingway, Field & Vontas 2002; Vontas, Ranson & Alphey 2010). As a consequence, the use of alternative methods such as natural products, predators and entomopathogenic fungi have been explored (Seye *et al.* 2009).

Biocontrol is typically defined as the use of natural enemies, such as predators, parasites, pathogens or the toxins produced by microorganisms (Woodring & Davidson 1996). Biological control aims to reduce and suppress the target pest population whilst avoiding adverse effects to the environment and non-target organisms.

During the late nineteenth century, natural predators, such as dragonflies and mosquito fish (*Gambusia affinis* and *G. holbrooki*) were successfully introduced into many countries and were utilised to control mosquito populations (Bellini, Veronesi & Rizzoli 1994; Legner 1995; Blaustein & Chase 2007; Walton, Van Dam & Popko 2009). The use of predaceous arthropods and their potential for large scale mosquito control is generally low. Some species have been used with success on a much smaller scale; this is particularly evident in the case of *Toxorhynchites*, a species of predatory mosquito. *Toxorhynchites* has a voracious appetite for the larvae of *Aedes*

and *Ochkerotatus*, and has been implemented successfully, in several field trial experiments (Focks, Seawright & Hall 1979; Collins & Blackwell 2000)

With the discovery and large scale use of synthetic insecticides during the 1940's and 1950's, biological control methods became less favourable and were no longer considered for controlling mosquito populations (Becker *et al.* 2010b). Over the past few decades, efforts on an international scale have led to the discovery of a great variety of pathogens, including entomopathogenic fungi, protozoa, bacteria and viruses (Chapman, Petersen & Fukada 1972; Davidson & Becker 1996)

1.3.1 Bacteria

Bacteria commonly implemented in mosquito control are the spore forming, rod-shaped bacteria belonging to *Bacillus* genus. These are typically soil borne terrestrial pathogens which include two of the most successful biocontrol agents to date, namely *Bacillus thuringiensis* (*B.t*) and *B. sphaericus* (*B.s*). For these bacteria to cause pathogenesis they must be ingested, the host is killed due to the release of one or several insecticidal toxins following ingestion (Lacey & Undeen 1986). *B.t* has a broad host range and is pathogenic to many insect crop and vector pests of the Lepidoptera, Coleoptera and Diptera (Lacey & Undeen 1986), however, changing the way *B.t* is formulated can improve specificity for a more targeted control (Mittal 2003; Boyce *et al.* 2013). The discovery of *Bacillus thuringiensis* var. *israelensis* (*B.t.i*) has revolutionised the use of *B.t* as a biocontrol agent. *B.t.i* is widely used in the control of mosquito larvae, however, there are concerns with its use due to the development of resistance in mosquito populations (Goldman, Arnold & Carlton 1986; Becker & Ludwig 1993; Paris *et al.* 2012).

1.3.2 Entomopathogenic Fungi and Oomycetes

Fungi are a phylogenetically diverse group occupying almost every niche in association with arthropods (Goettel, Inglis & Wraight 2000), they are an important component of soil microbial communities, where they function as decomposers, mycorrhizal mutualists and pathogens (O'Brien *et al.* 2005). Presently, there are thought to be over 1.5 million species of fungi (Hawksworth 2001; O'Brien *et al.* 2005) of which there are over 700, in 90 genera, that have been identified with entomopathogenic properties (Inglis *et al.* 2001; Faria & Wraight 2007; Roy *et al.*

2009). Virtually all insect species are susceptible to fungal infections, as a consequence, many species have been used in attempts to diminish insect pest populations (Table 1.3).

Table 1.3: Entomopathogenic fungi commonly used in insect pest biocontrol

Fungal Phyla	Fungal species	Insect host
Ascomycota	*Aschersonia aleyrodis*	Whitefly
	Beauveria bassiana	Broad host range
	B. brongniartii	Coleoptera, Hemiptera, Hymenoptera & Lepidoptera
	Hirsutella thompsonii	Hemiptera
	Isaria fumosorosea	Broad host range
	Lecanicillium lecanii	Broad host range
	Metarhizium acridum	Orthoptera
	M. album	Broad host range
	M. anisopliae	Broad host range
	M. brunneum	Broad host range
	M. flavoviride	Broad host range
	Nomuraea rileyi	Coleoptera, Lepidoptera
	Paecilomyces farinosus	Broad host range
	Tolypocladium cylindrosporum	Broad host range
Zygomycota	*Conidiobolus obscurus*	Hemiptera
	Empusa fresenii	Hemiptera
	Entomophaga aulicae	Lepidoptera
	E. gryli	Orthoptera
	Entomophthora muscae	Diptera
	E. thripidium	Thysanoptera
	Massospora cicadina	Hemiptera
	Pandora neophidis	Hemiptera
	Zoophthora radicans	Diptera, Hemiptera, Hymenoptera & Lepidoptera

*Red text highlights *Metarhizium* species.

These identified species are distributed across several taxa including two kingdoms. The phylum Oomycota (kingdom Chromista) (Leipe *et al.*, 1994) formerly classified within the kingdom Fungi, contains two genera, with the species *Lagenidium giganteum* and *Leptolegnia chapmanii,* which are known to be aquatic pathogens of mosquito larvae. Within Fungi, entomopathogens can be found in most taxonomic groups, excluding the higher Basidiomycetes. Although there are many important phyla of Fungi, namely the Chytridiomycota and Zygomycota, which have entomopathogenic properties, the most widespread pathogenic fungal genera are in

the Hypocreales of the Ascomycota. Within the Hypocreales lie two of the most important fungi, for use in biocontrol, *Beauveria* and *Metarhizium*. Several of these strains have now been registered commercially, primarily for the control of agricultural pests (Table 1.4).

Table 1.4: Examples of currently registered mycoinsecticides (adapted from Deacon 2006a)

Country	Registered product name	Fungus	Target Pest	Crop
Australia	BioGreen	*Metarhizium. flavoviride*	Cockchafer	Pasture, turf
Canada	Met52™	*M. anisopliae*	Black vine weevil, strawberry root weevil, thrips	Greenhouse crops and ornamentals,
France	Ostrinol	*Beauveria. bassiana*	Corn borer	Maize
Reunion	Betel	*B. bassiana*	Scarab beetle larvae	Sugar cane
South Africa	Green Muscle	*M. anisopliae*	Locust	Natural bush and
Switzerland	Engerlingspilz	*B. brogniartii*	Scarab beetle larvae	Pasture
Switzerland	Beauveria Schweizer	*B. brogniartii*	Scarab beetle larvae	Pasture
UK, Europe	Vertalec	*Lecanicillium lecanii*	Aphids	Greenhouse crops
UK, Europe	Mycotal	*L. lecanii*	Whitefly, Thrips	Greenhouse crops
USA	Mycotrol, Botanigard	*B. bassiana*	Whitefly, aphid, thrips	Greenhouse tomatoes and ornamentals
USA	Naturalis	*B. bassiana*	Sucking insects	Cotton, greenhouse crops
USA	NoFLY™ WP	*Isaria fumosorosea*	Whitefly	Greenhouse crops
USA	BioBlast	*M anisopliae*	Termites	Domestic houses
USA/Europe	PFR-97™	*I. fumosorosea*	Whitefly, thrips	Greenhouse crops

Strains of these fungi can be highly variable in their host specificity, as a consequence, the activity of any one strain cannot predict the activities of other

strains, emphasising the need for effective screening against target and non-target organisms.

1.3.2.1 *Beauveria* sp.

Beauveria is among the most commonly isolated entomopathogenic fungi, and is widely used to control a number of agri-forestry pests. *Beauveria* is known to be effective against both adult and larval stages of mosquito (Clark *et al.* 1968; Bukhari *et al.* 2010; Bukhari, Takken & Koenraadt 2011). The pathogen invades the larvae though respiratory apparatus i.e. siphon, spiracles and anal papillae, as well as entering through the mouth (Miranpuri & Khachatourians 1991; Bukhari *et al.* 2011). Limitations of *Beauveria* include the ability of this fungus to germinate without being in contact with the larvae, reducing infectivity, in addition, high dosage is required to be effective (Chapman 1974; Scholte *et al.* 2004). There are also serious concerns for the safety of vertebrates (Scholte *et al.* 2004; Zimmermann 2007).

1.3.2.2 *Coelomomyces* sp.

Coelomomyces genus contains over 70 obligatory parasitic aquatic fungi with a natural affinity for aquatic Dipteran insects, including mosquito larvae. Its life cycle is complex, with a developmental stage within an intermediate copepod host, followed by two mosquito generations (Lucarotti & Andreadis 1995). Following successful invasion, the fungus colonises the haemocoel and develops hyphae with sporangia at their tips, completely filling the haemocoel, killing the larvae (Roberts 1974; Federici 1981; Lacey, Lacey & Roberts 1988). *Coelomomyces* has had much interest as a biocontrol agent due to its ability to cause epizootics, persisting in larval populations for several years - providing there is a stable copepod population, as well as their relatively specific host range (Federici 1981; Lacey & Undeen 1986; Apperson *et al.* 1992; Kerwin & Petersen 1997).

1.3.2.4 *Culicinomyces* sp.

Culicinomyces is a facultative parasite of mosquito larvae and Dipteran larvae (Legner 1995). Pathogenesis of *Culicinomyces* is unusual among entomopathogenic fungi, its route of infection is through ingestion rather than direct penetration of the cuticle. Following successful invasion, the fungus colonises the body cavity with

mycelium of hyaline, septate, branched hyphae (Federici 1981; Sweeney 1983; Sweeney *et al.* 1983). Although larval control with *Culicinomyces* is good, storage can be problematic, with conidia losing virulence after 4 months at -20°C (Sweeney 1981; Lacey & Undeen 1986; Scholte *et al.* 2004).

1.3.2.4 *Metarhizium* sp.

Like *Beauveria*, *Metarhizium* is among the most common entomopathogenic fungi. *Metarhizium* is a soil-borne entomopathogen with a worldwide distribution, utilised in the control of many agri-forestry pests as well as arthropod vectors of disease (Kaaya & Hassan 2000; Smith, Wall & French 2000; Inglis *et al.* 2001; Bukhari *et al.* 2011).

Metarhizium, is characterized by green mycelia bearing asexual spores, termed 'conidia'. Often strains are more important than the species, as strains may be host and/or habit specific (Bidochka, Kamp & Amritha de Croos 2000). *Metarhizium* genus consists of two highly important species, widely utilised in biocontrol: *Metarhizium anisopliae* and *Metarhizium acridum*, these species are considered to be a generalist and a specialist species, respectively. *M. anisopliae* has a broad spectrum of target hosts. Although mosquito larvae are not considered to be a natural host of *M. anisopliae*, several strains have proved to be efficacious in the control of both adults and larvae (Roberts 1974; Al-Aidroos & Roberts 1978; Lacey *et al.* 1988; Alves & Alves 2002; Scholte, Takken & Knols 2007; Bukhari *et al.* 2011).

1.4 *METARHIZIUM* PATHOGENESIS

In contrast to bacterial control agents, typically entomopathogenic fungi do not have to be ingested in order to invade, colonise and subsequently kill their insect hosts, with the exception of *Culicinomyces*. The ability of entomopathogenic fungi to cause pathogenesis through direct penetration, removes the restriction of controlling one life stage or the reliance on a particular feeding mechanism, as required for *B.t.i.* As a result, entomopathogenic fungi can be used for the targeted control of non-

feeding stages, including both the eggs and pupae (Mochi *et al.* 2010a; b; Leles, D'Alessandro & Luz 2012).

In a terrestrial system, the fungus typically invades the insect though areas that are not heavily sclerotized, such as the intersegmental joints, mouthparts and also the spiracles, these regions offer conditions of high humidity which are favourable for germination and development of the fungi (Hajek and St. Leger 1994, Clarkson and Charnley 1996). *Metarhizium* is an opportunistic hemibiotroph, with two life stages during infection of its insect host. It lives parasitically in the living tissues of the host prior to switching to a saprophytic phase, colonising the cadaver post-mortem.

M. anisopliae pathogenesis follows a series of sequential steps, infecting susceptible hosts through a combination of enzymatic activity and mechanical pressure (Figure 1.4).

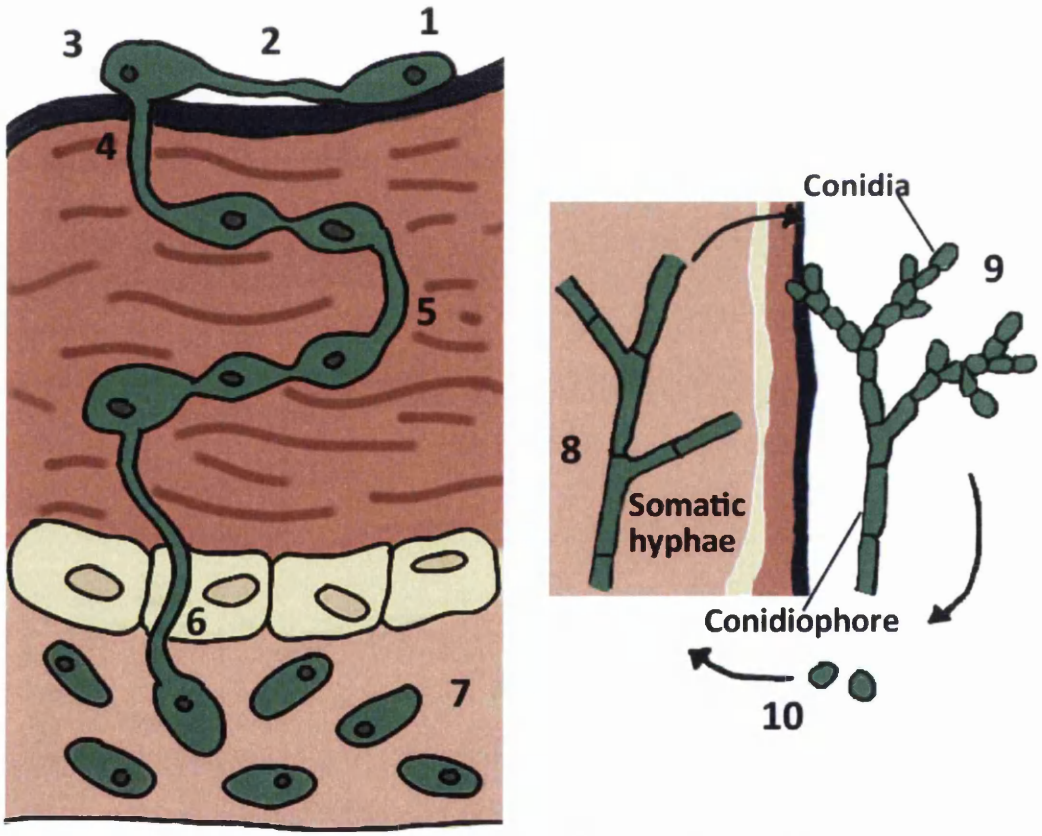

Figure 1.4: Typical infection process and life cycle of *Metarhizium anisopliae* (adapted from Deacon 2006; Thomas & Read 2007)

For an infection course to occur, initially the conidia must adhere to the host cuticle (Figure 1.4, **1**). Pathogenesis is a two-step process involving an initial passive attachment mediated though a combination of non-specific hydrophobic and electrostatic forces as well as attachment via specific ligands or specific adhesin protein interactions (Holder & Keyhani 2005; Wang & St. Leger 2007a).

Like other filamentous fungi, the conidia of *M. anisopliae* possess an assembly of hydrophobins, comprising an outer rodlet layer. These Hydrophobins mediate adhesion to hydrophobic substrates, such as arthropod cuticle (Wessels 2000; Wösten 2001; Holder & Keyhani 2005; Holder *et al.* 2007), through non-specific hydrophobic interactions, establishing the pathogenicity of the fungus (Zhang *et al.* 2011). In addition to non-specific hydrophobic interactions *M. anisopliae* is known to produce two adhesions, *Mad1* and *Mad2*, which are responsible for adhering the fungus to insect and plant surfaces, respectively (Wang & St. Leger 2007a; Barelli, Padilla-Guerrero & Bidochka 2011). *Mad1* has shown characteristics associated with pathogenesis (Wang & St. Leger 2007a) it is also thought that *Mad1* complements hydrophobins producing a tighter interaction providing a stronger adhesion. The second step includes the secretion of hydrolytic enzymes to establish an infection court.

From the hosts perspective, the cuticle acts as a protective barrier to the external environment as well as the attachment and invasion of fungal propagules (Boucias & Pendland 1991) (Figure 1.5).

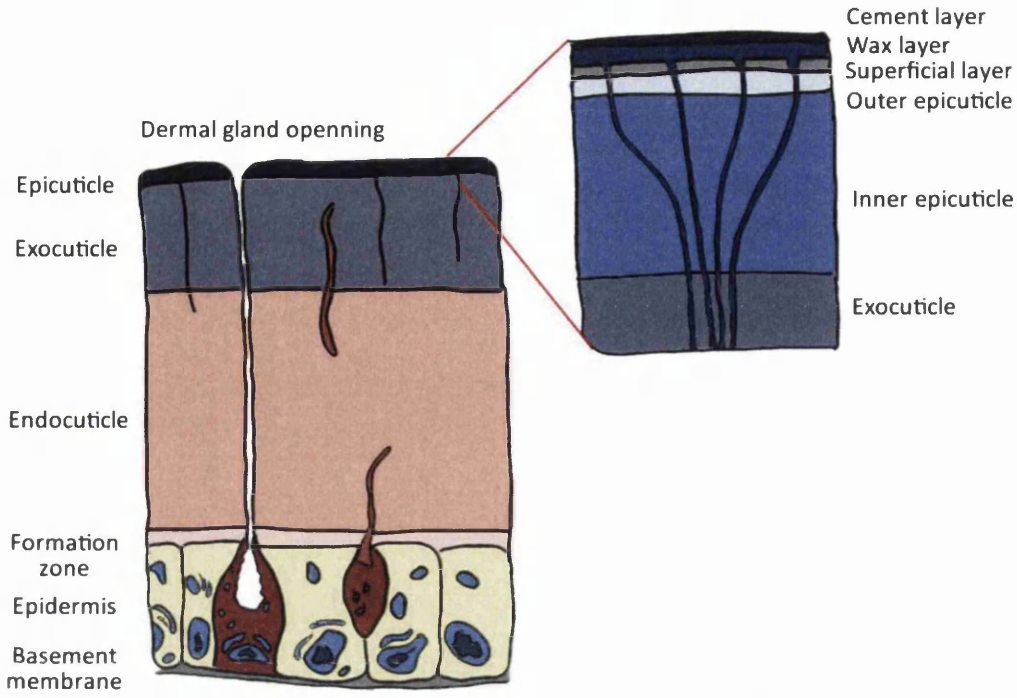

Figure 1.5: Insect cuticle structure (adapted from http://entomologyryk.blogspot.co.uk/2010/09/cuticular-appendages_06.html)

The epicuticle provides the substrate in which fungal conidia will first make contact, adhere and penetrate. The surface structure, topography and chemical composition is believed to play a role in the adhesion of fungal conidia to the host cuticle and therefore pathogenicity of fungus (Boucias & Pendland 1991; Lord & Howard 2004). Hydrophobic lipids within this layer play an essential role in attachment and germination of fungal propagules (Fargues 1984; Boucias & Pendland 1991; Pendland, Hung & Boucias 1993; James, Buckner & Freeman 2003). Hydrolytic enzymes secreted by the fungus degrade fungistatic fatty acids, release nutrients and facilitate penetration of the cuticle (St. Leger, Cooper & Charnley 1987; Paterson *et al.* 1994; Jarrold *et al.* 2007; Van Bogaert *et al.* 2011; Zhang *et al.* 2012), creating a more favourable environment for germination and production of a specialised infection structure, the appressoria (Boucias & Pendland 1991). Conidia require cuticular nutrients, namely long-chain fatty acids, lipids, sugars and amino acids to form a germ tube (Boucias & Pendland 1991; Bidochka *et al.* 2000) (Figure 1.4, **2**). Failure to germinate has been attributed to the presence of short chain fatty acids as well as inhibitory or fungistatic compounds, such as phenols and quinones (Hsiao, Bidochka & Khachatourians 1992). Differentiation to appressoria further promotes the production and secretion of cuticle degrading enzymes (St. Leger *et al.* 1989,

1996; Wang & St. Leger 2005) (Figure 1.4, **3**). Degradation of Lipids, within the epicuticle, to glycerol by the appressoria increases hydrostatic pressure providing greater mechanical pressure for penetration of the cuticle (Wang & St. Leger 2007b; Fang *et al.* 2009) (Figure 1.4, **4**), facilitated by the production of the subtilisin-like extracellular protease, Pr1 (St. Leger, Bidochka & Roberts 1994; Joshi, St. Leger & Roberts 1997; Wang, Typas & Butt 2002). Once the epicuticle (Figure 1.5) has been breached the hyphae proliferate within the cuticular layers (Figure 1.4, **5**) prior to invading the epidermal and hyperdermal layers (Figures. 1.4, **6** and 1.5) differentiating into yeast-like bodies or blastospores (Figure 1.4, **7**). Blastospores proliferate inside the insect haemolymph and colonise the haemocoel, producing a barrage of cytotoxic compounds, including a family of cyclic depsipeptide toxins, the destruxins (Samuels, Charnley & Reynolds 1988a; Samuels, Reynolds & Charnley 1988b; Kershaw *et al.* 1999). These Destruxins evoke the opening of the calcium channels, depolarizing body muscles within the insect, resulting in tetanic and flaccid paralysis or even death (Samuels *et al.* 1988a; b; Pal, St. Leger & Wu 2007).

Cell surface proteins and enzymes are pivotal in defining the host-pathogen interaction. Invasion by the fungi is typically recognised through the binding of proteins to β-1,3 glucans present within the fungal cell wall (Charnley 2003; Jiang *et al.* 2004). The insect humoral response is triggered by Pathogen Associated Molecular Patterns (PAMPs) and in the mosquito β-1,3 glucan recognition proteins stimulate the activation of the phenoloxidase cascade (Fabrick, Baker & Kanost 2003; Jiang *et al.* 2003; Kanost, Jiang & Yu 2004), resulting in melanisation and eventually death of the invading pathogen. In order to successfully colonise the insect host, the fungal pathogen must evade the host immune response, by camouflaging or altering β glucan composition on the surface. In *Metarhizium* a collagen like protein (MLC1) is known to prevent encapsulation and phagocytosis through altering the charge properties of the cell wall, masking the immunogenic β-1,3 glucans blocking pathogen recognition (Wang & St. Leger 2006). Inability of the insect host to recognise and remove short hyphal bodies and blastospores, allows the fungus to proliferate within the haemocoel, and establish itself causing death.

Insect haemolymph is rich in soluble sugars, predominantly comprised of the disaccharide, trehalose, which is an important nutrient source of *Metarhizium* (Xia,

Clarkson & Charnley 2002). The glyoxylate cycle plays an important role in both fungal and bacterial pathogenesis (Dunn, Ramírez-Trujillo & Hernández-Lucas 2009). In the presence of high sugar concentration, the glyoxylate cycle is repressed. Once available sugars have been metabolized and depleted *Metarhizium* reverts to a mycelial, saprotrophic phase, extensively colonizing the haemocoel (Figure 1.4, **8**). The glyoxylate cycle is up-regulated during re-emergence from the host cadaver, enabling the metabolism of lipids and fatty acids (Padilla-guerrero *et al.* 2011). In humid conditions, the fungus emerges through the intersegmental regions of the cuticle, producing conidiophores bearing numerous asexual conidia (Figure 1.4, **9**), which are later dispersed to infect another host (Figure 1.4, **10**).

M. anisopliae has shown great potential in its ability to control mosquito populations, both under field and a laboratory conditions (Riba *et al.* 1986; Scholte, Takken & Knols 2003b; Scholte *et al.* 2007; Bukhari *et al.* 2011; Paula *et al.* 2011b). It has been shown to work synergistically with chemical pesticides or when impregnated in black cloths (Howard *et al.* 2011; Paula *et al.* 2011a, 2013). Furthermore, *Metarhizium* will also kill mosquitoes that have developed resistance to conventional pesticides (Hancock 2009; Howard *et al.* 2011). There have been no reported cases of resistance to *Metarhizium* in mosquitoes, unlike *B.t.i.* and failure to germinate and persist in the aquatic environment, make *Metarhizium* a promising biocontrol agent. In addition to the control capabilities, conidia can be mass produced with ease on inexpensive substrates and stored for several months whilst retaining virulence (Daoust & Roberts 1983; Blanford *et al.* 2012).

Metarhizium, however, is not without disadvantages. Conidia are not produced in the water, as a consequence, frequent inundative applications are currently employed, unlike *Lagenidium* or *Coelomomyces* applications, which are able to maintain and persist in the mosquito habitat after a single application.

1.5 AIMS

The overall aim of this thesis is to investigate the underlying mechanisms conveying pathogenesis, virulence and specificity of *Metarhizium* sp. for use in mosquito larvae control

The aims for each chapter are as follows:

1) To identify the most efficacious strain of *Metarhizium* sp. for controlling larvae of three mosquito species (Chapter 2).

2) Investigate the underlying mechanisms contributing to the mode of pathogenesis (Chapter 3).

3) Investigate conidial attachment to mosquito larvae (Chapter 4).

4) Elucidate novel proteins for use as markers of specificity and virulence (Chapter 5).

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CHAPTER 2

IDENTIFICATION OF *METARHIZIUM* STRAINS HIGHLY EFFICACIOUS AGAINST *AEDES, ANOPHELES* AND *CULEX* LARVAE.

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Conceived and designed the experiments: **BPJG** & TMB.
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IDENTIFICATION OF *METARHIZIUM* STRAINS HIGHLY EFFICACIOUS AGAINST *AEDES, ANOPHELES* AND *CULEX* LARVAE.

2.1 ABSTRACT

Entomopathogenic fungi, such as *Metarhizium anisopliae* and *Beauveria bassiana*, have been shown to be highly efficacious in killing mosquito larvae of different mosquito species. Most often assays were conducted on a single genus of mosquito and/or specific instars. The current study compared the pathogenicity and efficacy of two formulations of three fungal strains against different instars of three mosquito species with the aim of identifying the most virulent strain for use under field conditions. Three strains of *Metarhizium,* ARESF 4556, ARSEF 3297 and V275, were assayed against early (L_{2-3}) and late (L_{3-4}) instar larvae of *Aedes aegypti, Anopheles stephensi* and *Culex quinquefasciatus.* Two formulations of the fungi were tested, dry conidia and aqueous suspensions (*i.e.* 'wet' conidia). Effects of all combinations of conidia, mosquito species, instar, fungal strain and concentration on mosquito mortality were analysed using Cox regression and Kaplan Meier analyses. Strain ARSEF 4556 was more virulent than ARSEF 3297 and V275, with LT_{50} values ranging from 0.3 to 1.1 days, with *Anopheles* and *Culex* being more susceptible than *Aedes.* Early and late instars were equally susceptible independent of species. Although the formulation did influence mortality rates, both 'wet' and 'dry' conidia applications were highly effective in controlling mosquito larvae. Viable spores were more efficacious than heat killed spores. The latter did cause mortality but only at high concentrations. *Metarhizium sp.* has proved to be effective in reducing survivability of all larval stages of *Aedes*, *Anopheles* and *Culex* under laboratory conditions. *Aedes* larvae were generally more tolerant than *Anopheles* and *Culex* irrespective of fungal strain.

2.2 INTRODUCTION

Mosquitoes of the genera *Aedes*, *Anopheles* and *Culex* are arguably the most important arthropod vectors of human and animal diseases worldwide and are increasing in importance due to their recent establishment in areas previously

unrecorded within Europe (Medlock *et al.* 2012). This increase in range, through a combination of climatic and environmental changes as well as the increase in international trade, poses considerable health risks (McMichael & Lindgren 2011; Medlock *et al.* 2012). Furthermore, there are increasing reports of resistance in mosquito populations to insecticides, presenting a serious threat to conventional chemical control efforts (Hemingway, Field & Vontas 2002; Vontas, Ranson & Alphey 2010). Mosquitoes are able to colonise temporary as well as permanent water sites and there are few aquatic habitats that do not lend themselves as a breeding site, often resulting in overlapping habitat range of these three genera (Yasuoka & Levins 2007; Becker *et al.* 2010).

Insect pathogenic fungi belonging to the genus *Metarhizium* have been developed to control a wide range of arthropods, including pests of crops and vectors of human and animal diseases (Frazzon *et al.* 2000; Orlando Beys Silva *et al.* 2005; Faria & Wraight 2007; Ansari *et al.* 2011). They are considered to offer an environmentally friendly alternative to chemical pesticides for the control of mosquitoes (St. Leger *et al.* 1996). *Metarhizium* is able to infect and kill all the developmental stages (eggs, larvae and adults) of *Aedes*, *Anopheles* and *Culex* mosquitoes (Alves & Alves 2002; Scholte *et al.* 2004; Albernaz, Tai & Luz 2009; Bukhari *et al.* 2010; Knols, Bukhari & Farenhorst 2010; Luz, Mnyone & Russell 2011). It has been shown to work synergistically with chemical pesticides or when impregnated in bed nets in the control of adult mosquitoes. Furthermore, *Metarhizium* will also kill mosquitoes that have developed resistance to conventional pesticides (Hancock 2009; Howard *et al.* 2011)

Fungal pathogenicity of arthropod hosts is influenced by a plethora of factors relating to the pathogen itself (e.g. virulence, specificity), environmental conditions (e.g. humidity, temperature) and the target host (Inglis *et al.* 2001; Wekesa *et al.* 2006). Previous studies have demonstrated that the hosts' developmental stage plays an important role in the efficacy of entomopathogenic fungi with early instar larvae usually being more susceptible to infection than older instars. This is clearly demonstrated for the European Corn Borer (*Ostrina nubilalis*) and the Diamondback moth (*Plutella xylostella*) (Feng *et al.* 1985; Vandenberg, Ramos & Altre 1998). There are some exceptions, for example Ekeski & Maniania found that legume

flower thrips (*Megalurothrips sjostedti*) larvae were less susceptible to *Metarhizium anisopliae* than the pupae and adults (Ekesi & Maniania 2000). Differences in susceptibility to the oomycete fungus *Lagenidium giganteum* were also noted for different instar mosquito larvae (Lord, Magalhães & Roberts 1987).

Various strategies have been used to control mosquito adults including release of sterile males, use of transgenic fungi with increased virulence and exploitation of synergies between the fungus and low dose insecticides (Farenhorst *et al.* 2010; Nolan *et al.* 2011; Fang *et al.* 2011; Gilles *et al.* 2013). The bacterium, *Bacillus thuringiensis israelensis* (*Bti*) is widely used to control larval stages of mosquitoes but there are concerns in its use due to the development of resistance in mosquito populations (Goldman, Arnold & Carlton 1986; Becker & Ludwig 1993; Paris *et al.* 2012). Currently there is much interest in the use of *Metarhizium* to control mosquito larvae with some strains killing as fast as *Bti* (Scholte *et al.* 2004; Butt *et al.* 2013; Boyce *et al.* 2013; Kroeger *et al.* 2013). Selection of a strain of *Metarhizium* that is stable and highly efficacious against all important mosquito genera will reduce both registration and application costs.

Until recently it was assumed the fungus propagated within the larvae, presenting the potential for inoculum to amplify within the host and horizontal transfer to occur (Riba *et al.* 1986; Lacey, Lacey & Roberts 1988; Bukhari *et al.* 2010). Recently we reported that the strains can kill without infection (Chapter 3: Butt *et al.* 2013), highlighting the need for a more in depth analysis of the relationship between dose and susceptibility. In light of these observations a re-evaluation of current control strategies is needed, as the route and mode of infection seriously impacts on rate and frequency of application and the overall control strategy.

This study provides the first comprehensive analysis of key parameters affecting fungus-induced mortality of mosquito larvae; it compares the efficacy of three strains of *Metarhizium* sp. against three major mosquito disease vectors using different doses and formulations of the fungus. We provide further evidence that the mode of pathogenesis is not the same as in a terrestrial system, although Pr1 undoubtedly plays a crucial role in pathogenesis, there are other factors that cannot be ignored, including the behaviour of the larvae and other cell wall components.

2.3 METHODS

2.3.1 Mosquitoes

Aedes aegypti (strain AeAe), *Anopheles stephensi* (strain Beech) and *Culex quinquefasciatus* (strain Muheza) eggs were obtained from the London School of Hygiene and Tropical Medicine, United Kingdom. All species were maintained in tap water and incubated at 25°C (± 2°C) in a 16L:8D photoperiod, and were fed on Tetramin® fish-food, whilst *An. stephensi* was also supplemented with fresh grass shoots.

2.3.2 Fungal Strains and Preparation

Aerial conidia of *Metarhizium anisopliae* isolates V275, ARSEF 4556 and *Metarhizium brunneum* ARSEF 3297 were produced through solid state fermentation using broken Basmati rice as previously described by Ansari and Butt (Ansari & Butt 2011). Conidial viability was assessed using the plate count technique on Sabouraud Dextrose Agar (SDA) (Vega *et al.* 2009), only those with viability above 90% were used in subsequent experiments.

All experiments were performed at room temperature (22°C ± 2°C) with a 16L: 8D photoperiod, in 250ml round containers (Diameter 92mm) with perforated lids. Each treatment was replicated three times with a control for each, and the whole experiment was independently repeated three times.

2.3.3 Pathogenicity of V275, 4556 and 3297 Using 'Wet' *Versus* 'Dry' Conidia

A series of experiments were conducted to determine whether larval susceptibility to fungal infection was influenced by 1) genera, 2) developmental stage, 3) fungus species/strain, 4) conidia concentration and 5) formulation of conidia

Mortalities were compared of early (L_{2-3}, 3-4 days old) and late (L_{3-4}, 4-8 days old) larval stages of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus* exposed to different concentrations ($1x10^5$, $1x10^6$, $1x10^7$ and $1x10^8$ conidia ml^{-1}) of *M. anisopliae* and *M. brunneum* conidia.

Metarhizium spp. conidia was either applied as a dry dust powder (dry conidia) or suspended in 0.03% Aqueous Tween 80 (wet conidia) and larvae were exposed to the fungal treatments for 12 days, to evaluate how the pathogenicity of fungal conidia and larval mortality is affected.

Ten larvae of either L_{2-3} or L_{3-4} were placed into round clear plastic containers containing 100 ml of conidia suspension or conidia dusted evenly over the water surface. Controls consisted of ten larvae exposed to 100 mL 0.03% Aq. Tween 80 or plain distilled water. Larval mortality was recorded daily for 12 days. To determine whether conidia caused blockage within the larvae or were required to actively bring about mortality, additional experiments were conducted with heat killed conidia, whereby the conidia were wrapped in aluminium foil and autoclaved for 15 min at 121°C, to denature enzymes and heat labile toxins.

2.3.4 Statistical Analysis

Effects of conidia, mosquito species, instar, fungal strain and concentration on mosquito mortality were analysed using Cox regression, resulting hazard ratio values were used to evaluate differential mortality rates. Survival curves were generated and Kaplan-Meier survival analysis was used to plot cumulative survival functions by treatment with pairwise comparison over log-rank test (Butt *et al.* 2013)

Mean lethal times, LT_{50} (for a concentration of $1x10^6$) and mean lethal concentrations LC_{50} (at day 6) were estimated for all combinations of conidia, mosquito species, instar and fungal species/strain by fitting probit regression models to the quantal response data. Log transformations were applied to the dosage and time independent variables. An additional parameter was fitted to the LC_{50} model, to account for the natural mortality rate of the trial mosquitos. Differences in LT_{50} and LC_{50} were compared by ANOVA and significance levels adjusted, where appropriate, by application of Tukey's multiple comparisons post-test.

All statistical analyses were carried out using SAS 9.3 (2011) and SPSS v16.

2.4 AIMS

1) To find a suitable strain of *Metarhizium* sp. for controlling the larvae of three mosquito species,

2) Identify the most effective concentration and application of *Metarhizium* sp.

3) Test whether larval instar or species has any influence of fungal efficacy

2.5 RESULTS

2.5.1 Virulence Strains (Based on Lt_{50} At 1 X 10^6 Conidia mL^{-1})

Significant differences in LT_{50} values were observed between treatments. 'Dry' and 'wet' conidia resulted in a significantly lower LT_{50} values than heat treated conidia [$F_{(2,60)}$ = 189.49; p<0.001] (Table 2.1). In terms of mosquito species, *Cx. quinquefasciatus* and *An. stephensi* were more susceptible to *Metarhizium* strains than *Ae. aegypti* [$F_{(2,60)}$ = 93.44; p< 0.001]. Larval instar had no overall effect [$F_{(1,60)}$ = 1.91; p=0.172]. For fungal species/strains, *M. anisopliae* ARSEF 4556 was more virulent, against all three mosquito species, than *M. brunneum* ARSEF 3297 and *M. anisopliae* V275 [$F_{(4,60)}$ = 3.92; p<0.007]

Significant interactions were also observed between the conidia application, fungal and mosquito species treatment Dry conidia of strain ARSEF 4556 were more virulent than ARSEF 3297 and V275, which had LT_{50} values ranging from 0.3 to 1.1 days , 0.3 to 3.1 days against L_{2-3} and L_{3-4} of *An. stephensi*, *Ae. aegypti* and *Cx. quinquefasciatus*, respectively (Table 2.1), all values being similar for heat killed conidia [$F_{(8,60)}$ = 3.37; p<0.003]. Similarly, lowest LT_{50} values were recorded for ARSEF 4556 when applied to *Cx. quinquefasciatus,* there being little effect of the various fungal species on *An. stephensi* and *Ae. aegypti* [$F_{(4,60)}$ = 3.92; p<0.007] (Table 2.1).

2.5.2 Dose Responses Test (6 Days after Treatment)

LC_{50} values of dry and wet conidia were significantly lower by 100 fold compared to heat killed conidia [$F_{(2,62)}$ = 112.78; p<0.001]. In general, lower LC_{50} values were

recorded against *Cx. quinquefasciatus* and *An. stephensi* compared with *Ae. aegypti* (Table 2.2; [$F_{(4,62)}$ = 5.54; p< 0.001] whilst main treatment effects of instar and fungal application and strain were not significant, ([$F_{(2,62)}$ =0.36; p=0.697], [$F_{(2,62)}$ =0.38; p=0.687] respectively).

The interaction between conidia application and mosquito species was significant (Table 2.2) [$F_{(4,62)}$ = 11.00; p< 0.001]. Under a dry application, all concentrations caused a significantly higher mortality than the control treatment ($p<0.05$); with the exception of *Cx. quinquefasciatus* larvae exposed to $1x10^5$ conidia mL^{-1} of 3297 (Figure 2.1, Table 2.3). A similar trend was observed under a wet application, all concentrations significantly reduced survivability, compared to the control ($p<0.05$), with the exception of *Ae. aegypti* larvae treated with $1x10^5$ conidia mL^{-1} of 4556 (Figure 2.2, Table 2.3). No significant differences in LC_{50} values were recorded between mosquitoes for dry conidia, while wet conidia significantly decreased LC_{50} for both *An. stephensi* and *Cx. quinquefasciatus* [$F_{(4,62)}$ = 11.00; p< 0.001]. The lowest LC_{50} values were observed for the combinations of dry conidia and *An. stephensi*, (all $<1.0 \times 10^5$, Table 2.2) whilst the highest LC_{50} values were observed for all strains against *Ae. aegypti*. (all $>1.7 \times 10^7$, Table 2.2). In the case where mosquitoes were exposed to heat killed conidia, LC_{50} values for *Cx. quinquefasciatus* were significantly lower than *An. stephensi* which in turn were significantly lower than *Ae. aegypti*.

Table 2.1. Mean lethal time (LT_{50}) in days for mosquito species treated conidial formulations of *Metarhizium anisopliae* (1×10^6 conidia/ml).

Formulation/conidia	Fungal strain	L_{2-3} (LT_{50})			L_{3-4} (LT_{50})		
		An. stephensi	*Ae. aegypti*	*Cx. quinquefasciatus*	*An. stephensi*	*Ae. aegypti*	*Cx. quinquefasciatus*
Dry	4556	1.1 (1.0-1.3)	1.5 (1.4-1.6)	0.3 (0.2-0.5)	1.2 (1.0-1.4)	3.1 (2.5-3.7)	1.0 (0.8-1.3)
	V275	1.4 (1.3-1.6)	3.2 (2.8-3.8)	4.1 (3.8-4.4)	1.2 (1.2-1.3)	4.5 (4.1-5.1)	3.5 (3.2-3.8)
	3297	2.8 (2.7-2.9)	5.7 (4.4-7.8)	1.9 (1.9-1.9)	1.2 (1.0-1.4)	6.3 (5.8-7.0)	1.8 (1.7-2.0)
Wet	4556	1.5 (0.9-1.7)	3.0 (2.2-4.9)	2.2 (1.3-3.3)	0.7 (0.5-0.9)	3.3 (2.8-4.3)	0.7 (0.6-0.8)
	V275	1.7 (1.2-2.0)	4.8 (3.9-5.6)	1.4 (0.9-1.8)	2.8 (2.6-3.0)	5.5 (4.4-7.2)	1.5 (1.2-1.7)
	3297	2.2 (1.4-2.8)	4.0 (3.1-4.9)	2.0 (0.9-3.3)	0.5 (0.2-0.8)	6.5 (6.2-6.8)	2.2 (1.6-2.8)
Heat killed	4556	15.5 (5.8- >30)	18.2 (14.2 - >30)	4.1 (2.8-7.2)	7.2 (5.7-11.0)	>30 ( )	3.6 (2.9-4.9)
	V275	7.9 (6.1-9.8)	15.7 (10.6-26.2)	11.6 (8.8-15.0)	5.9 (3.5-11.2)	23.1 (16.3-30.)	3.4 (2.6-4.5)
	3297	10.5 (8.2-16.0)	12.1 (8.5-17.8)	10.0 (2.0-18.6)	4.9 (2.9-10.3)	28.2 (20.8- >30	3.6 (3.3-4.1)

Mean lethal time (LT_{50}) for conidia formulations versus species, strain and larval life stage. 95% congenital intervals are given in parenthesis

Table 2.2 Mean lethal concentration (LC_{50}) for mosquito species treated conidial formulations of *Metarhizium anisopliae*

Formulation	Fungal strain	L_{2-3} (LD_{50})			L_{3-4} (LD_{50})		
		Ae. aegypti	***An. stephensi***	***Cx. quinquefasciatus***	***Ae. aegypti***	***An. stephensi***	***Cx. quinquefasciatus***
Dry	4556	5×10^4 (nc)	$<1.0 \times 10^5$ (nc)	$<1.0 \times 10^5$ (nc)	9.7×10^4 (4.7×10^4 - 1.5×10^5)	$<1.0 \times 10^5$ (nc)	9.2×10^4 (nc)
	V275	3.7×10^5 (1.7×10^5 - 6.6×10^5)	$<1.0 \times 10^5$ (nc)	2.3×10^5 (1.4×10^5 - 3.4×10^5)	1.8×10^5 (9.7×10^4 - 2.9×10^5)	$<1.0 \times 10^5$ (nc)	2.3×10^5 (1.4×10^5 - 3.4×10^5)
	3297	6.1×10^5 (2.4×10^5 - 1.1×10^6)	4.2×10^4 (nc)	1.2×10^5 (nc)	3.4×10^5 (1.4×10^5 - 6.4×10^5)	$<1.0 \times 10^5$ (nc)	1.2×10^5 (nc)
Wet	4556	1.8×10^5 (1.2×10^4 - 7.2×10^5)	6.2×10^4 (1.7×10^4 - 1.1×10^5)	5.1×10^4 (1.2×10^4 - 1.0×10^5)	7.5×10^5 (4.0×10^5 - 1.2×10^6)	1.1×10^5 (6.7×10^4 - 1.6×10^5)	9.0×10^4 (nc)
	V275	1.3×10^6 (5.0×10^5 - 2.6×10^6)	4.0×10^4 (4.0×10^3 - 1.0×10^5)	1.2×10^5 (nc)	1.2×10^6 (6.2×10^5 - 2.0×10^6)	1.3×10^5 (8.5×10^4 - 1.9×10^5)	7.2×10^4 (nc)
	3297	1.0×10^6 (4.5×10^5 - 2.0×10^6)	5.4×10^3 (2.9×10^3 - 5.3×10^4)	5.7×10^4 (1.5×10^4 - 1.1×10^5)	3.2×10^6 (1.5×10^6 - 5.5×10^6)	1.8×10^5 (1.1×10^5 - 2.8×10^5)	1.4×10^4 (3.6×10^2 - 4.7×10^4)
Heat killed	4556	5.8×10^7 (3.1×10^7 - 1.4×10^8)	1.0×10^7 (nc)	1.4×10^6 (6.2×10^5 - 2.4×10^6)	$<1.0 \times 10^8$ (nc)	1.8×10^7 (2.4×10^6 - 7.4×10^7)	4.7×10^5 (3.1×10^5 - 6.8×10^5)

	V275	1.7×10^7 (9.7×10^6 - 2.9×10^7)	6.0×10^6 (2.2×10^3 - 9.7×10^6)	7.4×10^6 (3.4×10^6 - 1.4×10^7)	$<1.0 \times 10^8$ (nc)	3.0×10^6 (4.1×10^5 - 1.4×10^7)	3.7×10^5 (1.6×10^5 - 6.7×10^5)
	3297	9.2×10^7 (2.1×10^7 - 1.7×10^9)	1.1×10^7 (nc)	1.6×10^6 (3.7×10^5 - 3.4×10^6)	$<1.0 \times 10^8$ (nc)	2.3×10^6 (4.2×10^5 - 7.2×10^6)	2.2×10^5 (7.2×10^4 - 4.8×10^5)

Mean lethal dose (LC_{50}) for conidia formulations versus species, strain and larval life stage. 95% congenital intervals are given in parenthesis

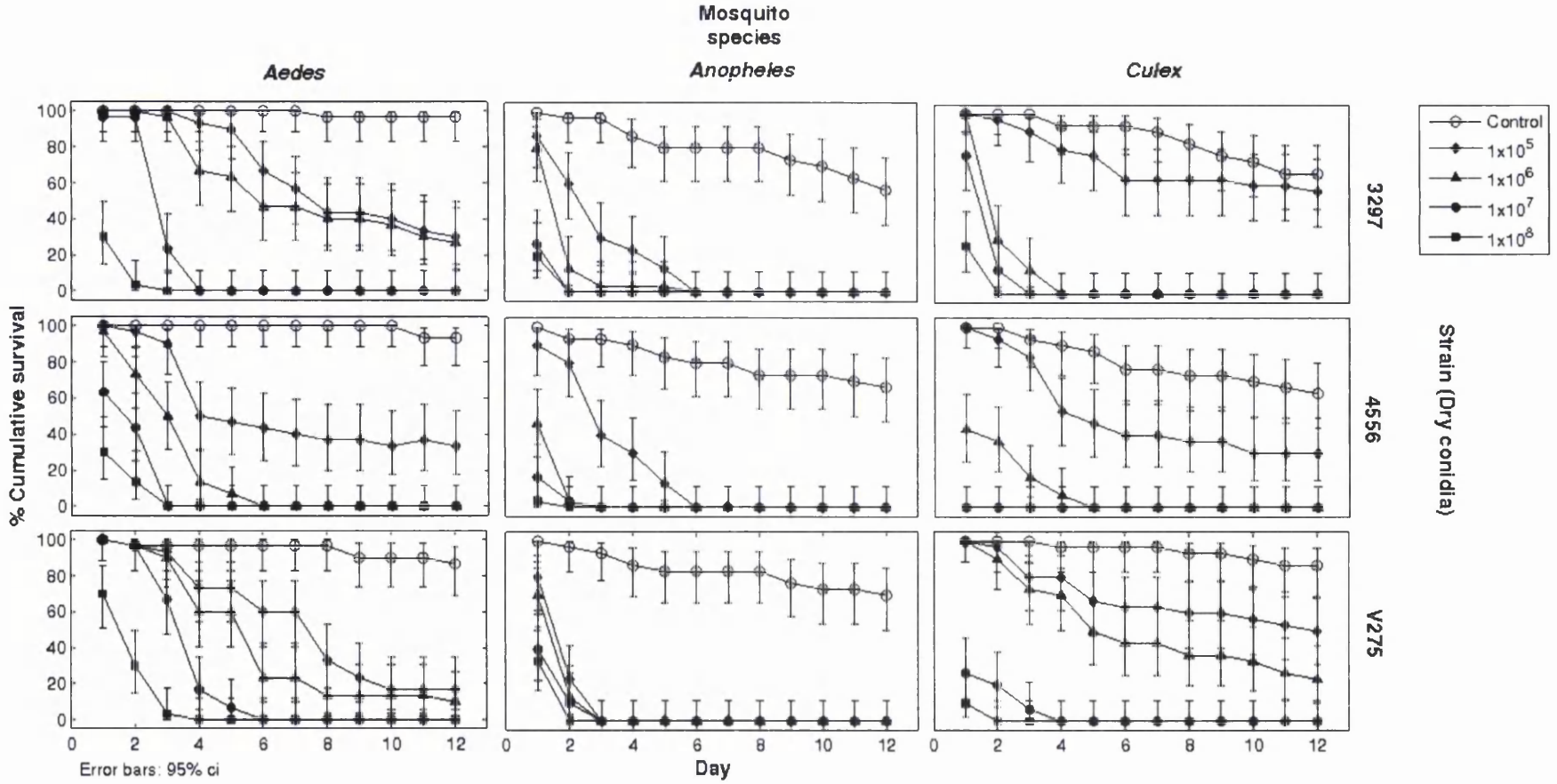

Figure 2.1: Survival curves of mosquito larvae exposed to different strains and concentration of 'dry' formulated *Metarhizium*..

Percentage cumulative survival curves of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* (L_{3-4}) exposed to different concentrations of 'dry' formulated *Metarhizium* sp. for 12 days. Error is represented as 95% ci

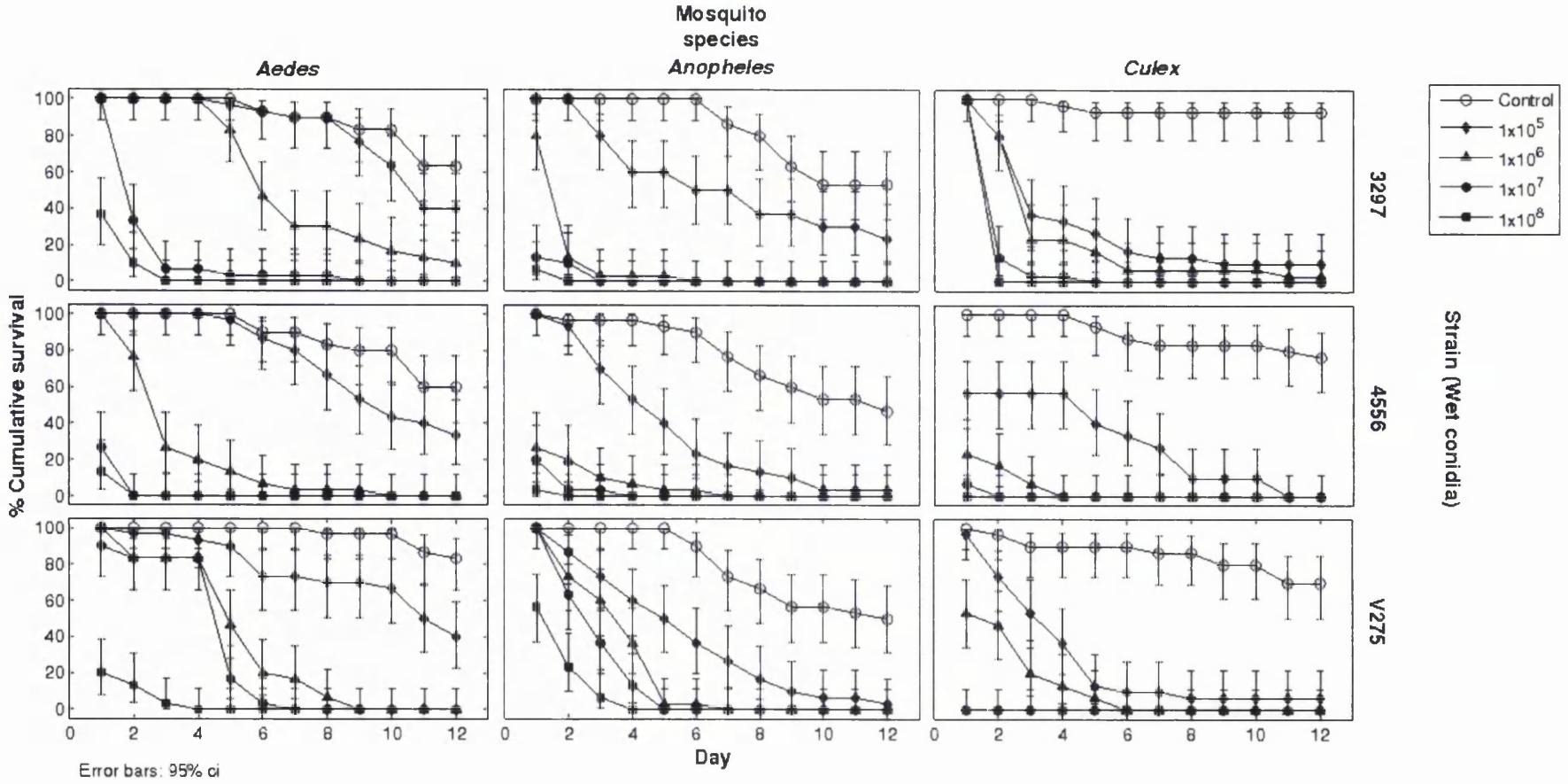

Figure 2.2: Survival curves of mosquito larvae exposed to different strains and concentration of 'wet' formulated *Metarhizium*.

Percentage cumulative survival curves of *Ae. aegypti*, *An. stephensi* and *Cx. quinquefasciatus* (L_{3-4}) exposed to different concentrations of 'wet' formulated *Metarhizium* sp. for 12 days. Error is represented as 95% ci

Table 2.3. Kaplan Meier log rank pairwise comparisons of conidia concentration

		Aedes				*Anopheles*				*Culex*			
Ap.	**Treat.**	**0**	**10^5**	**10^6**	**10^7**	**0**	**10^5**	**10^6**	**10^7**	**0**	**10^5**	**10^6**	**10^7**
W	10^5	ns				***				***			
	10^6	***	***			***	***			***	ns		
	10^7	***	***	***		***	***	*		***	***	***	
	10^8	***	***	***	***	***	***	**	ns	***	***	***	*
D	10^5	***				***				ns			
	10^6	***	ns			***	**			***	***		
	10^7	***	***	***		***	***	***		***	***	*	
	10^8	***	***	***	***	***	***	***	ns	***	***	ns	***
HK	10^5	ns				ns				***			
	10^6	ns	ns			*	ns			***	ns		
	10^7	***	***	***		*	ns	ns		***	***	***	
	10^8	***	***	***	*	***	***	***	***	***	***	***	***
W	10^5	*				***				***			
	10^6	***	***			***	***			***	***		
	10^7	***	***	***		***	***	ns		***	***	*	
	10^8	***	***	***	ns	***	***	**	*	***	***	**	ns
D	10^5	***				***				**			
	10^6	***	***			***	***			***	***		
	10^7	***	***	***		***	***	*		***	***	***	
	10^8	***	***	***	**	***	***	***	ns	***	***	***	ns
HK	10^5	ns				*				**			
	10^6	ns	ns			*	ns			***	***		
	10^7	***	***	***		***	***	***		***	***	**	
	10^8	***	***	***	***	***	***	***	ns	***	***	***	*

W	10^5	***	***			***				***			
	10^6	***	***			***	**			***	**		
	10^7	***	***	*		***	***	*		***	***	***	
	10^8	***	***	***	***	***	***	***	***	***	***	***	ns
D	10^5	***				***				**			
	10^6	***	ns	***	***	***	. ns			***	*		
	10^7	***	***	***		***	**	*		***	***	***	
	10^8	***	***			***	***	**	ns	***	***	***	*
HK	10^5	ns				ns				ns			
	10^6	ns	ns			ns	ns			***	***	***	
	10^7	***	***	***		ns	ns	ns		***	***	***	ns
	10^8	***	***	***	***	***	***	***	***	***	***		

Ae. aegypti, *An. stephensi* and *Cx. quinquefasciatus* (L_{3-4}) exposed to different concentrations of formulated *Metarhizium*. White = 3297, Light grey = 4556, Dark grey = V275, Ap. = Application, Treat. = Treatment. W = Wet, D = Dry, HK = Heat Killed * = $p<0.05$, ** = $p<0.01$, *** $p= <0.001$.

2.6 DISCUSSION

This study shows that all three strains of *Metarhizium* are highly pathogenic to *Aedes*, *Anopheles* and *Culex* larvae with ARSEF 4556 being the most aggressive. Selecting a highly virulent strain for all three important mosquito genera is important: to quickly reduce the pest population and cost of control, because less inoculum is required compared with less aggressive strains.

Differences in susceptibility were noted between mosquito species with *Ae. aegypti* being more resistant than either *Cx. quinquefasciatus* and *An. stephensi.* This has also been observed by other workers using different fungal species and strains (Scholte *et al.* 2003, 2004; Silva, Silva & Luz 2004). This trend is observed in both adults and larvae, demonstrating the robustness of *Aedes* species. Mortality in *Ae. aegypti* larvae due to *Metarhizium* was shown in earlier studies to be largely due to protease induced stress (Butt *et al.* 2013). Presumably *Anopheles* and *Culex* species are more easily stressed by the pathogen and, therefore, die more quickly.

Both early and late larval stages of all three mosquito species were equally susceptible to *Metarhizium,* independent of strain. Other researchers have reported differential susceptibility with early instar larvae being generally more susceptible (Bukhari *et al.* 2010). It is possible that in the case of the mosquito larvae there is an increase in protease binding receptors as the insect develops and results in a proportional increase in stress.

Heat killed conidia caused little larval mortality due to denaturation of Pr1 and this suggests that the mechanism of death reported for *Aedes* also applies to *Anopheles* and *Culex* (Butt *et al.* 2013). However, at relatively higher concentrations the heat killed conidia caused significant mortality suggesting factors other than Pr1 may also contribute to larval death. It is possible that the conidia are not nutritious and cause starvation stress. Previous studies by Butt *et al.* showed an increase in caspase activity in mosquito larvae treated with heat killed conidia (Butt *et al.* 2013), indicating that the cell wall components, other than Pr1, were causing stress. Cell surface proteins and enzymes are pivotal in defining the host-pathogen interaction. Invasion by the fungi is typically recognised through the binding of proteins to β 1-3 glucans present within the fungal cell wall (Charnley 2003; Jiang *et al.* 2004). The

insect humoral response is triggered by Pathogen Associated Molecular Patterns (PAMPs) and in the mosquito β 1-3 glucan recognition proteins stimulate the activation of the phenoloxidase cascade (Fabrick, Baker & Kanost 2003; Jiang *et al.* 2003; Kanost, Jiang & Yu 2004),. Through denaturing proteins and undoubtedly provoking conformational changes in the fungal conidia, an activation of the host humoral defences by cell wall sugars (β 1-3 glucans) may exacerbate the stress leading to host death. Indeed, fungal cell wall components will activate both cellular and humoral defences in most arthropods with the response being dose-related (Charnley 2003; Jiang *et al.* 2004; Gottar *et al.* 2006).

Slight differences in efficacy were noted between 'dry conidia' versus conidia suspensions or 'wet conidia', supporting the findings of Bukhari *et al.*, (Bukhari *et al.* 2010). In contrast, Alves *et al.*,(Alves & Alves 2002) noted the opposite for *Culex* larvae. The dry, hydrophobic conidia would largely remain concentrated at the surface, whereas the wet conidia would be suspended within the body of water. Exactly why the dry conidia should prove more virulent is unclear but this phenomenon has been reported for other insect hosts (Morley-Davies, Moore & Prior 1996; Rangel *et al.* 2005; Howard *et al.* 2010).

One possible explanation for dry conidia being more aggressive is the fact that spore bound enzymes, especially the virulence determining protease Pr1, would not have been removed by surfactants used to prepare conidia suspensions. Pr1 plays a key role in pathogenesis of both terrestrial hosts and mosquito larvae (Wang, Typas & Butt 2002; Fang & Bidochka 2006) removal or dilution of this enzyme would undoubtedly influence overall pest control. Passaged conidia of *Metarhizium anisopliae* has been shown to increase virulence for mosquito larvae (Daoust & Roberts 1982). This is because conidia from passaged pathogens are known to have higher levels of spore bound Pr1 (Frazzon *et al.* 2000; Shah, Wang & Butt 2005)

This studies show that a spore suspension was more efficacious at a lower concentration than a dry formulation, especially in respect to *An. stephensi* and *Cx. quinquefasciatus*. This difference in susceptibility could be attributed to the feeding habits adopted by the larvae. Typically *Anopheles* and *Culex* species are categorised as 'active' suspension-feeders, feeding actively on particulate matter within the water column via a collecting–filtering mechanism, as well as grazing from surface

biofilms (Dahl, Widahl & Nilsson 1988; Merritt, Dadd & Walker 1992; Merritt *et al.* 1996; Workman & Walton 2003). In contrast, *Aedes* are opportunistic feeders, preferentially feeding through a browsing mechanism, harvesting particulates attached to surrounding substrates. In addition *Aedes* are also able to filter feed (Eisenberg, Washburn & Schreiber 2000).

Mortality was dose-dependent, with all strains being more efficacious at higher concentrations, indicating the proportional increase in Pr1 as conidia concentration increases. Exposure of larvae to different concentrations of purified Proteinase K, which has over 70% homology with Pr1, also showed dose related mortality (data not shown). In the field, inoculum is typically applied at concentrations above $1x10^9$ conidia mL^{-1} (Kassa *et al.* 2004; Scholte, Takken & Knols 2007; Paula *et al.* 2011), since significant control was achieved at $1x10^6$ conidia mL^{-1}, this provides much latitude, allowing for dilution of the inoculum due to climatic and biotic factors.

2.7 CONCLUSION

Metarhizium sp. is highly pathogenic to mosquito larvae, independent of genus and larval developmental stage, providing a greater window of opportunity for the control of these important vectors of disease. Strain ARESF 4556 was highly aggressive, killing *Anopheles* and *Culex* larvae within 24 hrs. The formulation of the conidia will undoubtedly influence fungal field efficacy; central to this control strategy will be retention or enhancement of Pr1 activity since this enzyme plays a key role in pathogenesis (Butt *et al.* 2013).

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CHAPTER 3

METARHIZIUM ANISOPLIAE PATHOGENESIS OF MOSQUITO LARVAE

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Conceived and designed the experiments: TMB, **BPJG** & DCE.
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METARHIZIUM ANISOPLIAE PATHOGENESIS OF MOSQUITO LARVAE: VERDICT OF ACCIDENTAL DEATH

3.1 ABSTRACT

Metarhizium anisopliae, a fungal pathogen of terrestrial arthropods, kills the aquatic larvae of *Aedes aegypti*, the vector of dengue and yellow fever. The fungus kills without adhering to the host cuticle. Ingested conidia also fail to germinate and are expelled in faecal pellets. This study investigates the mechanism by which this fungus adapted to terrestrial hosts kills aquatic mosquito larvae. Genes associated with the *M. anisopliae* early pathogenic response (proteinases *Pr1* and *Pr2*, and adhesins, *Mad1* and *Mad2*) are upregulated in the presence of larvae, but the established infection process observed in terrestrial hosts does not progress and insecticidal destruxins were not detected. Protease inhibitors reduce larval mortality indicating the importance of proteases in the host interaction. The *Ae. aegypti* immune response to *M. anisopliae* appears limited, whilst the oxidative stress response gene encoding for thiol peroxidase is upregulated. *Cecropin* and *Hsp70* genes are downregulated as larval death occurs, and insect mortality appears to be linked to autolysis through caspase activity regulated by *Hsp70* and inhibited, in infected larvae, by protease inhibitors. Evidence is presented that a traditional host-pathogen response does not occur as the species have not evolved to interact. *M. anisopliae* retains pre-formed pathogenic determinants which mediate host mortality, but unlike true aquatic fungal pathogens, does not recognise and colonise the larval host.

3.2 INTRODUCTION

Mosquitoes vector a wide range of diseases (e.g. dengue, yellow fever, malaria, filariasis, and heartworm) which can have a devastating impact on human and animal health. Almost half the world's population is at risk to mosquito transmitted diseases and the range has expanded into Europe due to climate change and increased trade (Rezza *et al.* 2007; Medlock *et al.* 2012). Many chemical pesticides have been withdrawn due to the risks they pose to humans and the environment, and due to the

development of resistance in pest populations. Recent studies show that *Metarhizium anisopliae,* a soil borne fungal pathogen of terrestrial insects, offers an environmentally friendly alternative to chemicals for the control of mosquitoes. *M. anisopliae* will kill all the developmental stages (eggs, larvae and adults) of *Aedes*, *Anopheles* and *Culex* mosquitoes (Alves & Alves 2002; Scholte *et al.* 2004; Albernaz, Tai & Luz 2009; Bukhari *et al.* 2010; Luz, Mnyone & Russell 2011) yet the mechanism of how this terrestrial pathogen kills the aquatic larval stage is unclear. Strains of *M. anisopliae* have been developed to control a wide range of terrestrial arthropods including pests of agro-forests crops, stored produce and vectors of human and animal diseases (Blanford *et al.* 2005; Faria & Wraight 2007; Ansari, Carpenter & Butt 2010; Ansari *et al.* 2011). The pattern of infection of terrestrial arthropod hosts by *M. anisopliae,* like that of most other entomopathogenic fungi, follows a consistent pattern. Firstly, spores adhere to the surface of the host cuticle through hydrophobic forces followed by germination and differentiation of infection structures. The latter consists of an appressorium from which a narrow penetration peg is produced which penetrates the cuticle using a combination of enzymes and mechanical force (Goettel *et al.* 1989; St. Leger *et al.* 1989; Butt *et al.* 1995; Vestergaard *et al.* 1999; Toledo, Remes Lenicov & Lopez Lastra 2010). Following colonization of the haemocoel, the fungus erupts through the intersegmental membranes or natural orifices and differentiates conidiophores and conidia. The key pathogenicity determinants of *M. anisopliae* include cuticle degrading enzymes like *Pr1* (subtilisin protease) and toxic cyclic peptides like destruxins (Amiri-Bersheli *et al.* 2000; Charnley 2003; Schrank & Vainstein 2010). Fungal virulence appears to be correlated with *Pr1* and destruxin production; with hyper producers being more virulent (Wang & St. Leger 2007; Sree & Padmaja 2008). Equally important are the adhesins, *Mad1* and *Mad2*, which play an important role in adhesion of *M. anisopliae* to the insect cuticle (Barelli, Padilla-Guerrero & Bidochka 2011). Disruption of the *Mad1* gene reduces virulence by reducing adhesion of conidia to the host surface (Wang & St. Leger 2007). It is assumed that the pattern of infection outlined above occurs in aquatic mosquito larvae (Bukhari *et al.* 2010; Abdul-Ghani, Al-Mekhlafi & Alabsi 2012). This chapter demonstrates for the first time that *M. anisopliae* kills the mosquito larvae *via* a mechanism which does not entail the traditional infection process.

Based on the limited number of observational studies conducted on *M. anisopliae* infection of mosquito larvae, possible routes of invasion have been reported including entry *via* penetration of the cuticle, the respiratory siphon or alimentary canal, however, the precise mechanism remains elusive. Lacey *et al.*, (Lacey, Lacey & Roberts 1988) noted that when larvae of *Culex quinquefasciatus* broke the water tension with their perispiracular valves for air intake, floating conidia of *M. anisopliae* adhered to the inside surface of the valves, germinated and invaded the siphon tip tissue, then extended into and blocked the trachea resulting in suffocation and death. Lacey *et al.*, (Lacey *et al.* 1988) also noted that conidia suspended in the water were ingested and occluded the larval gut, initiating mortality within 6 to 24 hr after ingestion. In contrast, Riba *et al.*, (Riba *et al.* 1986) reported that high concentrations of *M. anisopliae* conidia killed *Ae. aegypti* within 1.1 days, before intra-haemocoelic invasion; but at lower concentrations typical mycosis occurred. Some workers suggest that death is due to colonization of the hemocoel by the fungus (Riba *et al.* 1986; Bukhari *et al.* 2010), others suggest it is due to toxins released by ingested conidia or during digestion of the conidia without colonisation of the hemocoel (Crisan 1971; Lacey *et al.* 1988; Silva *et al.* 2005).

The experiments in this chapter aimed to elucidate the underlying mechanisms that contribute to the mode of pathogenesis

3.3 MATERIALS AND METHODS

3.3.1 Fungal Strains and Production

M. anisopliae isolate ARSEF 4556, was identified in Chapter 2 as being highly pathogenic to all three species mosquitoes concerned in this thesis. *M. anisopliae* ARSEF 4556 was maintained on broken Basmati rice (Ansari & Butt 2011). Whereas, a green fluorescence protein (GFP) transformed strain of *Metarhizium brunneum* EAMa 01/58 Su*, obtained from Professor Enrique Quesada at University of Cordoba, was maintained on Sabouraud dextrose agar (SDA). Conidial viability was assessed using the plate count technique (Goettel & Inglis 1997). Only batches of fungi produced with >90% viability were selected for use in assays

*N.B. Attempts to transform ARSEF 4556 were made, see Appendix 1

3.3.2 Mosquito Source and Maintenance

Aedes aegypti (strain AeAe) *Anopheles stephensi* (strain Beech) and *Culex quinquefasciatus* (strain Muheza) were reared in distilled water to late (L_{3-4}, 4-8 days old) instars. The larvae fed on Tetramin® fish food, and maintained at room temperature (22°C ± 2°C).

3.3.3 Inoculation of *Aedes* Larvae with *Metarhizium* Conidia

Assays were performed using 6 well plates (Nunc, Roskilde, Denmark) with ten larvae per well. *M. anisopliae* ARSEF 4556 was assayed at 10^7 conidia ml^{-1} against L_{3-4} larvae, in concurrence with findings in Chapter 2. Additional assays were performed using heat killed conidia to determine the role of extracellular enzymes in pathogenesis. Extracellular enzymes were denatured by wrapping the conidia in aluminium foil and autoclaving for 15 min at 121°C. Control larvae were exposed to either 1ml 0.03% Aq Tween 80 or distilled water. Larval mortality was recorded daily up to 7 days. All assays were performed at room temperature with a 16L:8D photoperiod. There were 30 larvae per assay which was repeated three times. This format was used in subsequent assays to study host-pathogen interactions, in particular, insect defence responses and regulation of *M. anisopliae* pathogenicity determinants.

3.3.4 Microscopy

A number of different microscopy based techniques were utilised to visualise the physical effects, if any, the fungus was having on the mosquito larvae.

3.3.4.1 Light and Fluorescent Microscopy

Ae. aegypti larvae (n=20) were inoculated with conidia of ARSEF 4556 as outlined above (3.2.3) and examined at 0, 24, 48 and 72 hr post inoculation (pi). Healthy and infected larvae were examined by light microscopy (LM) to determine if there were preferential sites for spore adhesion and to monitor passage of the fungal conidia through the gut. Additional studies were performed using a GFP-transformed strain of *M. brunneum.* The surface and gut contents of infected *Ae. aegypti* larvae (n = 10)

as well as faecal pellets were examined by fluorescence microscopy (FM) using a Zeiss fluorescence microscope.

3.3.4.2 Cryo-Scanning Electron Microscopy (SEM)

Ae. aegypti larvae (n =20) were examined by Cryo- Scanning Electron Microscopy (SEM). Cryo-SEM imaging was conducted using a Hitachi S4800 field emission microscope equipped with a Quorum PPT2000 cryogenic stage and preparation chamber. The larvae were mounted on SEM sample holders using cryogenic glue, both horizontally for imaging of the cuticle and vertically for fracturing and cross sectional imaging. The specimen were plunged into nitrogen slush for rapid freezing then transferred to the cryogenic preparation chamber in vacuum. The specimens were warmed up to -90°C for 10 minutes to remove surface ice then returned to -130°C. Fracturing of the vertically mounted larvae was performed inside the preparation chamber at -130°C using a rotating knife. The specimen were then coated with approximately 5nm of Platinum then transferred to the SEM stage. Imaging took place at -130°C.

3.3.5 Mass spectrometry analysis of destruxins

3.3.5.1 Destruxin Extraction

Assays were performed using 24-multi-well plates (Falcon No. 3047, Hamburg, Germany) with ten *Ae. aegypti* larvae (L_{3-4}) per well containing 1ml aqueous suspension of $1x10^7$ conidia ARSEF 4556 or 1ml of distilled water (control). After 24 hr incubation, the larvae were removed from the fungal solution and washed three times in distilled water to remove spores adhering to the cuticle of the larvae, the ten larvae from each replicate were then put into separate microfuge tubes and homogenised with a micropestle. Microcentrifuge tubes were charged with 500 µL Dichloromethane (CH_2Cl_2)/ethyl acetate (EtOAc) (1:1, v/v) and agitated for 5minutes before leaving to rest for 30 minutes. After 30 minutes the supernatant was removed and discarded and another 500 µL CH_2Cl_2/EtOAc was added to the sample and the step before was repeated. Once the second supernatant was removed the remaining solution was evaporated using a speed vacuum. Extracts were stored at -20°C until required for analysis. Extracts were analysed by mass spectroscopy as

described by Butt *et al.*, (Butt *et al.* 2009). The effect of destruxins on *Ae. aegypti* larvae was also tested by introducing larvae to 1ml of distilled water spiked with 1nmole of destruxin A (Sigma-Aldrich) and determining larval survival.

3.3.5.2 Matrix Assisted Laser Desorption Ionisation Time of Flight (MALDI-ToF) Mass Spectrometric Analysis

The analysis of the outer surface proteins of the fungal spores utilised MALDI mass spectrometry. For this purpose, the spores were pelleted by centrifugation for 5 minutes at 3000 rpm and a small proportion of the spores redissolved in a matrix solution and 1 μL of the resulting mixture was spotted onto the MALDI plate and allowed to dry at room temperature. MALDI MS was also used to confirm the levels of detection below which any destruxins might be exhibited within the larvae themselves. For this purpose, larval extract was re-dissolved in a matrix solution and 1 μL of the resulting mixture was spotted onto the MALDI plate and allowed to dry at room temperature. For small peptides and proteins a matrix solution of 10mg/mL was used of alpha cyanocinnaminic acid (CHCA) in 50:50 0.1% trifluoroacetic acid (TFA): acetonitrile (ACN), whilst for larger proteins a 10 mg/mL solution of Sinapic acid in 70:30 0.1% TFA:ACN was used. MALDI-ToF analysis was carried out on a Voyager DE-STR instrument (Applied Biosystems, UK) in reflection mode with an acceleration voltage of 20,000 V, a grid voltage of 70% for peptides and 95% for larger proteins of the acceleration voltage, a delay time of 75 nsec or 200 nsec respectively and 50 laser shots per spectrum to analyse whether any peptides were present and a 1pmole standard of destruxin A applied to the plate as a control and as a method of determining the level below which any destruxins are present if signal intensity were below the standard.

3.3.5.3 Nano-Electrospray High Performance Liquid Chromatography Tandem Mass Spectrometry (nESI-LCMS/MS)

Destruxin extracts were analyzed by nano-reverse phase liquid chromatography (Ultimate Pump, LC-Packing, Dionex, The Netherlands) using an electrospray ion trap MS (LCQ Deca XP, ThermoElectron, Hemel Hempstead, UK). LC-ESI MS/MS separations were performed using a 10 cm x 75 mm I.D. pulled-tip capillary column, that was slurry packed in-house with 3 μm, 300 Å pore size C18 silica bonded

stationary phase (PepMap, Dionex, Camberley, UK). The autosampler was fitted with a 5 μL injection loop and was refrigerated at 4°C during analysis.

After injecting 5 μL of extract, the analytes were eluted from the analytical column, over which a 15min wash with 98% buffer A (0.1% formic acid in water v/v) was applied and any destruxins present were then eluted using a stepwise gradient of 0% solvent B (0.1% formic acid in acetonitrile v/v) to 65% solvent B in 100 min and then to 100% B in 10 min with a constant flow rate of 0.2 μL/min. The electrospray MS was operated in a data-dependent mode in which each full MS scan (*m/z* 400-1500) was followed by three MS/MS scans, in which the three most abundant peptide molecular ions were dynamically selected for collision-induced dissociation (CID) using a normalized collision energy of 35%. The temperature of the heated capillary and electrospray voltage was 160°C and 1.6kV, respectively. Post-acquisition processing utilised previously reported diagnostic mass losses during MS/MS analysis in order to select for destruxins from the mixture of separated ions

3.3.6 Enzymology

One much studied protease, Pr1, is an important virulence determinant. It is known to be regulated under stress conditions and can be induced by insect cuticle, de-repressed under starvation conditions and repressed if an excess of nutrients is present (Segers *et al.* 1995; Wang, Typas & Butt 2002)

The enzymatic activity was assayed in *Ae. aegypti* larvae exposed to *M. anisopliae* ARSEF 4556 as well as the fungal solution itself.

3.3.6.1 Pr1 Activity

Pr1 (subtilisin) activity was assayed using the chromogenic substrates N-Succinyl-Ala-Ala-Pro-Phe ρ-nitroanilide as described by St. Leger *et al.*, (1987). Briefly, 2 mM substrate was prepared in 0.1M Tris-HCl buffer pH 7.95 then 100 μL substrate, 80 μL buffer and 20 μL assay sample were mixed in a microplate at 350 rpm for 10 s, the absorbance was then read immediately at 405 nm, followed by recordings at 10 s intervals for 3 min. One EU was taken as the quantity of enzyme required to liberate 1 ρmol of ρ-nitroaniline/hr.

3.3.6.1 Protease Inhibition Assays

To determine if the extracellular proteases were responsible for larval mortality. Larvae were exposed to *M. anisopliae* ARSEF 4556 conidia containing either chicken egg white (0.1 mg/ml), EDTA (1 mM) or α2-macroglobulin (1 μg/ml) which are inhibitors specific for *Pr1*, metalloprotease and global (serine, cysteine, metallo-) proteases, respectively. All the inhibitors were purchased from Sigma-Aldrich. Controls consisted of buffer and buffered inhibitor. Mortality was recorded at 0, 12, 24, 36, 48 and 72 hr pi. In addition, assays were also performed using heat killed conidia at 10^7 conidia ml^{-1}.

3.3.6.2 Caspase Assays

Activity of caspases 2, 3, 7 and 8 was assessed in mosquito larvae infected with *M. anisopliae* (n = 10 per treatment), and incubated at room temperature (22°C ± 2°C) 24 and 48 hr. Non-infected control larvae also included at these time points. At each time point, larvae were removed from solution and frozen under liquid nitrogen and stored at -80°C before homogenizing mechanically using a micropestle and mortar. Total homogenates were resuspended in 500 μL 0.5% triton lysis buffer (Tris 20mM, NaCl 100mM, EDTA 500mM, 0.5% Triton X-100), and agitated gently before incubating on ice for 10 min. Homogenates were then centrifuged at 14, 000 g for 10 min. 35 μL aliquots of supernatant were added to 4 replicate wells in a white walled 96-well microtiter plate (Greiner Bio-one). Luminometric assays for caspase-2, caspase-3, caspase-7 and caspase-8 activity were performed in accordance with the manufacturer's guidelines using the Caspase Glo 2, Caspase Glo 3-7 and Caspase Glo 8 assay kits (Promega) by adding 35 μL of Caspase Glo reagent to the sample. Plates were agitated gently for 10 s at 180 cpm before incubating at 25°C. Endpoint luminescence was measured after 1 hr.

3.3.7 Transcript Quantification of Insect and Fungus-Derived Genes

3.3.7.1 Samples, RNA Extraction and cDNA Synthesis

Ten *Ae. aegypti* larvae per replicate, were either inoculated with 100 mL of $1x10^7$ conidia mL^{-1} solution of ARSEF 4556 in distilled water, or with distilled water alone,

or incubated at room temperature for 48 hr. Mosquitoes in distilled water harvested after this time were frozen under liquid nitrogen, termed time 0 Non-infected larvae, those inoculated in fungal solution were separated into living and dead mosquitoes before freezing in liquid nitrogen.

Faecal pellets were harvested from larvae previously starved for 48 hr and inoculated with 5 mL 1 x 10^7conidia mL^{-1} ARSEF 4556. Faecal pellets were harvested 24 hr pi, washed in distilled water three times with intervening centrifugation at 500 g for 5 min. The supernatant, free of fungal spores, was discarded, whilst the faecal pellet was frozen under liquid nitrogen.

A solution of non-ingested conidia was centrifuged at 18,000 g for 5 min post larvae removal, before freezing under liquid nitrogen.

Tenebrio molitor adults, were used as a positive control to assess gene expression changes in a typical terrestrial host. The adults were subjected to a submersion assay in 50 mL 1 x 10^7conidia ml^{-1} ARSEF 4556 for 20 sec, the insects were then incubated on moist filter paper at 27°C for 48 hr, before freezing under liquid nitrogen.

Each experiment, containing three replicates per treatment, was independently repeated three times. All samples frozen under liquid nitrogen were stored at -80°C until required.

All samples were ground with a micropestle and total RNA extractions carried out using the RNeasy Micro kit (Qiagen) following the manufacturer's instructions. RNA concentration and purity was assessed at 260 and 280 nm absorbance using a Nanophotometer (Implen). Total RNA (1 μg) was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen) with gDNA elimination reaction, for the experiment to quantify insect-derived transcripts and fungus-derived transcripts, respectively.

Relative cDNA quantity was analyzed by qRT-PCR using two reference genes for insect or fungal cDNA samples to ensure consistency between values: *Ae. aegypti*

ribosomal S7 (accession number: AAEL009496) and ribosomal protein 49/L32 (AAEL003396) and *M. anisopliae* 18S rRNA and elongation factor tEF (Table 3.1).

3.3.7.2 Quantitative Real Time PCR (qRT-PCR).

Transcript levels were determined using the CFX96™ Real-Time PCR detection system (Biorad) for both *Ae. aegypti* and *M. anisopliae* genes. Primers were designed to amplify key *Ae. aegypti* response genes and *M. anisopliae* pathology-related genes (Table3.1).

PCR reactions were performed in 10 µL volumes consisting of 1µM of each primer, 2 µL of cDNA sample, 5 µL SYBR Green Fastmix (Quanta) and 1 µL DEPC-treated water. All reactions were carried out in duplicate. PCR cycling conditions were as follows: one cycle of 45°C for 5 min and 95°C for 3 min followed by 39 cycles of 95°C for 10 sec, 60.3°C for 10 sec and 72°C for 30 sec. A dissociation step of 65-95°C over 5 sec was used for melt curve analysis for detection of non-specific products in the reaction. Primers for genes investigated (Table 3.1) were designed, using primer3 software, from an alignment of *Metarhizium* sp. gene sequences and primers were selected which showed the greatest conservation between genes.

A two-fold dilution series of cDNA pooled from each sample was included in each run to generate a standard curve for each primer set.

Bio Rad CFX Manager software was utilized to determine the cycle threshold of each sample (Ct-Target) which was normalised to the geometric mean cycle threshold (Ct) of the appropriate endogenous qPCR products (Ct-Control) for the same sample (Goidin *et al.* 2001; Lekanne Deprez *et al.* 2002; Eastwood *et al.* 2008). Relative gene expression was calculated using the comparative C_t method ($2^{-\Delta\Delta}C_t$), where $\Delta\Delta C_t = (C_{t.\ target} - C_{t.\ reference})_{Time\ x} - (C_{t.\ target} - C_{t.\ reference})_{Time\ 0}$, (Livak & Schmittgen 2001).

Table 3.1 *Metarhizium anisopliae* and *Aedes aegypti* loci used for expression analysis.

Putative function	Locus	Primer name	Forward primer	Reverse Primer	Sequence reference
Housekeeping (*Ae. aegypti*)	Ribosomal S7	Ada-Rb7	TCAGTGTACAAGAAGCTGACCGGA	TTCCGCGCGCGCTCACTTATTAGATT	(Telang *et al.* 2011)
	Ribosomal protein 49 /L32	Ad_RP49	ACAAGCTTGCCCCCAACT	CCGTAACCGATGTTTGGC	(Bonizzoni *et al.* 2011)
Antimicrobial peptides (*Ae. aegypti*)	AeDA, Defensin A	AeDA	CCGAAAGGACCAACCATGAA	ATTCCGACAGACGCACACCCT	
	AeDB, Defensin B	AeDB	TCATTTGTTTCCTGGCTCTGTG	GCGGCCTGATAGGTTTCCTC	
	Ada-Defensin D	Ada-DefD	CGGTGCTGGCGGACGAA	GCAATGAGCAGCACAAGCACTATC	(Telang *et al.* 2011)
	Cecropin A	AeCA2	TGGCTGTTCTTCTCCTGA	AAAACTCGTTTTCCTGCAC	
	Cecropin G	Ada-CcG	TCACAAAGTTATTTCTCCTGATCG	GCTTTAGCCCCAGCTACAAC	(Xi, Ramirez & Dimopoulos 2008; Telang *et al.* 2011)
Stress	Hsp70, heat shock protein 70	Hsp70	CCCGTCCTACGTGGCGTTCA	GGTGGCCTGACGTTGCGAGT	(Muturi *et al.* 2011)
(*Ae. aegypti*)	Hsp83, heat shock protein 83	Hsp83	AAGGCCGTTAAGGATCTGGT	CGCTAGTGTGGGGAAGAGAG	(Zhao *et al.* 2010)
Reactive oxygen	TPX, Thiol peroxidase	TPX	TCGACCGACAGTCACTTCAC	CTGGCGGAGATTCTGCTTAC	(Pan *et al.* 2012)
(*Ae. aegypti*)	CYP6Z6, Cytochrome P450	CYP6Z6	CTGCCTTATTTGGACTTATGC	ATCACAACACTGGATTCTGG	(Muturi *et al.* 2011)

	GPX, Glutathione peroxidase	GPX	ATATGGCGAAACGGAAGGTC	TCCCCGTTGACGTATATCTTG	(Pan *et al.*, 2012)
Proteases (*M. anisopliae*)	PR1a	PR1a	TCCGAGTCCTCTTGCCTATCA	GGCACCGTTGTAGGCAAGGTAGTT	
	PR2	PR2	TACGCCACATTGCCAGA	GCATGTCGCACGATCAA	
Adhesion genes (*M. anisopliae*)	MAD1	MAD1	CTCCTCACATCACCCAGGTT	GGGAGTAGGCATGACGATGT	
	MAD2	MAD2	CTATGTCCACCTTGCGACT	AGCAGCTGATGAGGGTCT	
Housekeeping genes (*M. anisopliae*)	18s		CGAAAGTCGCAATGGCTCA	CCGAAGTCGGGATTTTTAGC	
	Translation elongation factor	MaHKtEF	CGAGCGTGAGCGTGGTA	CAGCCTCGAACTCACCAG	

3.3.8 ROS Production, Lipid Peroxidation and Antioxidant System Activity

Three *Ae. aegypti* per sample were homogenized in 100 µL of ice-cold phosphate buffered saline (10 mM phosphate buffer, 150 mM NaCl, pH 7.2) containing PTU (1mg/ml). The homogenate was centrifuged for 5 min, 10,000 g at 4°C and activities determined as described by (Dubovskiy *et al.* 2013).

3.3.8.1 Reactive Oxygen Species (ROS) Activity

The 2′,7′-dichlorodihydrofluorescein ($DCFH_2$) was used for in vitro assay for measuring total ROS/RNS free radical activity in the homogenates (Chen *et al.*, 2010). 5 µL of samples were added to the wells with 200 µL of the $DCFH_2$ solution (10 µM in PBS) and the oxidation reaction is allowed to proceed 30 min 37°C. Samples were measured fluorometrically (Ex/Em = 485/530 nm). The excitation filter was set at 485 nm and the emission filter was set at 530 nm. The ROS generation is presented as fluorescence ((Ft_{30} - Ft_0), where Ft_{30} = fluorescence at time 30 min and Ft_0 = fluorescence at time 0 min) per mg protein.

3.3.8.2 Superoxide dismutase (SOD) Activity

Superoxide dismutase (SOD) activity was determined as the suppression of the reduction rate of NBT by the superoxide anion, generated as a result of xanthine oxidation by xanthine oxidase (McCord & Fridovich, 1969). 10 µL of the sample was mixed with 150 µL of the reacting solution (70 µM of NBT; 125 µM of xanthine; both dissolved in PBS) and 2 µL xanthine oxidase solution (10 mg of albumin bovine (BSA); 100 µL of xanthine oxidase (4.11 $unitmL^{-1}$); dissolved in 1 mL of PBS). The mixture was incubated in darkness at 28°C for 60 min. SOD activity is presented as the difference in absorbance between a sample containing the mixture and a clean reagent mixture at ΔA 560nm /min/mg protein.

3.3.8.3 Catalase Activity

Catalase activity was estimated as the decomposition rate of hydrogen peroxide (Wang *et al.*, 2001). 500 µL of substrate (1% hydrogen peroxide in PBS) was mixed with 1 µL of homogenate. The mixture was incubated at 28°C for 60 min. The catalase activity is presented as the ΔA at 240nm/min /mg protein.

3.3.8.4 Glutathione-S-transferase (GST) Activity

Glutathione-S-transferease (GST) activity was determined as the changing of the concentration of 5-(2,4-dinitrophenil)-glutathione (product of 2,4-Dinitrochlorobenzene (DNCB) and glutathione interaction) catalysed by GST (Habig *et al.*, 1974). The reaction mixture contained 1mM glutathione and 1mM of DNCB and 1 µL of the sample in 200 µL PBS. The mixture was incubated 30 min at 28°C. GST activity is represented as ΔA at 340nm/min /mg protein.

3.3.8.5 Lipid Peroxidation

The process of lipid peroxidation results in the formation of malondialdehyde (MDA). This is a later product in the sequence of lipid peroxidation reactions (Rael *et al.*, 2004). The thiobarbituric acid (TBA) assay was used to assess the MDA concentration, with some modifications as described in Dubovskiy *et al.* (2008). 20 µL of 20 % trichloroacetic acid was mixed with 40 µL of the sample, after which the mixture was centrifuged at 10000 g for 10 min at +4°C. 50 µL of supernatant was mixed with 150 µL of 0.8 % TBA, and the mixture was incubated at +100°C for 60 min. The MDA-TBA adduct was quantified fluorometrically (Ex/Em = 532/553 nm). The MDA concentration is presented as nmoles of MDA per mg protein using the 1,1,3,3-tetramethoxypropane as a standard.

3.3.8.6 Phenoloxidase (PO) Activity

Phenoloxidase (PO) activity was monitored in total larval homogenates (Ashida, Kinoshita & Brey 1990; Li *et al.* 2005; Ahmed 2013) throughout a time course study post *Metarhizium* infection until larval death (0hr, 10 min, 20 min, 30 min, 40 min, 50 min, 60 min, 6 hr, 12 hr, 24 hr, 48 hr, and 72 hr). Larvae were removed at each time point and frozen under liquid nitrogen.

15 *Metarhizium anisopliae* infected or control larvae were homogenised in 200 µL of phosphate buffer saline (PBS, pH 7.8) in a 1.5 mL microfuge tube, per replicate. Homogenate was made up to 800 µL with PBS and centrifuged at 3000 g for 20 min. Supernatants were removed and stored at -80°C until required. 5 replicates were run concomitantly, the experiment was independently repeated twice.

L-DOPA (3, 4-dihydroxy-L-phenyl-alanine, Sigma) was used as a substrate to determine PO activity. PO activity is expressed as a change in absorbance/min/mg protein (Dubovskiy *et al.* 2013). 20 µL of larval homogenate was added to 180 µL of L-DOPA (4mgmL^{-1}) in a 96-well plate and incubated at room temperature for 30min. End point absorbance was measured at 490nm.

The concentration of protein in the homogenates was determined by the Bradford method (Bradford, 1976); BSA was used to construct the calibration curve. All assays were made with Biotek Synergy H1 plate reader.

3.3.9 Statistical Analysis

Differences in mosquito larvae survival between live and heat killed conidia and protease inhibited conidia were analysed using Kaplan-Meier survival analysis to plot cumulative survival functions by treatment with pairwise comparison over log-rank test (Ansari *et al.* 2011). Biochemical and molecular data sets were analyzed using two-way Analysis of Variance (ANOVA) with Bonferroni's post-test. Prior to analysis gene expression data was logarithm (base 10) transformed, conforming to ANOVA assumption of homogeneity of variance (Eastwood *et al.* 2008). All statistical analyses were carried out using SPSS v21.0 (IMB corp 2012) and GraphPad Prism v5.0 (GraphPad Software, USA).

3.4 AIMS

1) Investigate whether destruxins have any role in mosquito pathogenesis,

2) Determine if there is the host-pathogen recognition and/or response of the key pathogenicity determinants in the presence of mosquito larvae.

3) Whether there is any host immune response to *Metarhizium*.

4) Investigate stress management systems in *Ae. aegypti*

3.5 RESULTS

3.5.1 Virulence of *Metarhizium anisopliae* ARSEF 4556 for *Aedes aegypti* Larvae

Ae. aegypti larvae were highly susceptible to *M. anisopliae* 4556 with the earliest mortalities being observed 24 hr pi and percentage mortality reaching 60%-90% between 72 and 96 hr pi (Figure.3 1). Heat killed conidia were used to determine whether conidia caused blockage within the larvae or were required to actively bring about mortality. Mortality was significantly higher in larvae exposed to live conidia than those exposed to heat killed conidia (p <0.001) and there was no significant difference in mortality between the heat killed conidia (p <0.153) and the untreated control (Figure.3.1).

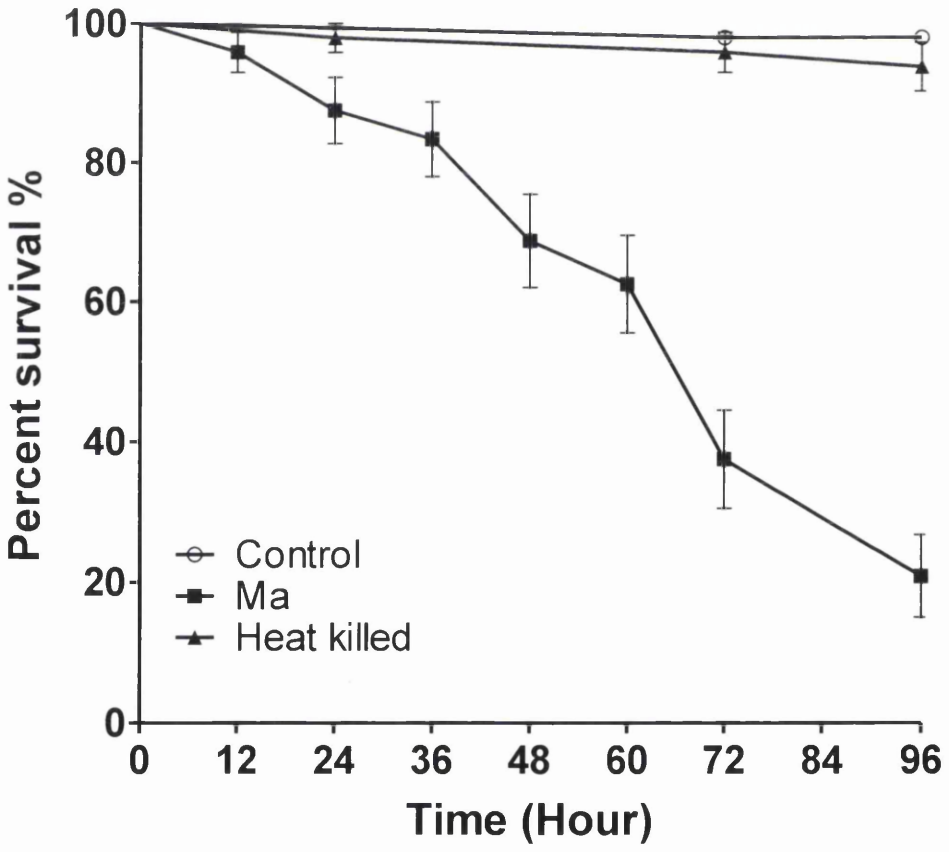

Figure 3.1 (overleaf). Heat killed treatment increased the survival of *Ae. aegypti* larvae.

Late 3rd-4th instar *Ae. aegypti* larvae (n = 72) were inoculated with live (Ma) and heat killed conidia of *M. anisopliae* at 1 x10^7 conidia mL^{-1}. Kaplan-Meier method was used to plot cumulative survival curves of larvae after inoculation, log-rank test was used to assess difference in survival between treatments. The curves of non-exposed and heat killed treatment show no statistical difference. Live conidia significantly decreased survival compared to heat killed conidia ($p < 0.001$). Larvae with no fungal treatment were used as a negative control.

3.5.2 *Metarhizium* Conidia Fail to Infect *Aedes* Larvae

Microscopy studies clearly showed little or no attachment of conidia to the surface of the mosquito body (Figures: 3.2A and B) with conidia being concentrated in the gut lumen (Figures: 3.2C-E). In some larvae, the conidia occluded the gut lumen while in others gaps were observed between the conidial clumps. None of the conidia in the gut had produced a germ tube and whereas some conidia were hydrated and swollen others appeared collapsed (Figure. 3.2E). Conidia had a prominent hydrophobin rodlet layer with little evidence of mucilage secretion FM showed that conidia in the gut and those present in faecal pellets expressed the GFP and were clearly viable and active (Figure.3.3). Most non-fluorescing GFP conidia were probably quiescent.

SEM examination of cross sections of abdomen showed no obvious differences in the appearance of the gut epithelial cells, peritrophic matrix and other structures of treated and untreated (control) larvae (Figures:3.2C-E). There was no visible evidence of damage to the internal organs. Compact faecal pellets were produced by control and infected larvae (Figure.3.3) suggesting the peritrophic matrix, peristalsis and other gut functions were intact at least until the time of death. The fungus never crossed the gut; it did not colonize the haemocoel as it would terrestrial arthropod hosts.

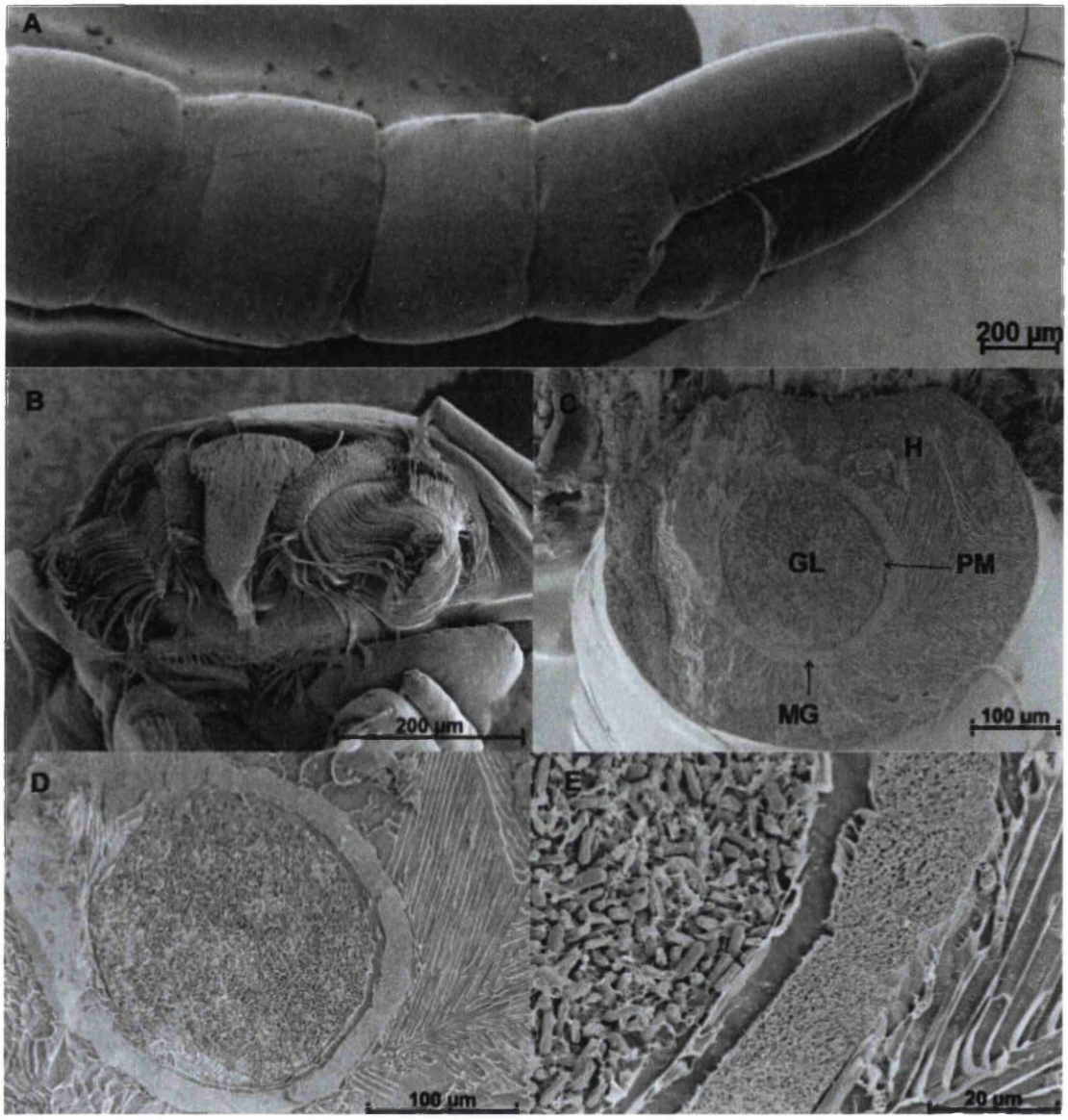

Figure 3.2. *M. anisopliae* do not attach to or invade mosquito hemocoel

Larvae inoculated with $1x10^7$ conidia ml^{-1} *M. anisopliae*, 48 hr post-inoculation, were subjected to Cryo-SEM to establish areas of attachment and penetration of the fungus. No conidia were observed attached to the surface of the head (A), abdomen and siphon (B). Cross section of control larva (C) and infected larva (D) showing the gut lumen (GL), midgut epithelium (MG), and peritrophic matrix (PM). Conidia of *M. anisopliae* were restricted to and appeared to occlude the gut lumen. (D-E). Both swollen and collapsed conidia were observed in the gut lumen with no evidence of conidia penetrating the gut wall (F) and invading the hemocoel (H).

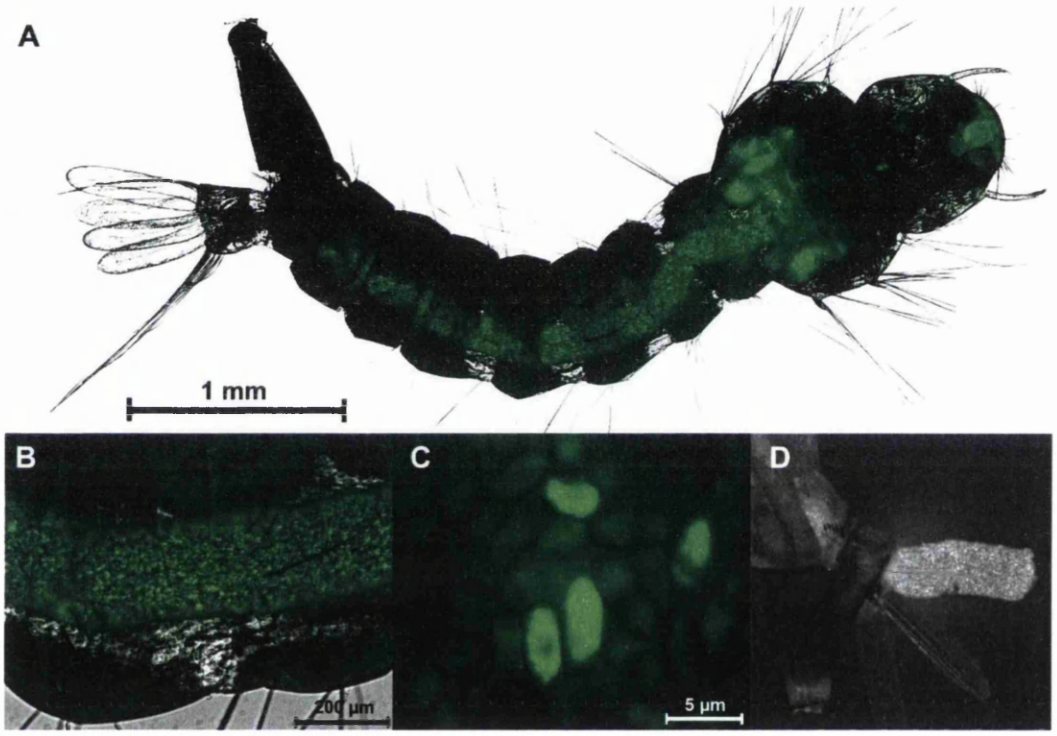

Figure 3.3. *Metarhizium* conidia expressing GFP in the gut and faecal pellets demonstrating activity and viability.

Larvae inoculated with conidia of a GFP transformed strain of *Metarhizium brunneum* were examined 48 hr pi to assess viability and activity of the conidia. (A) Conidia occlude most of the gut lumen, some autofluorescence is seen in the head and thorax region. (B) Numerous conidia are active and expressing the GFP within the gut lumen. (C) High magnification of conidia expressing GFP, the non-fluorescing conidia may be inactive or dead, (D) Faecal pellet being expelled from an infected larva showing many active conidia.

3.5.3 Destruxins Are Not the Cause of Larval Death

Destruxins, common virulence determinants of *M. anisopliae,* were not detected by mass spectroscopy in *Ae. aegypti* larvae that had ingested conidia of *M. anisopliae* The LCMS analysis was utilized to profile for any destruxin signals present using constant neutral loss signals established in our laboratories that highlight low levels of destruxins (Butt *et al.* 2009). No signals were detected which represent destruxins in any of the five replicate larval extractions (Figure. 3.4) and re-analysing the data for the m/z of specific destruxins also confirmed their absence (data not shown). The LCMS system was tested using a 10 fmole cytochrome C digest and 1 pmole destruxin A, however system test data was not routinely recorded and therefore the larval extracts were also analysed by MALDI ToF analysis alongside a destruxin A standard (Figure. 3.5). As can be seen the standard destruxin provides an excellent signal at the 1pmole level whilst the control experiment and larval extracts contain only those ions formed due to the MALDI matrix itself and no destuxin signals can be determined. This allows us to confirm the absence of destruxins, at least up to the 1pmole level per larval experiment.

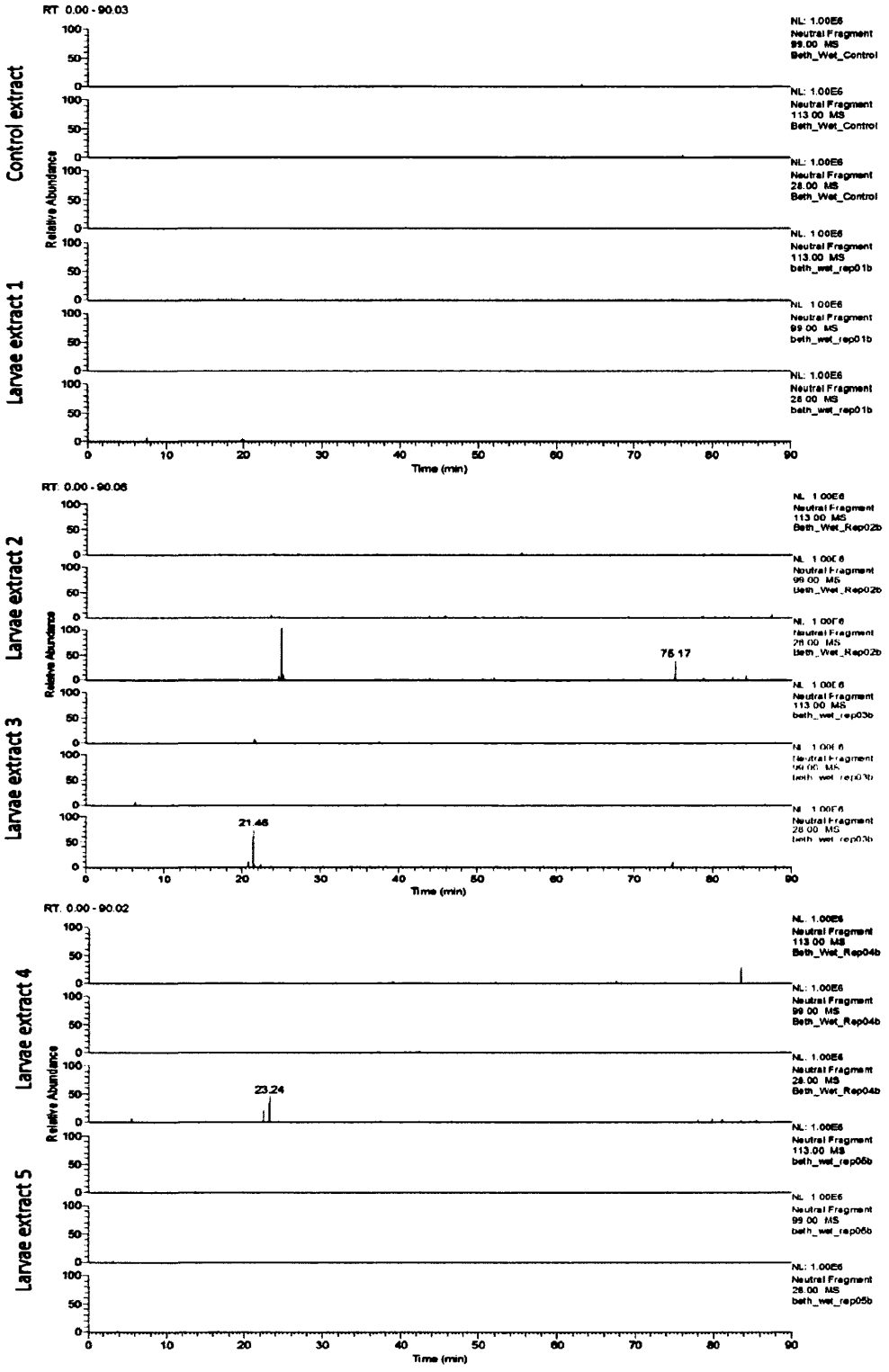

Figure 3.4. LCMS chromatogram showing no detectable *M. anisopliae* destruxin in *Aedes aegypti* larval extracts.

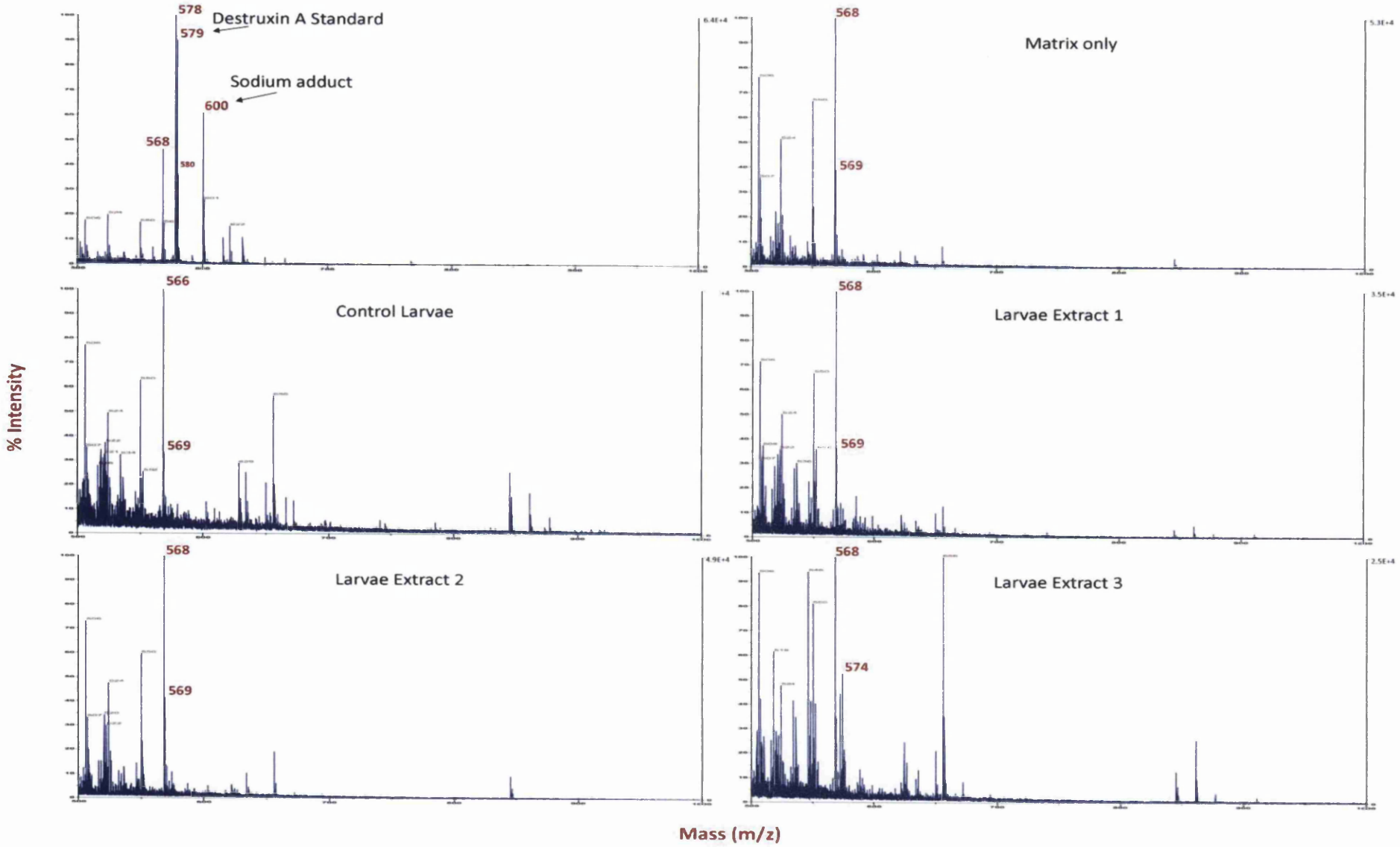
Destruxin A Standard
578
579
Sodium adduct
600
568
580
Matrix only
568
569
Control Larvae
566
569
Larvae Extract 1
568
569
Larvae Extract 2
568
569
Larvae Extract 3
568
574
% Intensity
Mass (m/z)

Figure 3.5. (overleaf) Destruxins not detected in larval extracts. Larval extracts were analysed by MALDI ToF alongside a destruxin A standard. No destuxin signals were detected with both larval extract and control larvae containing only those ions formed due to the MALDI matrix itself.

3.5.4 *Metarhizium* Pathogenicity Genes Expressed in Mosquito Gut and Faeces

Proteases (*Pr1, Pr2*) and adhesins (*Mad1, Mad2*) play a key role in fungal pathogenicity, expression of the genes for these components was analysed and was shown to be generally much higher in the gut lumen and remained high in the faecal pellets (Figure.3.6). Expression of *Pr1* was significantly lower in conidia in the presence of *Ae. aegypti* larvae compared with all the other treatments ($F_{(5, 47)} = 96.09$, $p < 0.01$) but was high in infected living, infected dead and faecal pellet samples ($F_{(5, 47)} = 96.09$, $p < 0.05$) even when compared with the *T. molitor* treatment ($F_{(5, 47)} = 96.09$, $p < 0.001$). *Pr1* expression was greatest in faecal pellets ($F_{(5, 47)} = 96.09$, $p < 0.001$) then infected larvae ($F_{(5, 47)} = 96.09$, $p < 0.05$) followed by infected dead larvae (Figure 3.6). All treatments had a significantly higher expression of *Pr2* than the spore pellets not exposed to larvae with expression being highest in infected living larvae ($F_{(5, 47)} = 96.09$, $p < 0.001$) then the faecal pellet ($F_{(5, 47)} = 96.09$, $p < 0.01$; Figure.3.6). Spore pellets exposed to larvae and infected dead larvae had lower and significantly similar expression levels. Conidia exposed to *T. molitor* had similar *Pr2* expression to the spore pellet exposed to the larvae, infected dead larvae and the faecal pellets (Figure. 3.6).

The pattern of expression of *Mad1* was comparable with the expression of *Pr2* with the exception of a slightly lower relative expression in the faecal pellet (Figure. 3.6). Infected living larvae showed significantly greater expression than any other treatment ($F_{(5, 47)} = 96.09$, $p < 0.01$; Figure.3.6). Greatest expression levels of *Mad2* were detected in faecal pellets ($F_{(5, 47)} = 96.09$, $p < 0.001$), *T. molitor* ($F_{(5, 47)} = 96.09$, $p < 0.001$) and infected dead ($F_{(5, 47)} = 96.09$, $p < 0.01$) when compared with the spores not exposed to larvae (Figure.3.6).

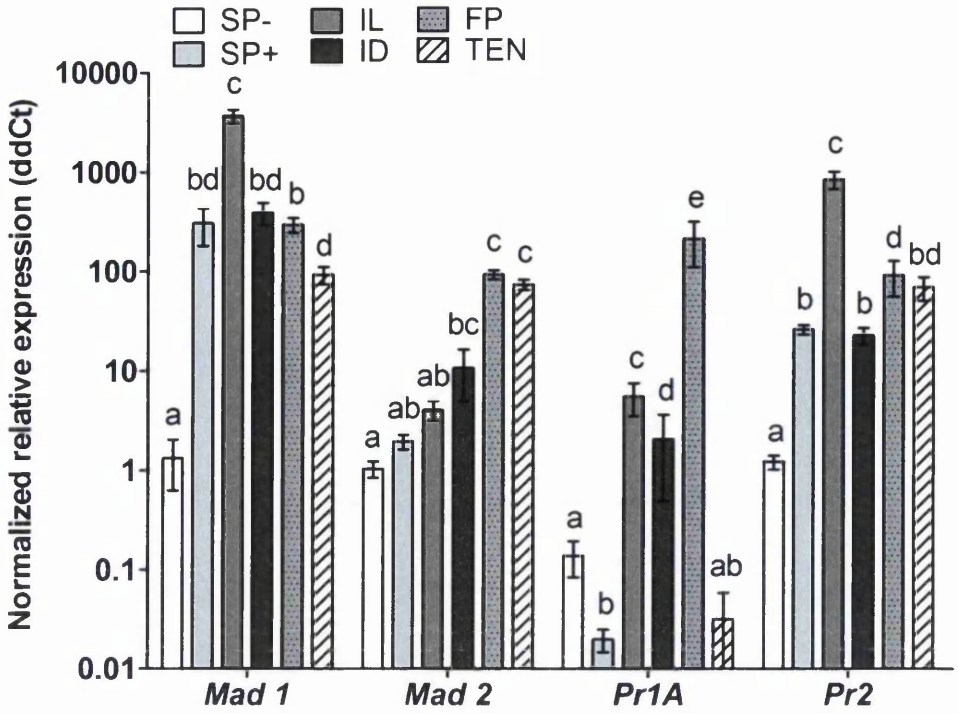

Figure 3.6. *Metarhizium* pathogenicity genes expressed in mosquito gut and faeces.

Expression of protease (*Pr1A, Pr2*) and adhesin (*Mad1, Mad2*) pathogenicity related genes by conidia of *M. anisopliae*, 48 hr pi, analysed by quantitative PCR. SP-: Spore pellet in the absence of *Ae. aegypti* larvae, SP+: Spore pellet in presence of larvae, IL: infected live larvae, ID: infected dead larvae, FP: mosquito faecal pellet, TEN: *Tenebrio molitor* (terrestrial host) positive control. Data was presented as mean (±SEM) means with different letters denoting statistical differences (two-way ANOVA). Data normalized to average dCt of SP-

3.5.5 Pr1 Active in Conidia.

Viable conidia used was shown to have good Pr1 activity, although presence of *Ae. aegypti* larvae did not show an increase in activity ($F_{(3,12)} = 23.73$, $p = 1.000$; Figure 3.7). Activity in Heat killed conidia was significantly reduced compared to the viable conidia control activity ($F_{(3,12)} = 23.73$, $p = <0.0001$; Figure 3.7), and increased in activity in the presence of mosquito larvae ($F_{(3,12)} = 23.73$, $p = 0.04$; Figure 3.7).

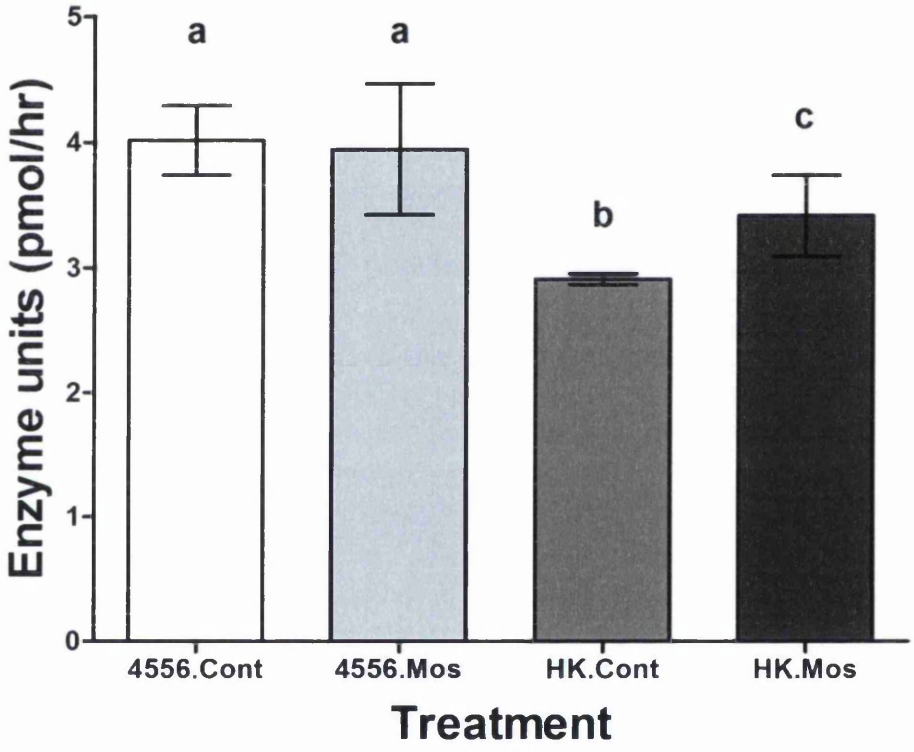

Figure 3.7. Confirmation of Pr1 activity in viable conidia.

Pr1 activity assayed in conidia with and without *Ae.aegypti* larvae present. Different letters represent samples that are statistically different. Error is represented as 95% ci.

3.5.6 Mortality Linked to Fungal Protease-Induced Apoptosis

One possible mechanism that may eventually lead to mortality of the larvae is the activation of apoptotic pathways in the larvae (involving caspase enzymes) by active agents released by the conidia. As the active agents identified were proteases, the

effect of inhibiting these enzymes on larval mortality was investigated and the larval caspase activity also monitored.

Mortality of larvae incubated with the fungus was significantly lower in the presence of protease inhibitors with the exception of EDTA which was not significantly different from the *M. anisopliae* treated larvae (Figure.3.8). The inhibition with chicken egg white increased percentage survival after treatment with the fungus from approximately 10% to 30%, whilst inhibition by α2-macroglobulin improved this value to 50% of the untreated larvae. As well as the effect of the proteases produced by the fungus on larval survival, the study of the effect of the inhibition of such bioactive entities on apoptosis was studied. Activity of caspases 2, 3/7 and 8 was significantly higher in *Ae. aegypti* larvae inoculated with live conidia of *M. anisopliae* without protease inhibitors than with inhibitors (Figure. 3.9). Interestingly, in the *M. anisopliae* treated larvae, activity increased dramatically, concomitant with larval mortality, between 36 and 72 hr pi (Figures: 3.8 & 3.9). Caspase activity was significantly lower in larvae in the presence of protease inhibitors for the whole period of the assay ($F_{(5,72)}$ = 661.39, $F_{(5,72)}$ = 90.4, $F_{(5,72)}$ =75.42 (caspase 3/7, 2 and 8 respectively) p <0.001; Figure. 3.9A). Caspase 2, 3/7 and 8 activity was generally lower 24-72 hr pi in the presence of EDTA than the other inhibitors ($F_{(5,72)}$ = 1359.03, $F_{(5,72)}$ = 486.01, $F_{(5,72)}$ =271.46 (caspase 3/7, 2 and 8 respectively) p<0.001; Figure. 3.9A).

Caspase activity, particularly caspases 3/7 and 8, was consistently higher in *Ae. aegypti* larvae exposed to live conidia compared to the heat killed conidia 48-72 hr pi ($F_{(5,54)}$ = 203.60, $F_{(5,54)}$ =71.15 (caspase 3/7 and 8 respectively) p<0.001; Figure. 3.9B-D). Caspase activity elicited by heat killed conidia increased over time up to 72hr pi.

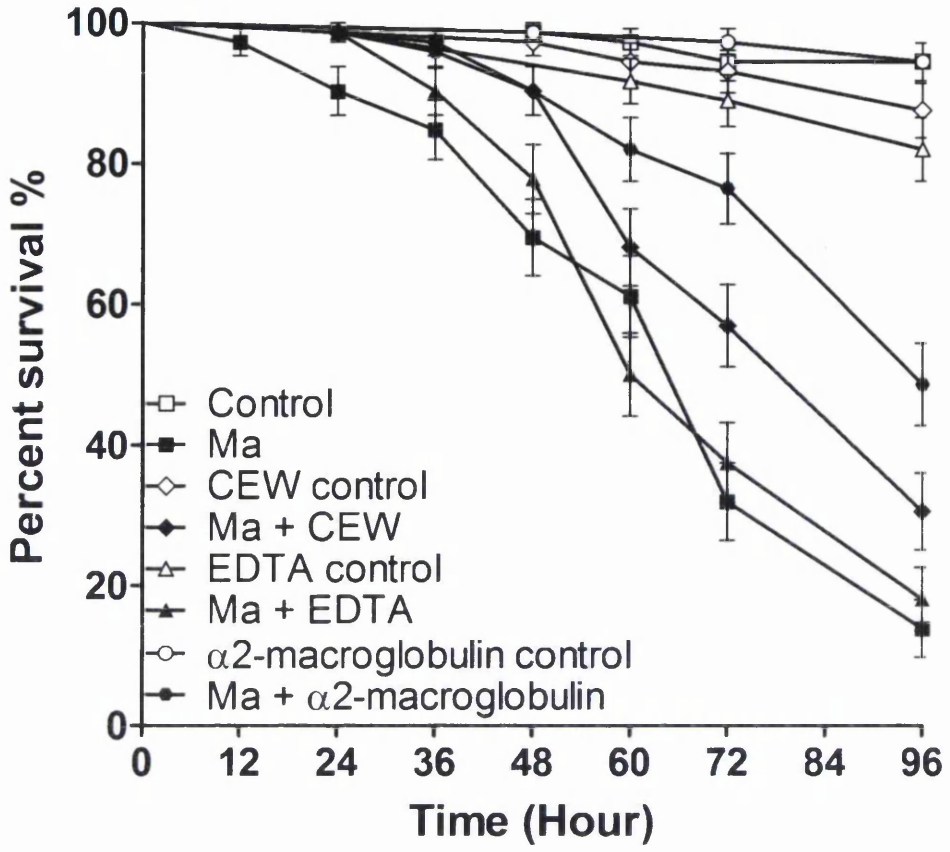

Figure 3.8. Survival of *Ae. aegypti* larvae in presence of protease inhibitor:

Ae. aegypti larvae (n = 72) were inoculated by *M. anisopliae* with and without protease inhibitors. CEW: Chicken Egg White a *Pr1* specific inhibitor, α2 mac: α2 macroglobulin a global protease inhibitor and EDTA a metalloprotease inhibitor. Kaplan-Meier method was used to plot cumulative survival curves of larvae after inoculation, log-rank test was used to assess differences in survival between treatments. Uninhibited conidia caused greater mortality than conidia treated with inhibitors with the exception of EDTA ($p < 0.001$). Controls consist of either 0.05% Aqueous Tween only, or 0.05% Aqueous Tween with protease inhibitor.

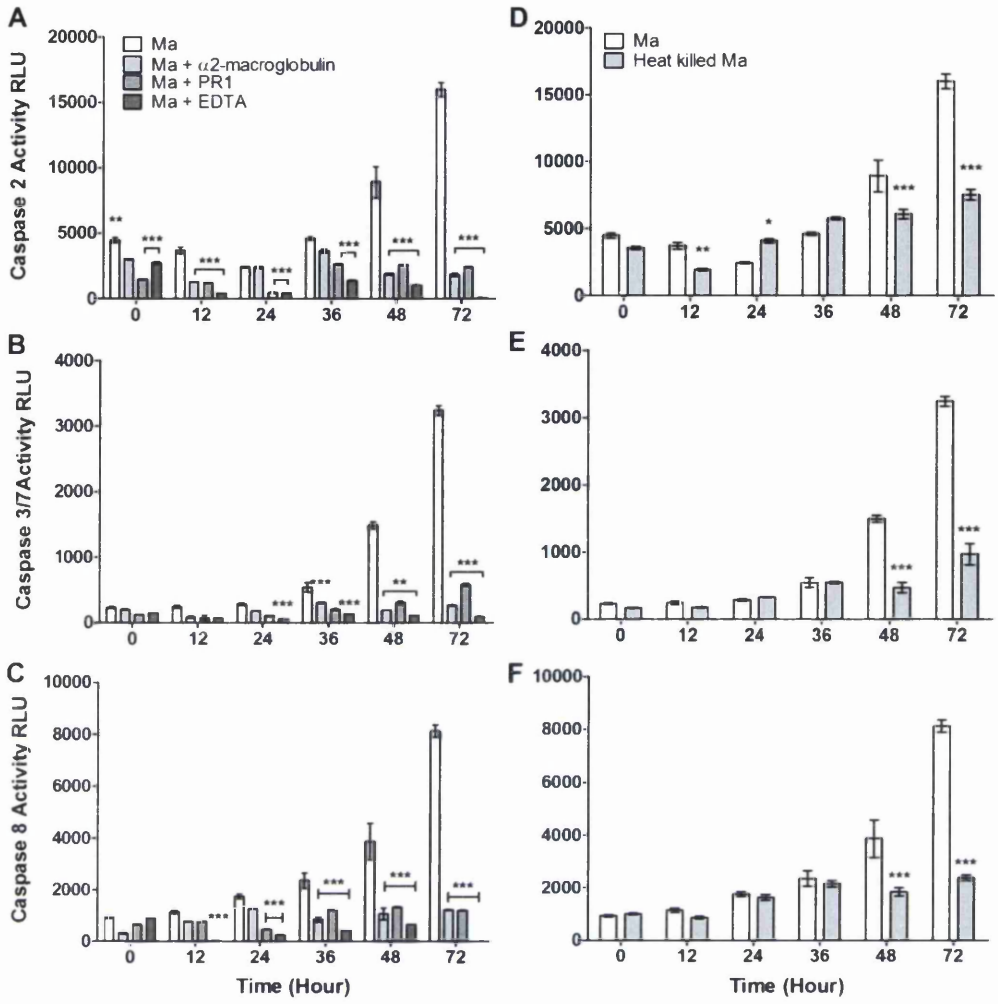

Figure 3.9. Caspase activity in *Ae. aegypti* exposed to *M. anisopliae* and protease inhibitors

Caspase activity in response to *M. anisopliae* with and without protease inhibitors (A-C) and exposed to live and heat killed conidia (D-F). Data was presented as mean (±SEM) (two-way ANOVA, ***- $p<0.001$, **-$p<0.01$, *-$p<0.05$, compared with *M. anisopliae* uninhibited control)

3.5.7 Oxidative Stress is Not an Obvious Mediator of Apoptosis

Oxidative stress within organisms can also be a trigger for the initiation of apoptosis and therefore various indicators of such stress were studied in larvae with and without conidia. Examination of reactive oxygen species generation, lipid peroxidation, catalase, superoxide dismutase and glutathione-S-transferase activity during two days of pathogenesis revealed no major differences between uninfected and *M. anisopliae* infected mosquito larvae except glutathione-S-transferase activity which was higher ($F_{(2,48)}$=12.10, p<0.01) 48hr pi in infected (Figures: 3.10 A-E). At 72 hr pi when most larvae were dead or dying we found the decrease in ROS generation ($F_{(2,60)}$=5.05, p<0.01); lipid peroxidation ($F_{(2,59)}$=1.66, p<0.05); catalase ($F_{(2,48)}$=6.77, p<0.01); and glutathione-S-transferase ($F_{(2,48)}$=12.10, p<0.01) activity in infected insects (Figure. 3.10A-E).

3.5.8 Metarhizium Fails to Induce a Phenol Oxidase Response

During early stages of infection (10min – 6hr) there was no significant change in Phenol oxidase (PO) levels compared to that of the control (Figure 3.10). PO activity became elevated above uninfected larval levels at 24hr and 48hr ($F_{(11,48)}$ = 17.72, p = <0.001, p = <0.01 respectively; Figure. 3.11). There was a significant decrease in PO activity, below that of the control (p = <0.001) at 72 hr, concurrent with larval death.

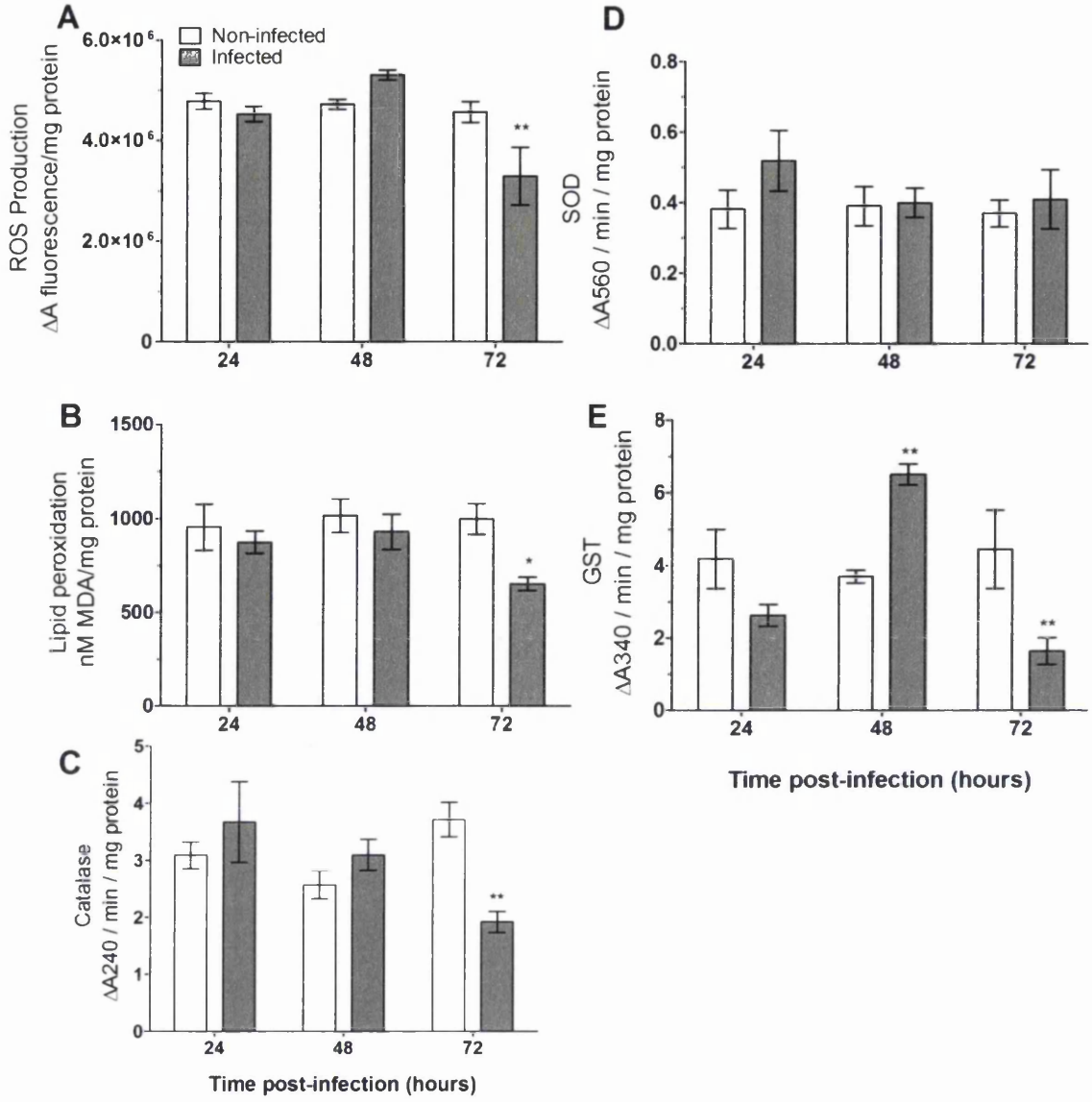

Figure 3.10. Limited antioxidant activity in mosquito larvae exposed to *M. anisopliae*.

Activity of mosquito larvae exposed and not exposed to conidia of *M. anisopliae*. (A) Reactive oxygen species (ROS) generation, and activity of (B) MDA (lipid peroxidation), (C) catalase, (D) Superoxide dismutase (SOD),and (E) glutathione-S-transferase (GST). Data presented as mean ± (SEM) (Two-way ANOVA

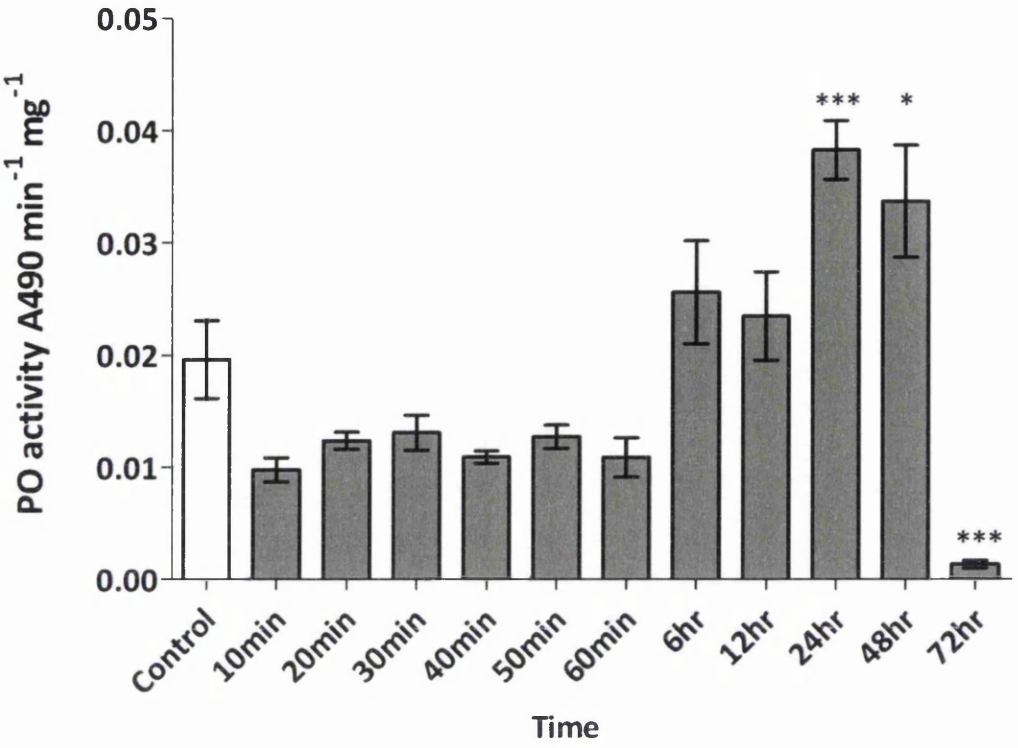

Figure 3.11: Fungus fails to induce phenol oxidase response before 24 hr post inoculation.

Phenoloxidase activity in total homogenate of *Ae. aegypti* larvae following time course application of *M. anisopliae* (data sets presented as mean +/- SEM; *** - p <0.001, *- p <0.05 compared with non-infected control insect)

3.5.9 Immune and Stress Management Systems Fail to Protect Mosquito Larvae

Given the release of active proteases by the fungus and elicitation of a pathogenic response when exposed to the larvae, the study of the larval response to the fungus was also undertaken. The analysis examined the larval defence mechanisms (predominantly antimicrobial peptides) as well as the stress response of the larvae. Expression of the antimicrobial peptide (AMP) genes *AeDA* and *AeDB* was not significantly up regulated following *Ae. aegypti* larval ingestion of live *M. anisopliae* conidia 24 hr pi (Figure. 3.12). While expression levels of *Ada-DefD* and *Ada-CcG* was significantly greater in samples exposed to *M anisopliae* after 24hr compared with time zero ($F_{(4, 50)} = 16.12$, $p < 0.05$) it was not significantly different from the 24 hr untreated control (Figure. 3.12). However, the gene was significantly down

regulated in larvae exposed to *M. anisopliae* 48hr pi compared with the other treatments ($F_{(4, 50)} = 16.12$, $p < 0.05$; Figure. 3.12). *AeCA2* was significantly down regulated in larvae at 48 hr pi compared with 24 hr pi ($F_{(4, 50)} = 16.12$, $p < 0.01$) but these did not differ from unexposed larvae at 48 hr and 24 hr, respectively (Figure. 3.12).

Of the five stress management genes examined, a significant increase in expression of the *TPX10* was observed in larvae exposed to *M. anisopliae* 24 hr compared with the time zero ($F_{(4, 40)} = 0.33$, $P<0.001$) and 24 hr untreated control ($F_{(4, 40)} = 0.33$, $p < 0.01$; Figure. 3.12). While there was a significant increase in expression of *TPX10* at 48 hr in exposed larvae compared with time zero ($F_{(4, 40)} = 0.33$, $p < 0.05$) this was not significant compared with the unexposed larvae at 48hr (Figure. 3.12). In contrast, the *Hsp70* gene was down-regulated in larvae exposed to conidia 48 hr pi compared with the untreated control ($F_{(4, 40)} = 0.33$, $p < 0.01$; Figure. 3.12). No significant changes were observed for both AMPs and stress genes in the untreated controls at 0, 24 and 48 hr (Figure. 3.12).

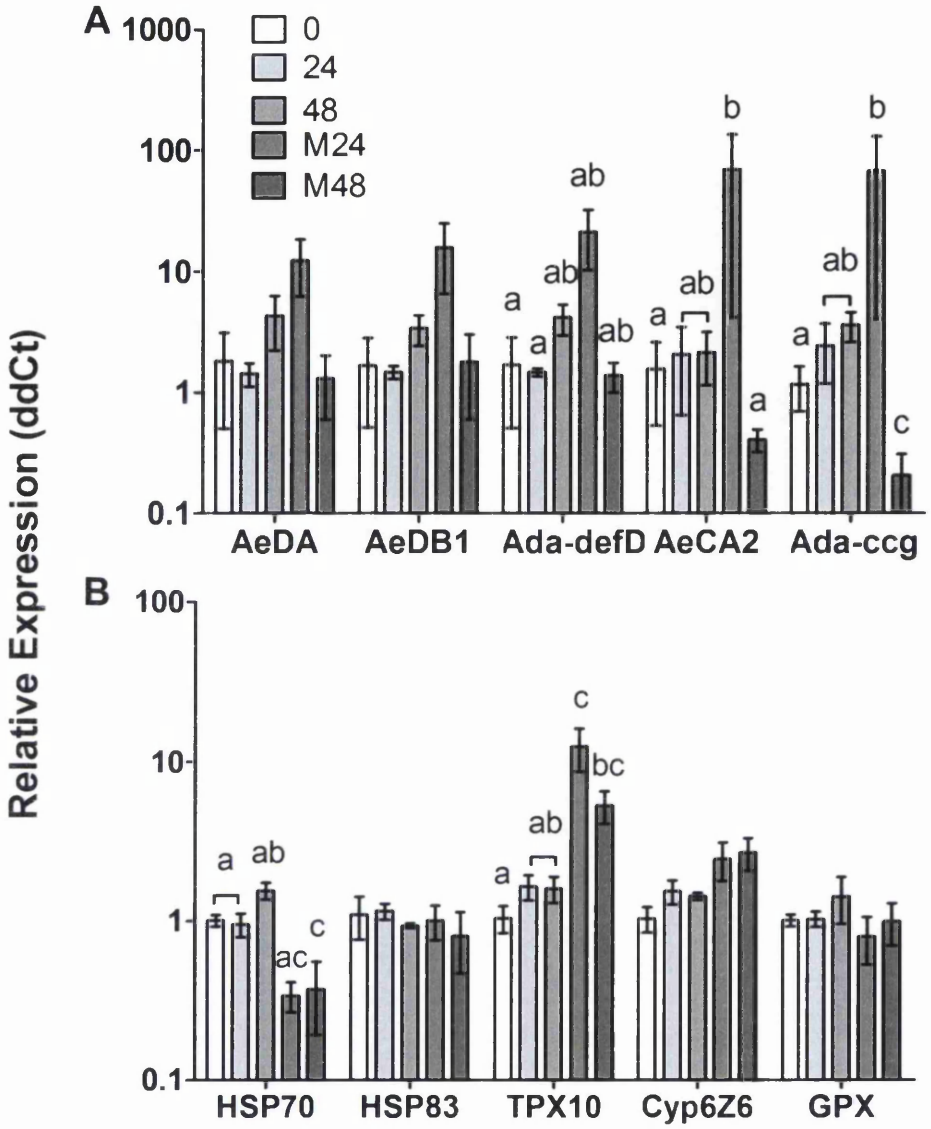

Figure 3.12. Expression of *Ae. aegypti* antimicrobial peptide and stress management genes during exposure to *M. anisopliae*

Expression of antimicrobial and stress management genes on *Ae. aegypti* were analysed in larvae inoculated with *M. anisopliae* 0, 24 and 48 hr pi by quantitative real time PCR. Antimicrobial genes included; *AeDA* (Defensin A), *AeDB1* (Defensin B), *Ada-defD* (Defensin D), *AeCA2* (Cecropin A), *Ada-ccg* (Cecropin G) and stress management genes; *HSP70* (Heatshock protein 70), *HSP83 (*Heatshock protein 83), *TPX10* (Thiol peroxidase 10), *Cyp6Z6* (Cytochrome P450), *GPX* (Glutathione peroxidase). Data was presented as mean (±SEM) means with different letters are statistically different (two-way ANOVA), **-$p<0.01$, *-$p<0.05$ compared with uninfected for the same time point)

3.6 DISCUSSION

This study shows that conidia of *M. anisopliae* do not firmly adhere to the surface of the cuticle of *Ae. aegypti* larvae and do not gain entry by penetrating the host cuticle. Conidia have been reported adhering to the cuticle, particularly the siphon and mouthparts of the fungus (Miranpuri & Khachatourians 1991), thereby infecting the larvae in a similar manner with which it infects terrestrial hosts (Bukhari *et al.* 2010).

Conidia attach to terrestrial hosts initially *via* passive hydrophobic forces followed by secretion of enzymes and adhesion compounds to anchor the spore to the cuticle surface (Shah, Wang & Butt 2005; Wang & St. Leger 2007). The failure of conidia to adhere to the cuticle of terrestrial hosts has been attributed to the cuticle chemistry, with some compounds altering hydrophobicity or being fungistatic (Butt *et al.* 1995; Lord & Howard 2004). It is feasible that the mosquito larval cuticle is not conducive for adhesion, with any mucilage produced by the fungus being diluted in the water. In contrast, aquatic fungal pathogens of mosquitoes such as *Lagenidium giganteum* and *Coelomomyces punctatus* produce zoospores that can attach to and penetrate the larval cuticle before colonizing the haemocoel. *Culicinomyces clavisporus*, an aquatic Sordariomycete related to *M. anisopliae*, produces conidia which, following ingestion by the larvae, adhere to and penetrate through the chitinous wall in the fore- and hindgut (Sweeney 1983).

In conidia of *M. anisopliae, Mad1* expression in the presence of mosquito larvae suggest the fungus had responded to cuticular cues in a similar manner to a terrestrial host despite its failure to adhere through passive hydrophobic forces. *Mad1* was up regulated particularly inside the gut of live insects suggesting that the fungus had perceived additional cues. *Mad2* was not upregulated in the same manner, however, expression of both these genes was significantly higher in the gut of dead insects and faecal pellets possibly due to nutritional stress which would also explain why no germ tubes were produced. Nutrient starvation is known to up regulate *Mad2* (Barelli *et al.* 2011). The concomitant upregulation of *Mad1, Mad2, Pr1* and *Pr2* by the ungerminated conidia of *M. anisopliae* suggest that the fungus is mounting a response to infect but fails to progress due to the lack of stimuli normally present in the terrestrial arthropod host. Conidia of *M. anisopliae* are readily ingested by mosquito larvae with some workers suggesting this to be the main route of infection

(Scholte *et al.* 2004). Our studies show that the conidia fail to produce germ tubes and penetrate the gut wall, nor do they cause inflammation of the midgut epithelium or interfere with gut function, allowing the insect to remove conidia in compact faecal pellets at least until death. Toxins, particularly destruxins, have been implicated as the cause of mosquito larval death, produced by ungerminated conidia on the cuticle, inside the gut or released following digestion of *M. anisopliae* conidia (Crisan 1971). In our study, no destruxins were detected in *Ae. aegypti* larvae that had ingested conidia of *M. anisopliae* 4556, even though this strain is known to produce destruxins, thus discounting these compounds as the cause of death.

Extracellular proteases of *M. anisopliae,* with the exception of metalloproteases, contribute significantly to *Ae. aegypti* larval mortality which appears to be mediated through stress induced apoptosis. *Pr1* and *Pr2* were expressed during passage through the insect gut, in the faecal pellet and recently killed larvae. Chicken egg white, an inhibitor of *Pr1*, significantly improved survival of *Ae. aegypti* larvae but not to the same extent as the global protease inhibitor, α2 macroglobulin, suggesting that several proteases working in concert were contributing to larval mortality. Not all proteases contribute to mortality since inhibition of *Pr2* did not improve survival (unpublished). EDTA treated insects posed an anomaly as these exhibited low caspase activity but high larval mortality. It is possible that EDTA, besides inhibiting metalloproteases, interfered with cation dependent cellular processes such as signalling, homeostasis, and caspase activation (Squier *et al.* 1994; Tantral *et al.* 2004) which would exacerbate the stress caused by the fungal pathogen. This is clearly an area for further investigation. Mortality in heat killed conidia and untreated control was statistically similar suggesting that extracellular proteases contributed significantly to larval mortality. Proteases will accrue with time as more conidia pass through the gut. The high survival of larvae in the presence of protease inhibitors and heat killed conidia show that death does not arise due to blockage of the mouthparts or breathing apparatus as suggested by some previous studies (Lacey *et al.* 1988).

Upregulation of *A. aegypti* antimicrobial peptide (AMP) genes, peaking 24 hr pi, is the typical immune response of insects exposed to pathogens, stress or injury (Dubovskiy *et al.* 2013). The mosquito larvae did not mount a strong AMP mediated

defence response to *M. anisopliae;* the only significant activity was downregulation of cecropins A and G, 48 hr pi, which coincided with a significant increase in caspase activity and larval mortality. Indeed, mortality appeared to be correlated with caspase activity. Activities of initiator (caspases 2 and 8) and effector (caspases 3 and 7) caspases increased with time suggesting an increasing number of cells undergoing apoptosis. Once a threshold of dead cells had been reached the insect would be unable to sustain life functions resulting in death. Apoptosis is known to be induced by oxidative damage either from oxygen free radicals or hydrogen peroxide directly or from their generation in cells by injurious agents (Cooper, Granville & Lowenberger 2009). Insects, like many other organisms, actively produce reactive oxygen intermediates as signalling molecules to control processes such as, apoptosis, abiotic stress responses, and pathogen defence (Whitten *et al.* 2001). Cellular antioxidant mechanisms countering oxidative stress include soluble free radical scavenger molecules such as glutathione and enzymes like superoxide dismutases, catalases and peroxidases. Most of these enzymes were not elevated in *M. anisopliae* infected *Ae. aegypti* larvae, with the exception of glutathione-S-transferase, 48 hr pi but at 72 hr pi they had all fallen possibly due to insects being close to death. Expression of the stress management genes at the critical 48 hr pi was not as extensive as reported in terrestrial insects (Dubovskiy *et al.* 2013), presumably due to the mosquito larvae never encountering the fungus and evolving an appropriate response. Most notable was the downregulation of *Hsp70* and upregulation of *TPX10*. *Hsp70* has vital housekeeping functions, maintaining homeostasis and protecting cells against thermal and oxidative stress (Mayer & Bukau 2005). It can directly inhibit apoptosis upstream of caspase 3 activation (Li *et al.* 2000; Beere & Green 2001). *Hsp70* is activated by a wide range of factors including cytokines, energy (ATP) depletion and reactive oxygen species (Mayer & Bukau 2005) The downregulation of *Hsp70* would predispose the mosquito larvae to apoptosis. Thiol peroxidases (TPx) play an important antioxidant role in a wide range of organisms including insects. They utilize thioredoxin as a substrate to carry out detoxification of reactive oxygen species (Hambarde, Singh & Chandna 2013). Thiol peroxidases can inhibit apoptosis (Wood, Poole & Karplus 2003; Hambarde *et al.* 2013) , therefore, upregulation of *TPX10* may be an attempt by the *M. anisopliae* infected larvae to contain apoptosis.

This study shows for the first time that mortality of mosquito larvae exposed to *M. anisopliae* is multifactorial. It is not due to invasion and colonisation of the host, as proposed by other workers, but entails *M. anisopliae* proteases triggering stress induced apoptosis which ultimately leads to host death, hence the verdict of accidental death. The fungus has the machinery to infect terrestrial insect hosts and although some of this apparatus is expressed in the mosquito larvae it is ineffective in the aquatic environment. Likewise, the mosquito larvae did not mount a strong defense response as for *C. clavisporus* (Sweeney 1983). Presumably, mosquito larvae have either not evolved appropriate pathogen recognition receptors to identify *M. anisopliae* derived pathogenicity associated molecular patterns, as is the case for terrestrial hosts (Gottar *et al.* 2006) or alternatively, the lack of success with regard to the fungal colonization limits the insects ability to recognise the attempted infection. Failure of *M. anisopliae* to colonize and sporulate on the mosquito host would result in no horizontal transfer of inoculum and for biocontrol management strategies would require regular application unlike the aquatic pathogens which can cause epizootics because of their ability to reproduce in mosquitoes and other aquatic invertebrates (Scholte *et al.* 2004). Genetic or physiological manipulation of *M. anisopliae* to over produce proteases could accelerate larval mortality and pose little environmental risks because of the inability of the fungus to infect or reproduce in mosquito larvae.

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CHAPTER 4

CONIDIA OF THE INSECT PATHOGENIC FUNGUS, *METARHIZIUM ANISOPLIAE*, FAIL TO ADHERE TO MOSQUITO LARVAL CUTICLE

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Conceived and designed the experiments: **BPJG**.
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CONIDIA OF THE INSECT PATHOGENIC FUNGUS, *METARHIZIUM ANISOPLIAE*, FAIL TO ADHERE TO MOSQUITO LARVAL CUTICLE.

4.1 ABSTRACT

Adhesion of conidia of the insect pathogen, *Metarhizium anisopliae*, to the host cuticle initially involves hydrophobic forces followed by consolidation facilitated by the action of extracellular enzymes and secretion of mucilage. Gene expression analysis and Atomic force microscopy were used to directly quantify recognition and adhesion between single conidia of *M. anisopliae* and the cuticle of the aquatic larval stage of *Aedes aegypti* and a traditional terrestrial host, *Tenebrio molitor*. Gene expression data indicated recognition by the pathogen of both hosts, however adhesion to the mosquito was approximately 5 times lower than forces measured for *Tenebrio*. Although weak forces were recorded in response to *Aedes*, *Metarhizium* was unable to consolidate firm attachment. An analysis of the cuticular composition revealed an absence of long chain hydrocarbons, thought to be required for fungal development, in *Aedes* larvae. This study provides the first evidence that *Metarhizium* does not form firm attachment to *Aedes aegypti* larvae *in situ*, therefore preventing the normal route of invasion and pathogenesis from occurring.

4.2 INTRODUCTION

Metarhizium anisopliae, is a widespread, soil-borne fungal pathogen of insects, ticks and mites (Kaaya, Mwangi & Ouna 1996; Smith, Wall & French 2000; Inglis *et al.* 2001) and therefore, there, is much interest in this fungus as an environmentally friendly alternative to conventional chemical pesticides for the control of pests of socio-economic importance (Inglis *et al.* 2001). *Metarhizium* infection processes are similar to those of other entomopathogenic fungi; they entail conidial attachment to the host surface followed by germination and penetration of the host cuticle through a combination of enzymatic activity and mechanical force (Quintela & McCoy 1998; Arruda *et al.* 2005; Thomas & Read 2007). The fungal cell wall is critical in the infection process and are involved in numerous essential functions including protection, osmotic stability, morphogenesis and cell-cell interactions, specifically

host recognition and adhesion. It is mainly comprised of many proteins associated with host-pathogen interactions located in the outer cell wall bound covalently to β-1,6-glucans. These proteins include; the superoxide dismutases, phospholipases, aspartyl proteases and adhesins (Pitarch, Nombela & Gil 2008; de Groot *et al.* 2013) Molecular recognition through these proteins is a key event in pathogenesis, with the initiation of infection process occurring between fungal adhesins and specific receptors in the host cuticle (Gaboriaud & Dufrêne 2007).

From the hosts perspective, the arthropod cuticle is the primary and possibly most important barrier to the external environment as well as the attachment and invasion of fungal propagules (Boucias & Pendland 1991; Dubovskiy *et al.* 2013). It's surface structure, topography and chemical composition can influence spore adhesion and therefore fungal pathogenicity (Boucias & Pendland 1991; Lord & Howard 2004), with hydrophobic lipids and fungistatic compounds within the outermost epicuticular wax layer playing an essential role in attachment and germination of fungal propagules (Fargues 1984; Boucias & Pendland 1991; Pendland, Hung & Boucias 1993; James, Buckner & Freeman 2003). Firm adhesion is crucial for the success of the pathogen as this can influence specificity and virulence.

Conidial attachment is a two-step process. The first step involves passive attachment mediated through a combination of non-specific hydrophobic and electrostatic forces as well as attachment via specific ligands or specific adhesin protein interactions (Holder & Keyhani 2005; Wang & St. Leger 2007). Hydrophobins in particular, found in the outer layer of the spore cell wall, mediate adhesion to hydrophobic components of the arthropod cuticle (Wessels 2000; Wösten 2001; Holder & Keyhani 2005; Holder *et al.* 2007). The second step involves secretion of enzymes to create an infection court. Hydrolytic enzymes degrade fungistatic fatty acids, release nutrients and facilitate penetration of the cuticle (St. Leger, Cooper & Charnley 1987; Paterson *et al.* 1994; Jarrold *et al.* 2007; Van Bogaert *et al.* 2011; Zhang *et al.* 2012). Mucilage is often secreted to consolidate the attachment of the fungus to the host surface. The adhesins, *Mad1* and *Mad2*, assist in attachment of the fungus to insect and plant surfaces, respectively with *Mad1* also contributing to pathogenesis (Wang & St. Leger 2007; Barelli, Padilla-Guerrero & Bidochka 2011).

Recently, strains of *Metarhizium* were reported to be highly pathogenic to mosquito larvae (Silva, Silva & Luz 2008; Bukhari, Takken & Koenraadt 2011), with death following the ingestion of conidia as opposed to adhesion and penetration of the cuticle (Butt *et al.* 2013). Exactly why the conidia did not adhere is unclear. This study therefore sought to study the attachment process of an individual spore to host cuticle for both the mosquito larvae and a traditional terrestrial invertebrate host. The attachment process for both was studied in the mosquito's usual aqueous environment in order to determine whether non-specific factors such as water interferes with the attachment process, thereby allowing for the determination of fungus-host specific determinants of successful or unsuccessful attachment. Typically spore adhesion is measured utilising count methods, however, recent advances in atomic force microscopy (AFM) have enabled the direct measurement of the adhesion forces of a single particle or cell and their interacting surfaces (Binnig, Quate & Gerber 1986). The present study combines the use of AFM, gene expression and lipidonomic analyses to further investigate the host-pathogen interactions of *Metarhizium* to its insect host. The expression of the *Metarhizium* adhesin genes (*Mad1* and *Mad2*) in response to terrestrial insect cuticle and aquatic mosquito larvae was utilised as an indicator of the recognition of the cuticle as a cue for pathogenesis to be initiated by the fungus. The AFM measurements then were used to determine whether this cue was allowing traditional infection processes to be successful whilst the lipidomic analysis studied the two cuticle "trigger" sources from a chemical perspective. The integrated data allows for a more detailed study of the processes and mechanisms that influence the infection process in aquatic mosquitoes compared with the more commonplace, terrestrial insects targeted by the fungus.

4.3 MATERIALS AND METHODS

4.3.1 Samples, RNA Extraction and cDNA Synthesis.

Ten *Ae. aegypti* larvae per replicate were either incubated in 50 mL of $1x10^7$ conidia mL^{-1} solution of ARSEF 4556 or with distilled water and incubated at room temperature for 24 hr. After 24 hr the mosquitoes were harvested, washed in distilled water and frozen under liquid nitrogen. In addition, three *T. molitor* larvae,

per replicate were subjected to an immersion assay in 50 mL of 1 x 10^7conidia ml^{-1} ARSEF 4556 for 20 sec, the insects were then incubated on moist filter paper at 27°C for 24 hr, before freezing under liquid nitrogen. All samples were prepared in triplicate.

Samples were ground with a micropestle and total RNA extractions carried out using the RNeasy Micro kit (Qiagen) as per section 3.3.7.1.

4.3.2 qRT-PCR Assay for Expression of Adhesin Genes.

Relative cDNA quantity was analyzed by using the qPCR method previously described (Butt *et al.* 2013). The same protocol was followed as per section 3.3.7.2.

4.3.3 Insect Cuticle Preparation.

Aedes aegypti (strain AeAe) were reared at room temperature (22°C ± 2°C) in distilled water to late (L_{3-4}) instar and fed Tetramin® fish food. *Tenebrio molitor* larvae were maintained at 27°C (± 2°C) on wheat bran. *Ae. aegypti* larvae were removed from water and dried on Whatman N°1 filter paper before flash freezing under liquid nitrogen. *T. molitor* larvae were washed in distilled water and flash frozen under liquid nitrogen before cutting the abdominal cuticle into 5 x 10 mm sections. The mosquito and *T. molitor* cuticle were subsequently fixed, with a small amount of glue (Epoxy resin), to a glass slide.

4.3.4 Spore Probe Preparation.

M. anisopliae isolate ARSEF 4556 spores were harvested from cultures maintained on broken Basmati rice (Ansari & Butt 2011). Individual fungal spores were immobilized at the end of a V-shaped tipless AFM cantilever (Thermomicroscopes) (Figure 4.1). Fungal spores were dusted onto a glass slide, previously cleaned with ethanol. Glue (Homebase glass glue) was applied to the AFM cantilever and a single conidium fixed to the AFM cantilever using the atomic force microscope itself. To confirm attachment the spore colloid probe was visualised under a VHX-600 Keyence microscope and later imaged with a Scanning Electron Microscope (SEM).

4.3.5 Atomic Force Microscopy.

A JPK nanowizard II AFM (JPK instruments, Berlin) was utilised to measure the adhesion forces between the spore probe and sample, by the vertical deflection of the cantilever. Cantilever deflection is measured as a change in the reflected laser path, resulting in a cantilever deflection – displacement curve (Figure 4.2). Prior to force measurements, the cantilever was calibrated, to convert the deflection into a quantitative force measurement. The spring constant and sensitivity of cantilever were measured directly, by lowering the cantilever onto a clean glass surface submerged in deionised water, generating a spring constant of 0.26 N m^{-1} and sensitivity of 53 nm V^{-1}, these values were used in subsequent force calculations following Hooke's law. Experiments were carried out in a fluid cell containing deionised water. All forces curves were normalized resulting in tip deflection of 0 nm where there was no interaction.

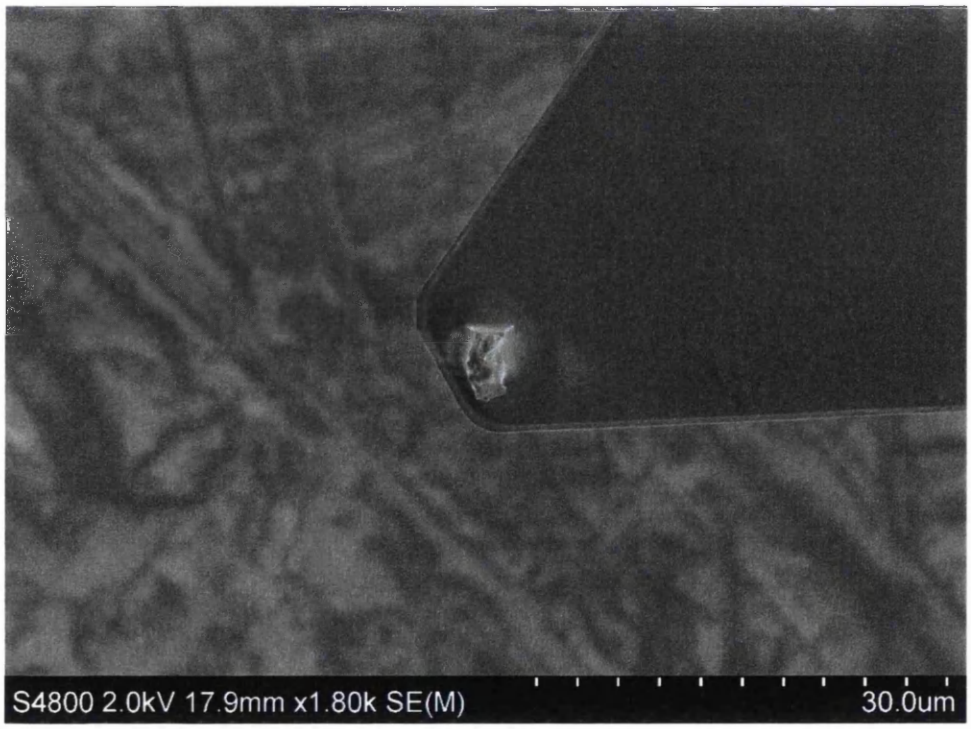

Figure 4.1. *Metarhizium* conidium immobilized at tip of AFM cantilever
SEM image of a spore probe after force measurements.

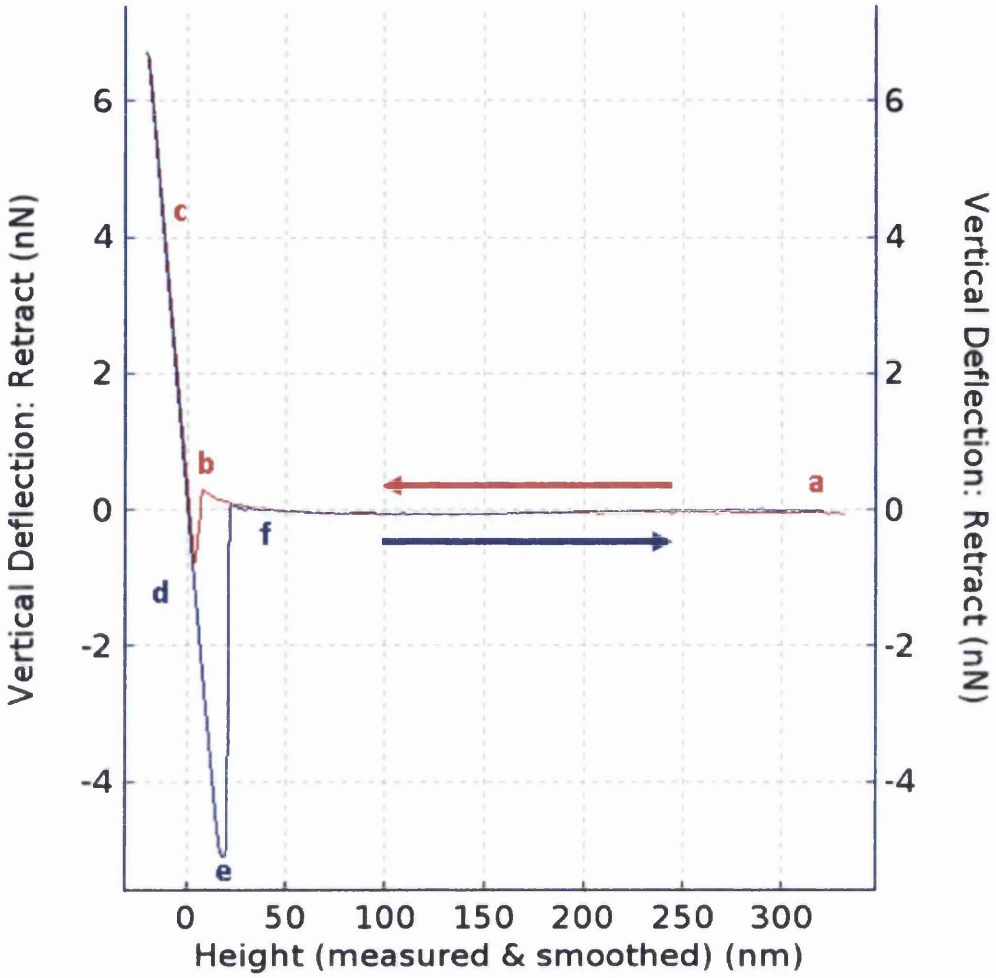

Figure 4.2. Typical force–distance curve showing a single adhesion event of *Metarhizium anisopliae* to glass.

The red line is the approach curve and the blue line is the retraction curve. The cantilever is away from the sample surface (a), as the cantilever approaches the sample, initially the force is too small to provide a measureable deflection and remains in a neutral position at 0 nm, until reaching the contact point (b) where attractive forces (Van der Waals) overcome the cantilever spring constant and the tip jumps to contact with the sample. Once in contact the tip remains in contact as the separation between cantilever and sample decreases, causing a deflection of the cantilever (c). As the cantilever is retracted the tip remains in contact (d), due to adhesion and the cantilever is deflected down (e). Eventually the force on the cantilever is enough to overcome adhesion and the tip breaks free, returning to a neutral position (f).

4.3.6 Epicuticular wax extraction.

All insects were washed in deionised water and dried at 30°C (± 2°C) prior to solvent extraction. Six replicates of Five *Ae. aegypti* or two *T. molitor* larvae were washed in hexane for 10 s before discarding, the hexane fraction was subsequently dried under Nitrogen gas before resuspending in 1 mL transesterification solution: HCl/MeOH (5% v/v) containing 0.01% butylated hydroxyltoluene (w/v). Samples were heated to 90°C for 90 min and allowed to cool to room temperature before incubating in 2 mL hexane with gentle agitation for 30 min. The hexane extract was collected and the sample extracted with hexane a further three times. All hexane fractions were pooled before drying under Nitrogen gas. Heptadecanoic acid (C17:0) was added prior to extraction to act as an internal standard. Dried samples were stored at -80°C until required.

4.3.7 GC-MS.

Prior to GC-MS analysis, samples were resuspended in hexane and 1 µL of each sample injected onto a Thermo TR-5ms SQC GC column (15 m*0.25 mm* 0.25 µm) (Themo Scientific, UK) which was eluted by a Trace GC Ultra (Thermo Scientific, UK). There was a 0.5 s pre-injection dwell time and a single wash with 1 µL chloroform followed by one pre-injection rinse. The temperature gradient for elution used an initial temperature of 50°C which was held for 1 minute and then increased at 30°C per minute up to a final temperature of 300°C which was held for 5 minutes before returning to starting conditions. The eluent from the GC column was analysed by a DSQ II mass spectrometer which was used in positive ionisation mode and a mass range for 33-650 amu in electron impact mode. An ion source temperature of 200°C, a multiplier voltage of 1215 V, 4.06 scans/second, 2534 amu/second and a scan range of 33-600 amu were used. For compound identification in EI analysis, mass spectra from individual components were submitted to the NIST database within the Xcalibur software (ThermoScientific, UK) and identifications confirmed by probability score and manual inspection of the experimental and theoretical data.

4.3.8 Statistical Analysis.

Comparison of adhesion force measurements between samples was analyzed using a Kruskal-Wallis H test. Pairwise comparisons were performed using Dunn's post-hoc with a Bonferroni correction for multiple comparisons. GC-MS data was analysed using Mann Whitney U test and Molecular data sets were analyzed using two-way Analysis of Variance (ANOVA) with Tukey HSD post-test. Prior to analysis gene expression data was subjected to a Box-Cox transformation, conforming to ANOVA assumption of homogeneity of variance (Eastwood *et al.* 2008). All statistical analyses were carried out using MatLab R2013b and R 3.0.2 (R Core Team 2013).

4.4 AIMS

1) Identify and quantify level of adhesion of *Metarhizium* conidia to mosquito larvae

2) Determine whether the chemical composition of mosquito larval cuticle is the same as that of a terrestrial insect.

4.5 RESULTS

4.5.1 Gene Expression of *Mad* Genes Confirms Recognition.

Adhesins (*Mad1* and *Mad2*) are fundamental in fungal adhesion and as a consequence pathogenicity, the genes for these components were analyzed and shown to be constitutively expressed in conidia but up-regulated in the presence of both *Aedes* and *Tenebrio* with no difference in expression of *Mad1* irrespective of insect host (Figure 4.3; $F_{(1,12)} = 7{,}195$, $p = 0.326$). The only significant differences in expression between genes were recorded for *Aedes* (Figure 4.3; $F_{(2,12)} = 11.649$, $p = 0.0199$) with expression of *Mad2* being significantly lower in *Aedes* compared with the same genes expression in *Tenebrio* (Figure 4.3; $F_{(2,12)} = 49.792$, $p = 0.0199$) .

4.5.2 Conidia Fail to Adhere to *Aedes* Cuticle.

The force interactions between the abdominal cuticle segments and an immobilised spore were determined using AFM in order to determine whether the elevated expression of the adhesion genes determined allowed for effective attachment of the

spore to the cuticle, compared with non-attachment to an inert glass surface. Figure 4.4 shows typical force-distance curves, with a single adhesion event, of *Metarhizium anisopliae* to glass, *Aedes* and *Tenebrio*. *Tenebrio* approach curves show a large jump to contact peak, indicative of strong attractive forces, occurring at a distance of approximately 0.05 μm. Typically, the cantilever snaps to the sample surface if the attractive force on the probe reaches or exceeds the value of the spring constant, in this case 5 nN, nearly 20 fold greater than the measured spring constant (0.26 nN). In contrast to *Tenebrio*, force-distance curves measured between glass and *Aedes* show no noticeable attractive interactions within the approach curve (Figure 4.4).

Similar forces were measured between glass and *Aedes* (Figure 4.4). Forces generated on *Tenebrio* cuticle were an order of magnitude greater than those of *Aedes* ($\chi^2(2) = 65.366$, $p = <0.001$). Indeed, compared to the control sample, the adhesion forces measured for *Tenebrio* were 5 fold greater than glass or *Aedes* (Figure 4.5; Mdn = 25.855, $p = <0.0001$). There was no significant difference in forces measured between glass and *Aedes* (Figure 4.5; Mdn = 4.1505, 2.9980 respectively, $p = 0.130$) illustrating an absence of specific attachment to the cuticle of the mosquito.

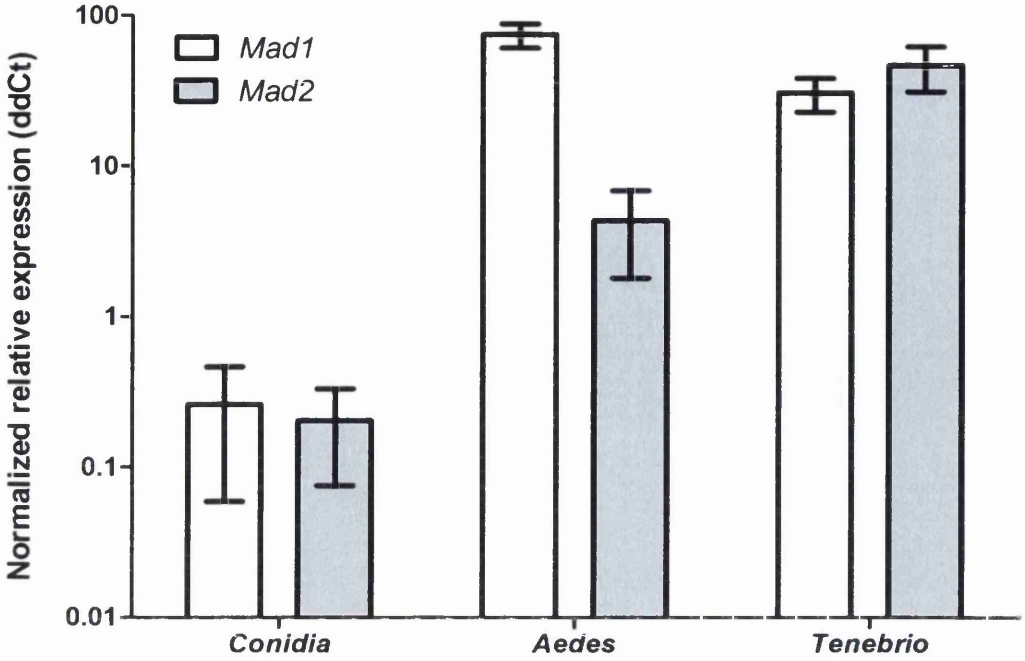

Figure 4.3 Upregulation of *Mad* genes indicative of a host-pathogen response.

Expression of Adhesin genes, 48hr post inoculation, analysed by qRT-PCR. *Mad 1* genes are constitutively expressed in conidia, regardless of insect host. Data was presented as mean (±SEM). Data normalized to average dCt of Conidia.

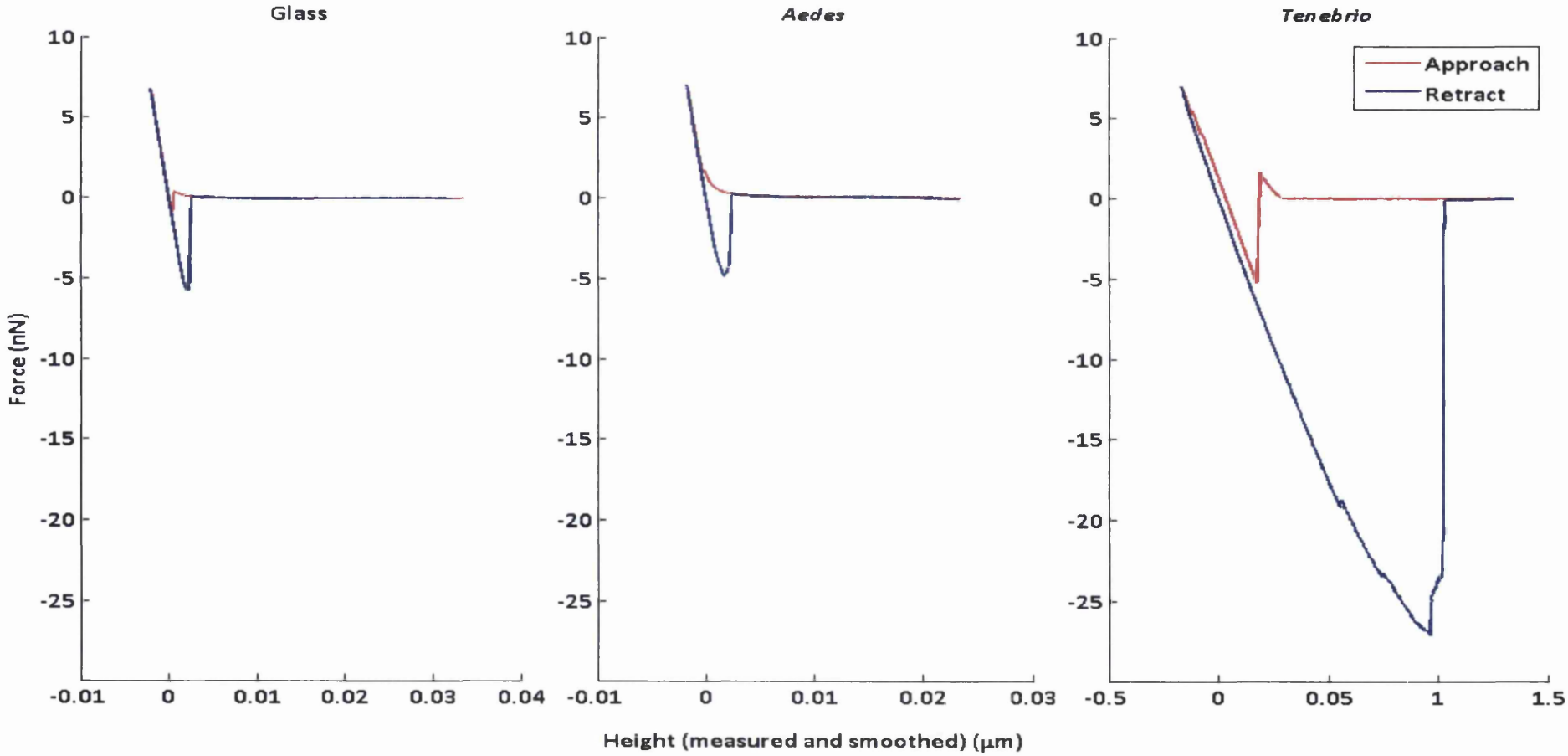

Figure 4.4 *Metarhizium* conidia fail to adhere to *Aedes* cuticle

Typical Force-distance curves observed in for *Metarhizium* conidia to glass or *Aedes* and *Tenebrio* cuticle. Large force measurements, obtained in *Tenebrio*, indicative of a strong interaction are absent in glass and *Aedes* samples.

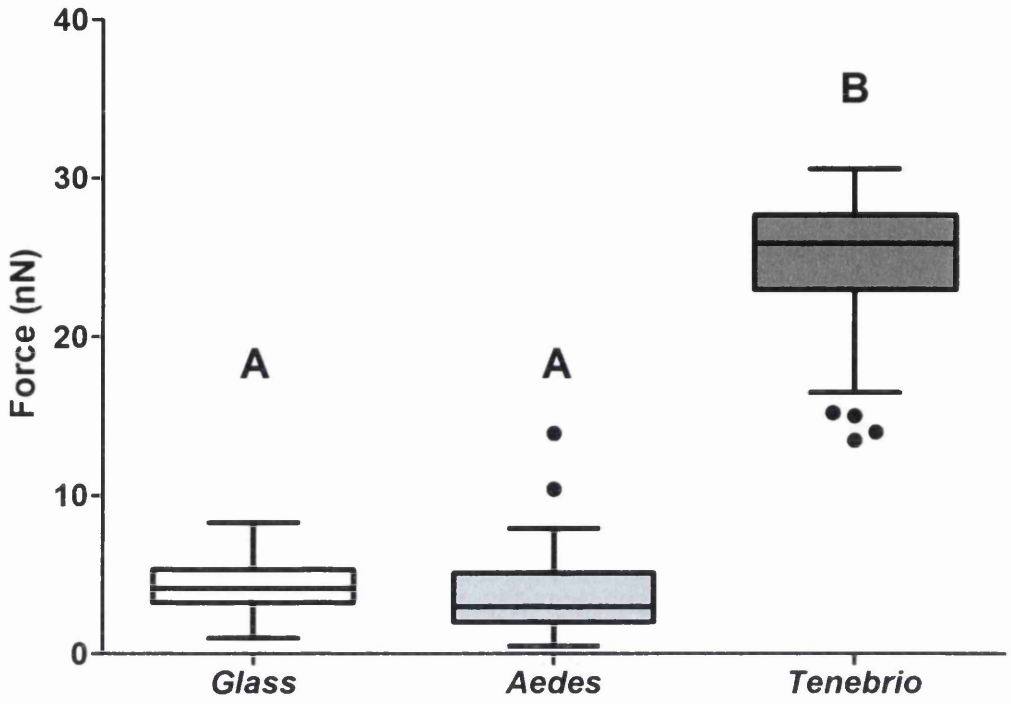

Figure 4.5. Average adhesion forces measured.

Adhesion forces measured between *Metarhizium* conidia and *Aedes* (n = 115), *Tenebrio* (n = 121), and glass (n = 100). Letters denote significant differences, Tukey whisker (25th and 75th percentile). Boxes denote interquartile range, bisected horizontally by median values; whiskers extend to 1.5× interquartile range beyond boxes; outliers are represented with black dots outside of whiskers.

Retraction curves demonstrated interesting differences, *Tenebrio* force-distance curves characteristically showed one adhesive peak over a large area with a sharp, de-adhesion to a neutral position, after a distance of approximately 1 μm. In contrast to this, retraction curves with multiple peaks and constant force plateaus at lower adhesion forces of approximately 0.5 nN were measured for *Aedes*, compared to weakest forces recorded in *Tenebrio* being between 15-20 nN. This indicates the possibility of other short range molecular interactions, which are much weaker than those formed with *Tenebrio* (Figure: 4.6).

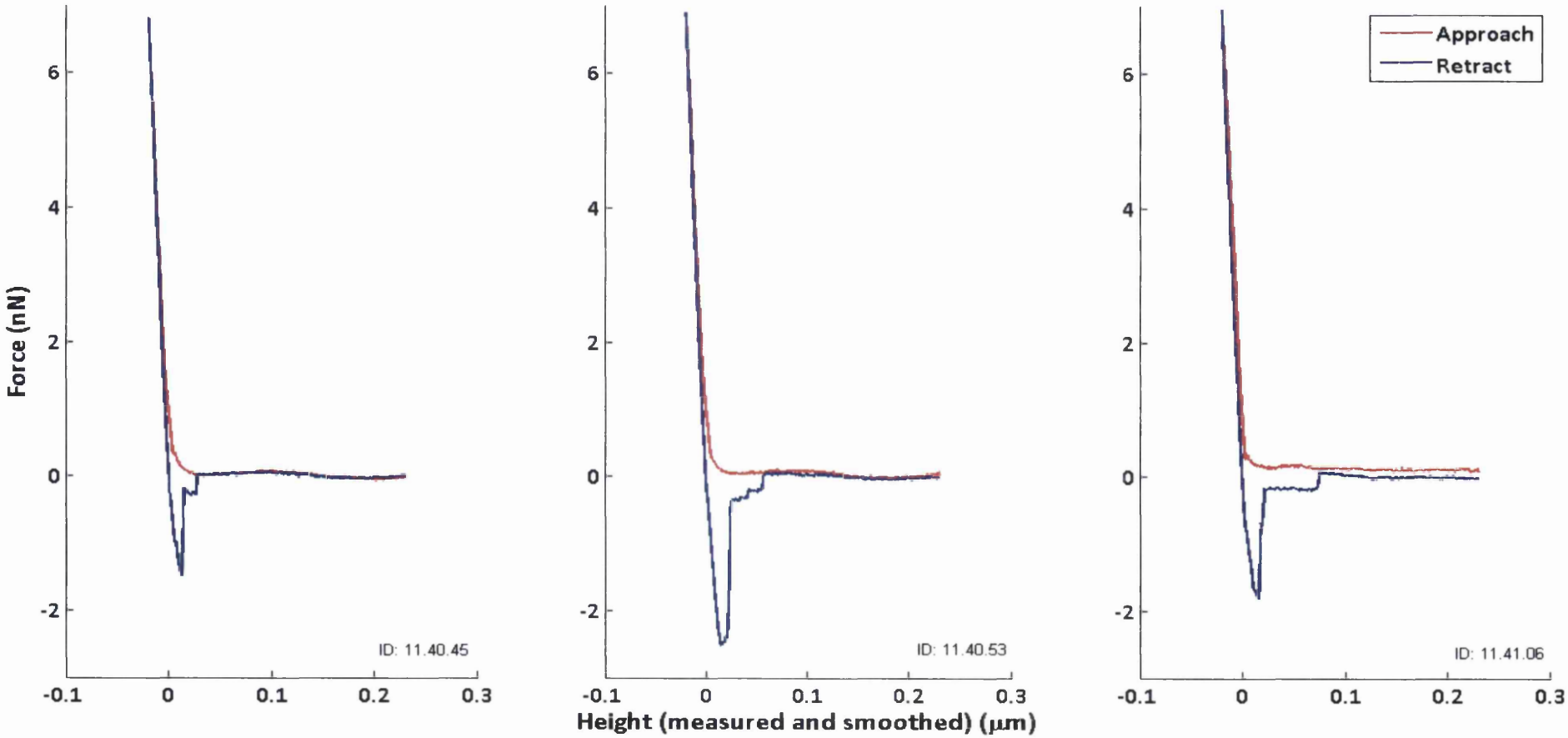

Figure 4.6. Presence of short range interactions. Force-distance curves for *Aedes* presenting multiple peaks and constant force plateaus at lower adhesion forces indicative of short range molecular interactions.

4.5.3 Epicuticular Wax Analysis.

In order to determine whether the chemical make-up of the cuticle itself may – at least partially – be a contributing factor with regard to successful attachment or not of the spore to the different tested insects, the majority lipid composition of cuticle was analysed by GCMS analysis (Figure 4.7). Seven hydrocarbons were identified whose abundance was similar across the two insects (Figure. 4.8). The *Tenebrio* cuticle contained the long chain hydrocarbons, Tetramethylheptadecane and 9-Methylnonadecane whereas the *Aedes* cuticle did not (Figure 4.8; $U = 23.00$, $z = 2.263$, $p = 0.031$ and $U = 25.00$, $z = 2.785$, $p = 0.008$, respectively).

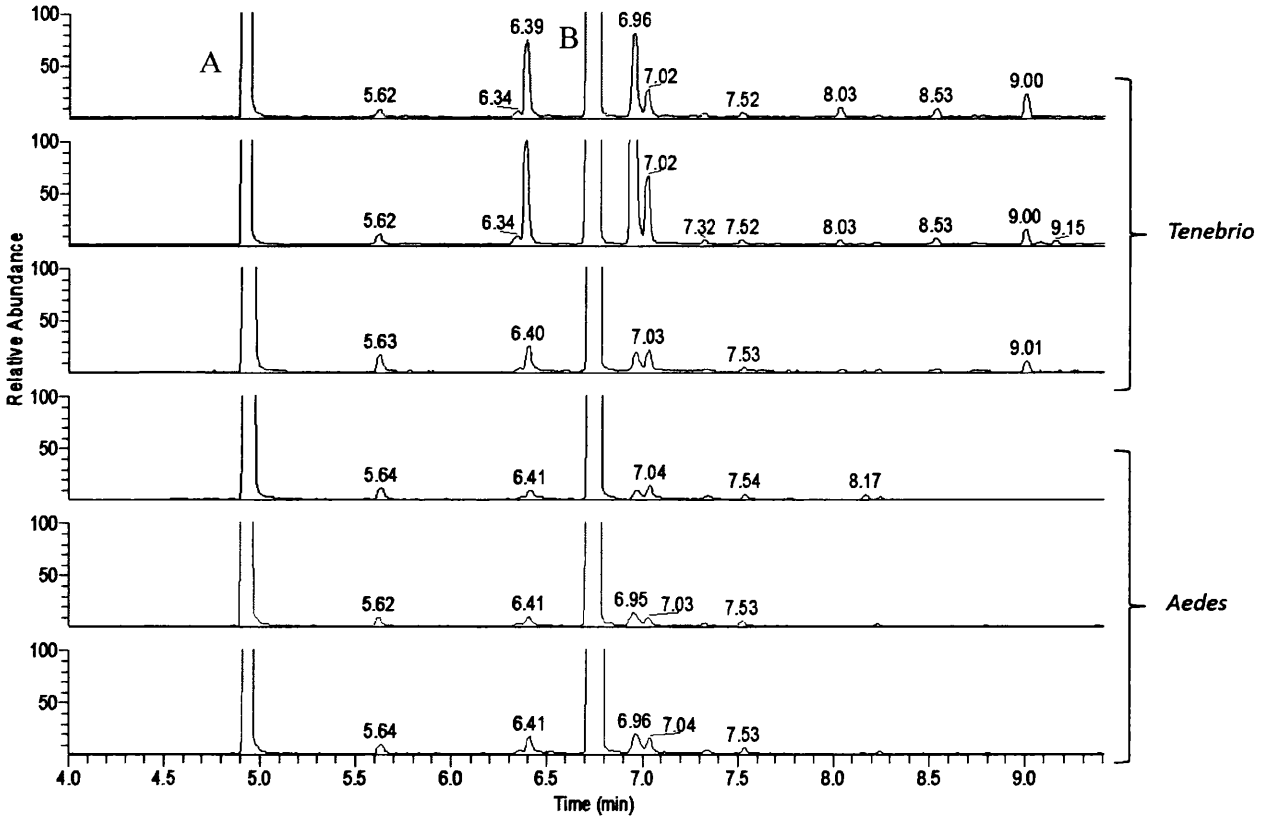

Figure 4.7 GC-MS Chromatogram of insect cuticle fatty acids

Cuticular fatty acids analysed using GC-MS. Internal standard Heptadecanoic acid (A) and FAME mix standard (B).

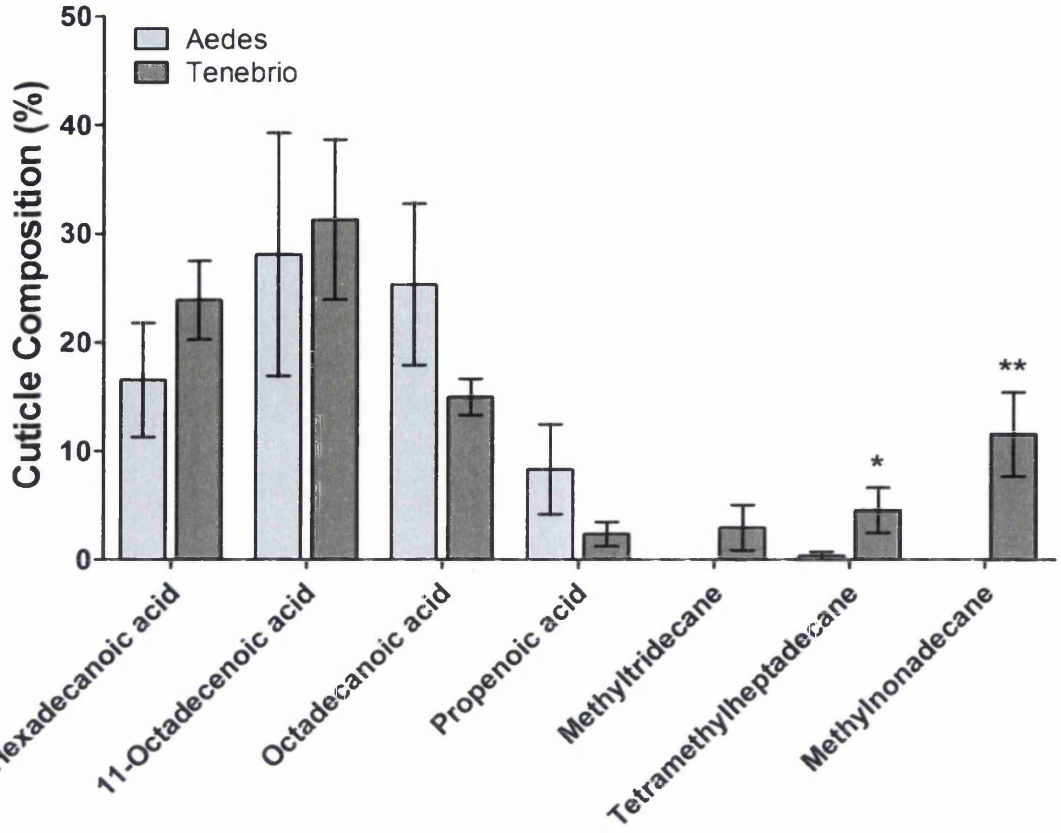

Figure 4.8. Long chain fatty acids absent in mosquito larvae
Fatty acid composition in *Aedes* and *Tenebrio* larvae. Data presented as ±SEM. (* = <0.05, ** = < 0.01)

4.6 DISCUSSION

Adhesion of entomopathogenic fungi to host cuticle is considered to involve an initial binding of the conidia to the cuticle, largely through weaker hydrophobic forces, followed by consolidation and firm attachment (Fargues 1984; Boucias & Pendland 1991).

This study found that there is an upregulation of *Mad* genes in response to exposure to *Aedes* cuticle which, further corroborates work in chapter 3 (Butt *et al.* 2013) and is indicative of host-pathogen recognition. However, the inability of the fungus to establish firm attachment prevents the typical infection course from proceeding as the AFM data demonstrates that conidia of *M. anisopliae* fail to firmly adhere to the mosquito larvae cuticle, discrete force steps observed in both *Tenebrio* and *Aedes* force curves, but absent in the glass force curves, may be attributed to the breaking force of a receptor- ligand pair (Müller *et al.* 2009). Whilst no firm attachment was determined for *Aedes,* the presence of weak short-range molecular interactions in *Aedes* further emphasises recognition as suggested by the adhesion gene up-regulation, Formation and strength of attachment is dependent upon the stability of molecular interactions, between the spore cytostructure and cuticle surface receptors, under force (Evans & Calderwood 2007), suggesting the initial recognition and binding to *Aedes* is extremely weak and not readily maintained. It has been shown that cells are able to regulated their receptor-ligand interactions, adapting them to their environments they are subjected to (Müller *et al.* 2009).

Although preventing adhesion to the cuticle may be a rare defense mechanism, it is well documented that hydrocarbon content of the waxy layer can influence fungal pathogenesis, with some compounds altering hydrophobicity or being fungistatic (Butt *et al.* 1995; Sosa-Gomez, Boucias & Nation 1997; Lord & Howard 2004), in addition some insects actively secrete a variety of antimicrobial compounds on the cuticular surface. GC-MS analysis revealed no notable fungistatic compounds within the mosquito cuticle. There was, however, an absence of long chain hydrocarbons within the mosquito larval cuticle. For germination to occur, conidia require cuticular nutrients, namely long-chain fatty acids, lipids, sugars and amino acids (Boucias & Pendland 1991; Bidochka, Kamp & Amritha de Croos 2000) it is therefore feasible that the mosquito larval cuticle is not conducive for fungal development.

Metarhizium has demonstrated the ability to form firm attachment to a terrestrial host cuticle, under water, indicating the ability of this fungus to adapt and form associations in an aquatic environment. Although the route of infection is more similar to the aquatic fungal pathogen *Culicinomyces clavisporus*, which is ingested rather than penetrates the cuticle (Sweeney 1983), the upregulation of adhesin genes suggest there is recognition and an attempt to form attachment to the mosquito larvae. As a consequence it can only be postulated that the cuticle itself is not providing an adequate substrate to further develop and, therefore initiate full pathogenesis via traditional means.

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CHAPTER 5

COMPARATIVE PROTEOMIC AND GENE EXPRESSION ANALYSIS OF *METARHIZIUM ROBERTSII* AND *METARHIZIUM ACRIDUM*

In Preparation:

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AUTHOR CONTRIBUTIONS
Conceived and designed the experiments: **BPJG,** ED & DCE.
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COMPARATIVE PROTEOMIC AND GENE EXPRESSION ANALYSIS OF *METARHIZIUM ROBERTSII* AND *METARHIZIUM ACRIDUM*

5.1 ABSTRACT

Metarhizium sp. is widely used as a biocontrol agent for a number of arthropod pest species. *Metarhizium acridum* and *Metarhizium robertsii* are categorized as being a specialist and generalist species, respectively. Despite their importance as biocontrol agents there are still several aspects relating to their respective specificity and virulence that are not yet known. A comparative study of proteomics and gene expression analysis was performed to investigate determinants of specificity, virulence and pathogenesis in *M. acridum* and *M. robertsii*. Analysis combined the use of 1-dimensional gel electrophoresis and matrix assisted laser desorption ionization time-of-flight mass spectrometry with quantitative real-time PCR. Comparison of gene expression profiles presents a greater expression of virulence factors in *M. robertsii* reflective of a generalist habit. Comparison of protein profiles for the two species identified that 28% of the proteins were unique to *M. acridum*, 41% were unique to *M. robertsii* and 31% were common to both species. Those exclusive to *M. acridum* contained proteins involved in reactive oxygen resistance as well as several involved in metabolic functions. *M. robertsii* contained those linked with primary and secondary metabolite production and cuticular degradation. Multiple proteins implicated in signalling and pathogenesis were common to both species.

5.2 INTRODUCTION

The entomopathogenic fungi encompass a wide group of fugal species which are able to infect an insect host to acquire nutrients. These strains can be broadly divided into those which show a wide host range or those with a specific host to infect. There has been much interest entomopathogens as alternative to chemical insecticides, in both agricultural and disease-vector control and *M. robertsii* and *M. acridum* are at the forefront of development of these biocontrol strategies (Lomer *et al.* 2001; Faria & Wraight 2007; St. Leger & Wang 2010). *M. robertsii* and *M.*

acridum are characterised as being a generalist and specialist species, respectively. The ability of entomopathogenic fungi to infect and kill its insect host is generally determined through conducting bioassays and observing the mortality rates in response to the fungus. Recently, research focus has moved towards the presence of virulence markers to determine infection potential and their expression in response to host cues. Virulence genes, such as *Pr1* and *Pr2*, are well established and the up/down regulation of these genes has been shown to greatly impact on the ability of a strain to infect and kill its target host (Paterson *et al.* 1994; Wang, Typas & Butt 2002; Freimoser, Hu & St. Leger 2005; Mohanty, Raghavendra & Dash 2008). The use of genetic markers (transcriptomics), for identifying novel genes involved in the infection process has become a staple of molecular mycology, with quantification of gene expression showing the upregulation of several virulence genes, during individual infection stages (Freimoser *et al.* 2005; Fang & Bidochka 2006; Wang & St. Leger 2007a; Fang *et al.* 2009).

The following chapter aims to elucidate novel proteins that are essential components of host specificity, recognition and infection between generalist and specialist fungal pathogens through the use of proteomic screening on host and non-host induced media. Analysis of transcript levels of known pathogenesis related genes (virulence genes: *Pr1 Pr2*, Adhesion genes; *Mad1* and *Mad2*) as well as those indicated in research by other workers will provide new insights into the host-pathogen interactions. In addition, it is envisaged that a proteomic approach will characterise various biochemical pathways that might be involved in adaptation to changing environments. To date, many key genes have been identified that are involved in the infection process, including the adhesins and hydrophobins, responsible for attachment to host cuticle (Wang & St. Leger 2007a), numerous cuticle degrading enzymes utilized to breech the cuticle (St. Leger, Cooper & Charnley 1986; Bagga *et al.* 2004), genes involved in adapting the fungus to different environments encounter during penetration (Fang, Pei & Bidochka 2007; Fang *et al.* 2009; Wang & St. Leger 2007b; Wang, Duan & St. Leger 2008; Duan *et al.* 2009; Liu, Peng & Xia 2012) as well as those implicated in evading the host immune response (Wang & St. Leger 2006). Despite great advances, over recent years, in elucidating the underlying mechanisms of pathogenesis there is still much to learn particularly regarding their role in host specificity.

Proteomic analysis is important for the study of complex biological systems, due to the direct role proteins have in both form and function of the organism. Proteomics has previously been applied to other fungal systems, and has become a valuable tool in identifying proteins related to antibiotic/fungal responses, host–pathogen interactions, osmoregulation and catabolite repression (Kim, Nandakumar & Marten 2007; Kniemeyer, Lessing & Brakhage 2009; Rizwan *et al.* 2010; Crespo-Sempere, Gil & Martínez-Culebras 2011). In addition, this approach also has great potential for deciphering regulatory circuits and the role of post-translational regulatory mechanisms, such as phosphorylation and glycosylation affecting signalling pathways as well as their in involvement in fungal stress response (Pandey & Mann 2000; Kroll, Pähtz & Kniemeyer 2014). The wood-degrading fungi, *Phanerochaete chrysosporium* and *Lentinula edodes*, were used as the first models for proteomic studies of filamentous fungi, identifying proteins pivotal to ligninolytic processes, namely the biosynthesis and/or transport of the iron-complexing agents (Hernández-Macedo *et al.* 2002). In addition, a number of proteins have been identified that are involved in a metabolic shift from the glyoxylate cycle to the tricarboxylic acid cycle in *P. chrysosporium* (Shimizu *et al.* 2005). Similarly, a metabolic flux toward glycerol biosynthesis during osmoadaptation has been identified in *Aspergilus nidulans* (Kim *et al.* 2007).

Metarhizium produces conidia both during a saprophytic phase and during pathogenesis. In contrast to mycelium, conidia show low levels of metabolic activity, as a consequence transcription and protein synthesis does not occurs until germination, thus limiting adaptation and response to environmental changes (Braga, Destéfano & Messias 1999; Barros *et al.* 2010), although penetration of the insect cuticle requires the differentiation of penetration structures, the events prior to appressoria production including host recognition, activation of macromolecule biosynthesis and differential gene expression (St. Leger, Bidochka & Roberts 1994) are of interest. In order to determine protein expression profiles and identify those proteins and potential genes which are pivotal in the development, virulence and pathogenesis of these strains, a comparative proteomic and gene expression study was performed on the mycelia of *M. robertsii* (ARSEF 23) and *M. acridum* (ARSEF 7486) grown on different insect cuticle induced media.

The analysis utilised a combination of 1- dimensional gel electrophoresis (1-DE) and Matrix-Assisted Laser Desorption Ionization Time-of-Flight Mass Spectrometry (MALDI-ToF MS) as well as qRT-PCR for gene expression analysis.

5.3 MATERIALS AND METHODS

5.3.1 Fungal Strains and Production

M. robertsii (ARSEF 23) and *M. acridum* (ARSEF 7486) were incubated for 12 days at 27°C (±2°C) on Sabouraud dextrose agar (SDA; 4% D- glucose, 1% Mycopeptone and 1.5% agar). After the initial 12 day growth a 1cm diameter plug was cut and placed on pre-sterilized 9 cm cellophane disc, on the surface of SDA medium and grown for a further 21 days.

Cellophane discs with mycelia were transferred to either fresh SDA media or an induction medium comprising minimal media (0.2% KH_2PO_4, 0.03% $MgSO_4$, 1.4% $(NH_4)_2SO_4$, 1.5% agar and $FeCl_3$ as trace) with 0.5% dehydrated insect cuticle; *Galleria mellonella* (Wax moth larvae), *Tenebrio molitor* (Meal worm, Darkling beetle larvae) or *Schistocerca gregaria* (Desert locust adult) for 24 hrs. Nine replicates of each treatment were prepared, for each extraction method.

5.3.2 RNA Extraction and cDNA Synthesis

Ground mycelia stored for transcript quantification (from section 5.3.5.6) was divided in to 100 mg aliquots, with three replicates per sample. RNA extraction and cDNA synthesis was performed using Qiagen kits and followed as per Chapter 3 (section 3.2.7.1).

5.3.3 qRT-PCR Analysis of Key Determinants of Virulence

Transcript levels of key virulence determinants were assessed in *M. acridum* and *M. robertsii* mycelia grown on the insect cuticle induction media, as per Chapter 3 (section 3.2.7.2); to further investigate their involvement in host recognition and pathogenesis. Primers for genes investigated (Table 5.1) were either designed from an alignment of *Metarhizium* sp. gene sequences, or obtained from published articles.

Table 5.1: Key virulence determinants in *Metarhizium* sp

ID	Annotation	Function	Fungus *	Forward	Reverse	Reference
18s	Ribosomal RNA	Housekeeping gene		CGAAAGTCGCAATGGCTCA	CCGAAGTCGGGATTTTTAGC	Designed in house
tEF	Translarion elongation factor 1	Housekeeping gene		CGAGCGTGAGCGTGGTA	CAGCCTCGAACTCACCAG	Designed in house
crr1	Carbon response regulation gene	Carbon regulation of Pr1 enzyme		AAGACCATGACGACCACTAC	GATACTTGGCAACTCGTACC	Designed in house
Cwp10	Call wall Protein	Hydrophobicity and adhesion		TCAGCCTCACTATTGCCAGC	GTAGAAGTACGTGCCAGCGA	Designed in house
Cyp52	Cytochrome P450 monoxygenases 52	Up-regulated upon the first stages of infection, involved in alkane and insect epicuticle degradation		ATGCAAGTCACCGTCCTGGT	CTACTCGGCGAAATGGAGGC	(Lin *et al.* 2011)
DtxS1	Non-ribosomal peptide synthetase	Biosynthesis of destruxins		AGATTTGCCGCAGCTACCTA	CACCAGATGCGAGTTCTCAA	(Wang *et al.* 2012)
MaAC	Adenylate cyclase	Regulating the fungal responses to hyperosmolarity, high temperature and oxidation		GGACGAAGGACTTGACAGACC	CACAGCATCTCCAGACGAGG	(Liu *et al.* 2012)
MaATL	Autophagy related Lipase	Hydrolase activity and lipid metabolic process, virulence		AAGACGCTGCCGATTGGTTA	TGATGCCAAGTCCACTGTCC	Designed in house
Mad1	*Metarhizium* adhesin 1	Host surface adhesion, cell differenciation and virulence		CTCCTCACATCACCCAGGTT	GGGAGTAGGCATGACGATGT	Designed in house (Wang & St. Leger 2007a)
Mad2	*Metarhizium* adhesin 2	Plant surface adhesion		CTATGTCCACCTTGCGACT	AGCAGCTGATGAGGGTCT	Designed in house (Wang & St. Leger 2007a)
MaFKS	1,3-glucan synthase	Cell wall integrity, hyperosmotic tolerance and conidiation		AAGCAGTGGTATCAACGCAGAGT	CGACCGTAGTAGTCCATTTCAGC	(Yang, Jin & Xia 2011)

Mamrd 1	Pre-rRNA processing gene	Hyphal growth, conidiation and virulence Fungal	CGAAGAACGACGGCGAGAAG	CGAGAAGATGAGTGTCCTTGAGC	(Cao, Li & Xia 2011a)
Mapmi	Mannose 6 Phosphate Isomerase	Fungal growth, conidiation, germination and virulence Cell	GCTCGGCGGTAGACCTGTACG	TCCCTCCAGCGGCTCAAACG	(Cao, Li & Xia 2011b)
MaHOG1	Mitogen-activated protein kinase 1	Regulating the fungal responses to hyperosmolarity, high temperature and oxidation	TTTGCTGAGATGCTGGAGGG	TGTTCTTCAAGGGTTGGCGT	Designed in house (Jin, Ming & Xia 2012)
Mos1	*Metarhizium* osmosensor	Mediates cell adaptation in hyperosmotic conditions, once the hyphae have penetrated and colonised the haemocoel	ACTGCCTGACAAGGACAACC	CATTGCTTGGTTATGCATCG	(Wang *et al.* 2008)
MaPK1	PKA AMP-dependent protein kinase catalytic subunit	Mapka1 is required for sensing host-related stimuli and transduction of these signals to regulate many infection processes	CACCCAGACCTGATTGCCTT	TCGTCGCCCTCAACAAAGAA	Designed in house (Fang *et al.* 2009)
Mpl1	Metarhizium perilipin-like protein	Regulates lipolysis, turgor pressure, and formation of infection structures	CCCTCCCTTCTTTCACCACC	ATTGAGAATGTGGTGGGCGT	(Wang & St. Leger 2007b)
Nrr1	Nitrogen regulator response gene	Germination, conidiogenesis and pathogenesis	ACTATTGATGAGCGTCGTAAC	TGCGTCGTTGTCCATGAAG	(Screen *et al.* 1998)
Pr1A	*Metarhizium protease 1*	Extracellular chymoelastase fundamental in insect cuticle penetration and degradation	TCCGAGTCCTCTTGCCTATCA	GGCACCGTTGTAGGCAAGGTAGTT	Designed in house (St. Leger, Cooper & Charnley 1987)
Pr2	*Metarhizium protease 2*		TACGCCACATTGCCAGA	GCATGTCGCACGATCAA	Designed in house

* = *M. acridum*, = *M. anisopliae*, = *M. robertsii*. Colours represent strain specificity of primers, primers designed in house from sequences/primers provided in references

5.3.5 Protein Extraction

5.3.5.1 Isolation of Cell Wall

The following procedure disrupts the cell wall and plasma membrane of filamentous fungi, allowing for the fractionation of cell walls from other cell components through differential centrifugation (Pitarch, Nombela & Gil 2008). After 24 hr, cellophane disks were removed from the insect cuticle induction media and subjected to the following steps: Firstly, mycelia was suspended in 1 mL ice-cold water and centrifuged for 5 minutes at 4,500g before decanting the supernatant and resuspending the pellet in 1 mL ice-cold lysis buffer (10mM Tris-HCl, pH 7.4, 1 mM PMSF) centrifuging again using the same conditions and retaining the pellet. The pellet was resuspend in 3 volumes of ice-cold lysis buffer, e.g. 300 uL: 100 mg of pellet, ground twice with a micropestle and glass beads at a maximum speed for 60 s, and incubated on ice for 2 minutes. The supernatant was collected and stored whilst before spinning at 2, 000g for 10 minutes whilst cell homogenates were centrifuged at 2, 000 g for 10 minutes. After discarding the supernatant, the cell pellet was sequentially washed twice with 1 mL Wash solution A (1 mM PMSF), 1 mL Wash solution B (5% (w/v) NaCl, 1 mM PMSF), Wash solution C (2% (w/v) NaCl, 1 mM PMSF) 1 mL ice-cold Wash solution D (1% (w/v) NaCl, 1 mM PMSF), with intermittent centrifuging at 4, 500g for 2 minutes and removal of supernatant, before resuspending the pellet in 1 mL ice-cold Wash solution A with a centrifuge step of 4, 500g for 5 minutes. After the first wash, cell pellet was transferred to a pre-weighed tube and resuspended in Wash solution A and centrifuged under the same conditions as before, after which the pellet was retained and weighed.

5.3.5.2 Solubilisation Using Detergents and Reducing Agents

Sodium dodecyl sulphate (SDS) is used to dissociate cell wall proteins that are non-covalently bonded to other wall components. The use of dithiothreitol (DTT) allows for the solubilisation of those cell wall proteins with weak associations, primarily those that are linked via disulphide bridges or through non-covalent bonds.

Purified cell walls were resuspended in 1 mL ice-cold Wash buffer A (50 mM Tris-HCl, pH 8.0, 1 mM PMSF) and centrifuged at 1,000g for 10 min at 4°C, the supernatant was carefully decanted into sterile 15 mL tubes. 5 µL of extraction

buffer (50 mM Tris-HCl, pH 8.0, 0.1 M EDTA, 2% (w/v) SDS, 10 mM DTT) was added to each 1 mg of cell wall and boiled at 100°C for 10 min, before centrifuging at 1, 000 g for 10 min. The supernatant was collected in a sterile microfuge tube and the pellet was stored for further extraction. The supernatant, containing SDS/DTT extracted cell wall proteins, was precipitated with Trichloroacetic acid (TCA) and stored at -80°C.

The stored pellet was resuspended in 5 µL of extraction buffer and boiled again for 10 min at 100°C before centrifuging at 1, 000 g for 10 min. The supernatant was discarded and wet cell wall pellets, containing SDS/DTT resistant cell walls, were weighed and aliquoted into two tubes for use in further extractions.

5.3.5.3 Extraction of Cell Wall Proteins Linked to β-1,3-Glucans

Cell wall proteins linked to β-1,3-glucans can be dissociated under mild alkali conditions, through the disruption of alkali-sensitive linkages by the β-elimination process. Enabling the extraction of glycosylphosphatidylinositol (GPI) cell wall proteins, implicated in the cellular recognition and binding.

One aliquot of purified SDS/DTT resistant cell walls was resuspended in 1 mL ice-cold Wash solution A and centrifuged at 1,000g for 10 min at 4°C; the supernatant was discarded and washed again in wash solution A. Cell walls were washed 5 times, with intermittent 10 min centrifuge steps at 1,000 g, in 1 mL ice-cold Wash buffer B (0.1 M Sodium acetate, pH 5.5, 1 mM PMSF). 400 µL of ice-cold Extraction solution (30 mM NaOH, 1mM PMSF) was added to every 100 mg of wet cell wall and resuspend, before Incubating at 4°C for 17 h with gentle shaking. The reaction was stopped after 17 hr with acetic acid and centrifuged at 1,000 g for 10 min at 4°C to collect supernatant. Proteins were the precipitated using TCA and stored at -80°C

5.3.5.4 Extraction of Intracellular Proteins

Mycelia was removed from cellophane disks after 24 hr and washed with ultrapure water in a Buchner funnel, before freeze drying. Freeze dried mycelia was then subjected to mechanical grinding in a pestle and mortar before transferring ground material into sterile 1.5 mL microfuge tubes. Samples were rehydrated in 1 mL Tris-glycine buffer (1x; 25 mM Tris-Cl, 250 mM glycine, 0.1% SDS) and mixed

thoroughly by pipetting. Resuspended samples were then centrifuged at 10, 000 g for 40 min at 4°C, supernatant was carefully transferred to a fresh microfuge tube, ready for protein precipitation.

5.3.5.5 Protein Precipitation

Protein fractions are required to be concentrated prior to proteomic analyses, by precipitating with TCA.

Protein pellets were resuspended in 10% TCA and vortexed thoroughly before incubating on ice for 30 min. Samples were then centrifuged at 10, 000 g for 15 min, after which supernatants were carefully removed. Protein pellet was washed twice in 300 µL of ice cold acetone to remove residual TCA and subsequently air dried for 30 min at room temperature. 0.1M NaOH was added to neutralise samples before freezing at -80°C

5.3.5.6 Preparation of Total Protein Extracts, a Different Approach

Mycelia were removed from each cellophane disk and frozen under liquid nitrogen before freeze drying. They were mechanically ground, into a fine powder, under liquid nitrogen utilising a pestle and mortar and sterilized sea sand (Riedel de Haen). Approximately 500 mg of ground mycelia, per replicate, was transferred into a sterile 1.5 mL microfuge tube, remaining sample was stored for RNA extraction. To the samples aliquoted for protein extraction, 1 mL of Phenol: chloroform: isoamyl alcohol (PCI; 25:24:1) was added and samples were ground further to ensure a homogenous solution. The samples were vortexed before incubating on ice for 5 minutes. After incubating on ice, the samples were centrifuged at 12, 000 *g* for 10 min to pellet the cell debris and the upper phase was transferred carefully, without disturbing the white interface between the aqueous and phenol layer, to a sterile 15 mL centrifuge tube before adding 5 volumes of ice cold ammonium acetate/methanol solution. The samples were then agitated gently before incubating at -20°C overnight, to precipitate the phenol extracted proteins. The samples were then centrifuged at 6, 000 *g* for 15 minutes to collect the precipitate. Precipitated proteins were pelleted by centrifuging at 12, 000 *g* for 10 minutes. The protein pellet was washed twice in ice-cold methanol, with intermittent vortexing to ensure the pellet was fully resuspended with each wash. The pellet was subsequently dried at room

temperature for 20 minutes and resuspended in 500 µL sample lysis buffer (7 M Urea, 2 M Thiourea, 4% CHAPs, 0.5% ampholytes, 20 mM Tris and 65 mM DTT) and stored at -80°C until required.

5.3.4.7 Protein Quantification using Bradfords Assay

All protein extract concentrations were determined by the Bradford method, briefly 5 µL of sample was mixed with 250 µL Bradfords reagent and incubated for 5 minutes at room temperature before taking an endpoint absorbance measurement at 595 nm.

5.3.5 SDS-PAGE

Total protein extracts resuspended in sample lysis buffer were diluted 2:1 in Laemmle sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 25% glycerol, 0.01% Bromophenol blue, 5% 2-Mercaptoethanol) and boiled briefly for 10 minutes at 95°C, in a heat block. After boiling, the samples were gently agitated and allowed to cool before applying 10 µL of sample (20 µg μL^{-1}) to a 10–20% 1-D SDS-PAGE gel.

Prior to staining the gels were fixed for 1 hr in a 50% (v/v) methanol: 10% acetic acid solution and rinsed twice with ultrapure water (Gibco). Gels were stained with colloidal Coomassie G-250 stain (34% (v/v) Methanol, 2% (v/v) Phosphoric acid, 17% (w/v) Ammonium sulphate and 0.066% (w/v) Coomassie G-250) overnight on a rocking table and de-stained for an hour in ultrapure water (Gibco).

5.3.6 Image Analysis

Protein band detection was performed using the BioRad Image Lab™ software (v 5.1) package. Bands were detected utilising automatic band detection with custom sensitivity of 100% and manually adjusted for individual gels. Band intensity and contrast levels were manually altered to intensify appearance of bands.

5.3.7 Protein Digestion

Protein bands, identified in the Image lab™ software, were excised and washed in 100% acetonitrile (ACN) prior to drying using a SpeedVac centrifuge (Eppendorf) for 20 minutes and in-gel digested overnight at 37°C with Trypsin Gold (20 µg mL^{-1} in 40 mM NH_4HCO_3/10% ACN), following manufacturers guidelines (Promega).

5.3.8 MALDI-ToF MS

Prior to MS analysis, digested protein extracts were resuspended in 10 µL of 0.1% TFA. The eluate was mixed with 10 mg ml Cyano-4-hydroxycinnamic acid (CHCA) in 50% ACN and 0.1% Trifluoric acid (TFA) for spotting onto the sample plate, and air dried for MALDI-ToF MS analysis.

MALDI-ToF analysis was performed with a Voyager DE-STR instrument (Applied Biosystems, UK) in reflection mode with an acceleration voltage of 20,000 V, a grid voltage of 70% for peptides and 95% for larger proteins of the acceleration voltage, a delay time of 75 nsec or 200 nsec respectively and 50 laser shots per spectrum.

Peaks were detected and spectrums calibrated, to trypsin autolysis peptides, using Data Explorer. Peptide mass fingerprints were examined with the MASCOT programme (www.matrixscience.com) with the following parameters: Swiss-Prot sequence database, taxonomy – other fungi, enzyme – trypsin, allowed missed cleavages – 1, fixed modifications – none, variable modifications – oxidation (M), peptide tolerance – 10 ppm. Proteins with hit scores and random number match probabilities lower than 0.05 were considered to be successful identifications and used in subsequent comparisons with *M. robertsii* and *M. acridum* sequences.

Best matched peptides were aligned to and searched against *M. robertsii* and *M. acridum* specific sequences using the NCBI protein BLAST programme (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Samples with an E value below $1x10^{-4}$ were considered homologous, those that did not meet this threshold but showed query and percent coverage above 80% were considered likely candidates.

5.3.9 Statistical Analysis

Prior to statistical analysis band area was converted to total percentage of lane. Comparison of species was performed with Wilcox ranksum test, whereas differences in band distribution was analysed with a Kruskal-Wallis H test. Pairwise comparisons were performed using Dunn's post-hoc with a Bonferroni correction for multiple comparisons.

qRT-PCR data sets were analysed using the comparative ddCt method as per chapter 3 (section 3.3.7.2). Prior to analysis gene expression data subjected to a BoxCox

transformation, conforming to ANOVA assumption of homogeneity of variance (Eastwood *et al.* 2008). All statistical analyses were carried out using MatLab R2012b and R.

5.4 AIMS

1) Elucidate novel proteins for use as markers of specificity and virulence

2) Analyse known pathogenicity determinants in the fungi grown on different insect cuticle mediums

3) Identify candidate proteins for transcript analysis and gene knock out

5.5 RESULTS

5.5.1 Gene Expression

The genes selected (Table 5.1) play a crucial role in fungal virulence and adaptation to the environments they are in; as a consequence they were used to assess how the fungus responds to different insect host cuticles.

5.5.1.1 *Metarhizium acridum* Specific Genes

There was no significant change in expression of any of the genes specific to *M. acridum* (*MaMrd1, MaPmi, MaHOG1*) irrespective of insect host ($F_{(3,72)} = 2.192$, $p = 0.1000$, Figure 5.1).

5.5.1.2 *Metarhizium robertsii* Specific Genes

Expression of *M. robertsii* genes (*Cwp10, Cyp52, DtxS1, MaFKS, MaPKA, Mos1, Mpl1)* were analysed and found to be constitutively expressed, irrespective of insect cuticle, with the exception of *MaPKA* and *Mad1* which were down regulated when grown on *Tenebrio* cuticle ($F_{(3,112)} = 12.726$, $p = <0.0001$, Figure 5.2).

5.5.1.3 Genes Common to Both Fungi

Genes conserved between *M. robertsii* and *M. acridum* (*Crr1, MaAC, Mad1, Mad2, MaATL, Pr1*) were analysed to provide a direct comparison of the two species.

There was no difference in expression between insect cuticle treatments ($F_{(15,96)}$ = 0.706, p = 0.7729, Figure 5.3) nor was there any difference in expression, between species, for those genes associated with environmental stress. Interestingly, *M. robertsii* presented a greater expression of genes associated with virulence and pathogenicity than *M. acridum* ($F_{(5,96)}$ = 5.676, p = <0.0001, Figure 5.3), with a higher expression of *Mad1* ($F_{(5,96)}$ = 5.676, p = 0.0289, Figure 5.4), *Pr1* ($F_{(5,96)}$ = 5.676, p = <0.0001, Figure 5.3) and *MaATL* ($F_{(5,96)}$ = 5.676, p = 0.0198, Figure 5.3).

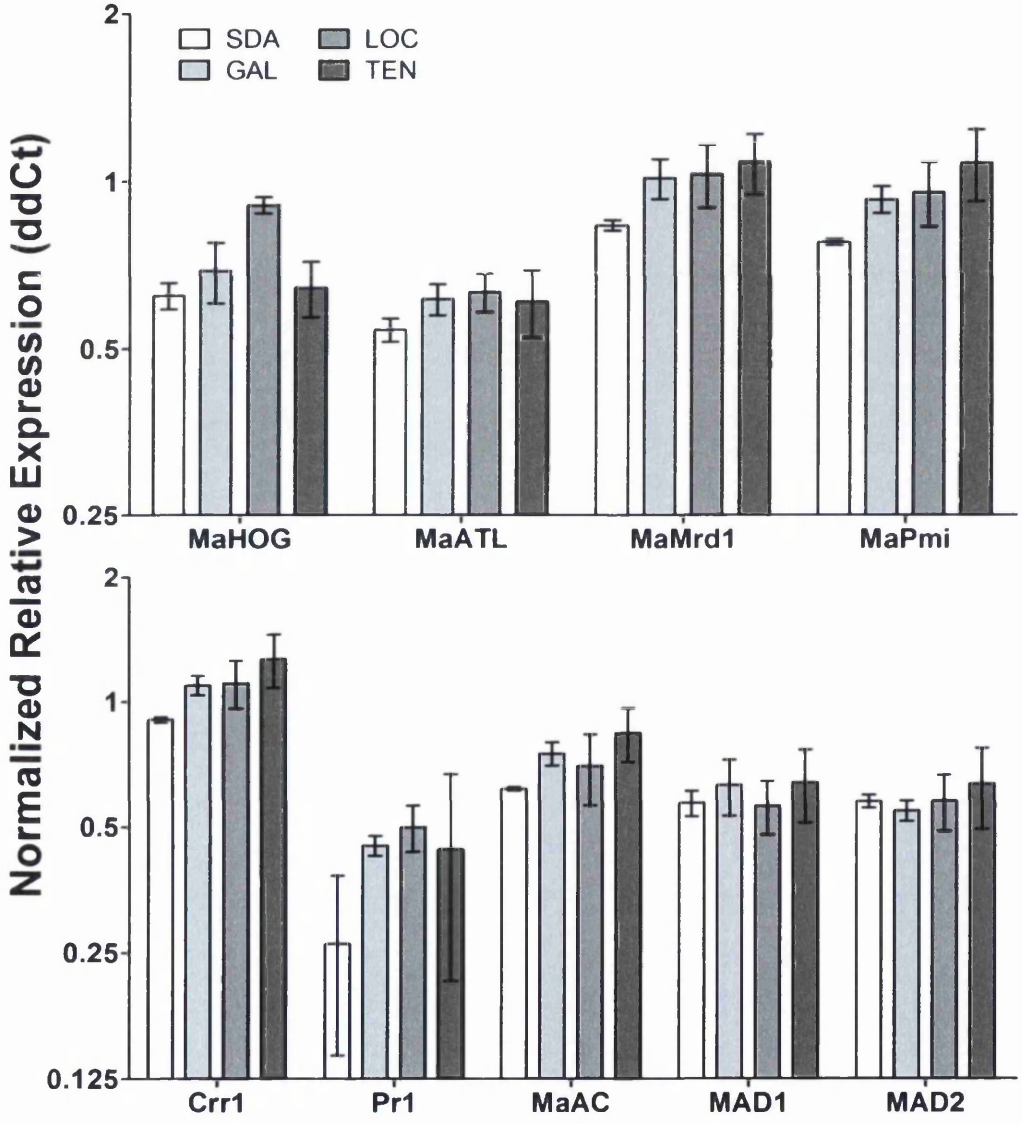


Figure 5.1. *Metarhizium acridum* specific genes. Expression of key virulence genes in *M. acridum*, 24hr post inoculation, analysed by qRT-PCR. No change in gene expression irrespective of insect host treatment. SDA: Control, GAL: *Galleria*, LOC: locust and TEN: *Tenebrio*. Data was presented as mean (±SEM). Data normalized to average dCt of SDA.

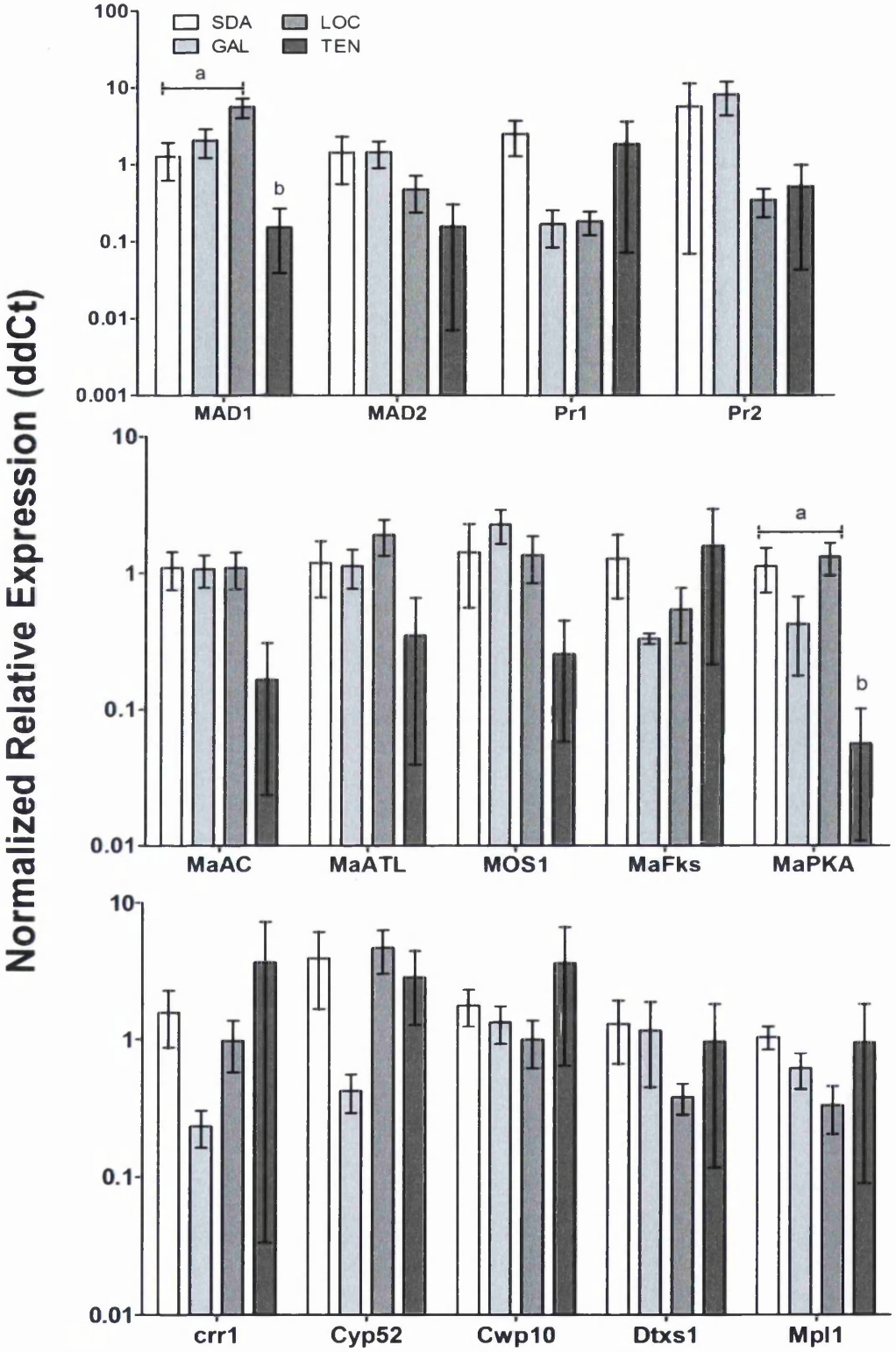


Figure 5.2 *Metarhizium robertsii* specific genes.

Expression of key virulence genes in *M. robertsii*, 24hr post inoculation, analysed by qRT-PCR. *MAD1* and *MaPKA* genes down regulated in presence of *Tenebrio* cuticle. SDA: Control, GAL: *Galleria*, LOC: locust and TEN: *Tenebrio*. Data was presented as mean (±SEM). Means with different letters denoting statistical differences (two-way ANOVA). Data normalized to average dCt of SDA.

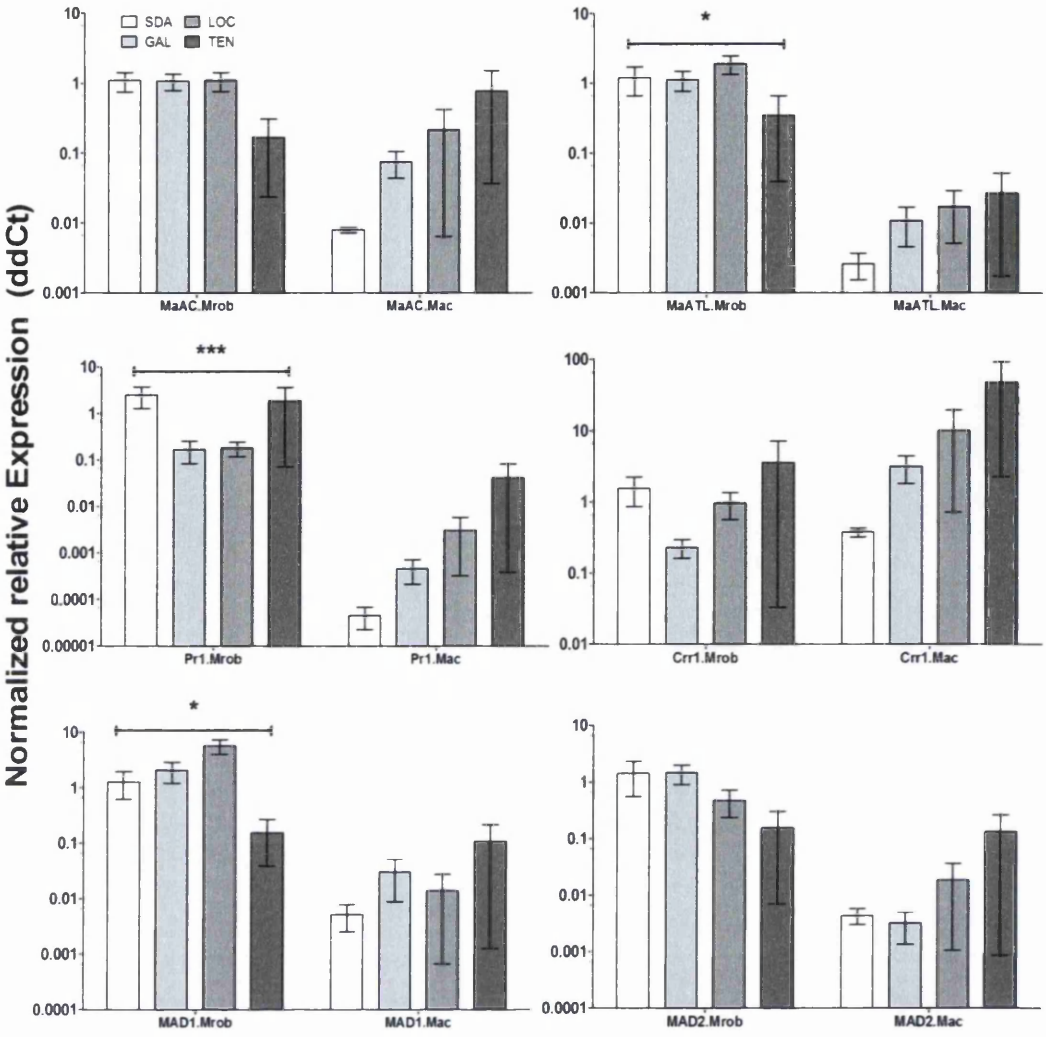


Figure 5.3: Expression of genes common to *M. robertsii* and *M. acridum*

Expression of key virulence genes conserved between *M. acridum* and *M. robertsii*, 24hr post inoculation, analysed by qRT-PCR. No change in expression of genes related to environmental stress. Virulence genes are expressed more in *M. robertsii*. SDA: Control, GAL: *Galleria,* LOC: locust and TEN: *Tenebrio.* Data was presented as mean (±SEM). Means with different letters denoting statistical differences (two-way ANOVA). Data normalized to average dCt of SDA.

5.5.2 Protein Analysis

For the comparison of protein expression in *M. robertsii* and *M. acridum*, 1-DE was performed and proteins were identified using MALDI-ToF MS. The methodologies used for Cell wall (4.3.2.1, 4.3.2.3) and intracellular (4.3.2.4) proteins had limited success in extracting proteins, both methodologies produced a 'protein' pellet, however, re-suspension in TCA was poor, as consequence a different approach was taken to extract proteins (4.3.2.6). The following results are solely based on protein extracts obtained from the methodology in 4.3.2.6.

5.5.3 Gel Analysis

Comparison of 1-DE images demonstrated qualitative differences in the protein profiles both between species and in response to different insect hosts. A total of 37 protein bands in *M. acridum* and 33 in *M. robertsii* were resolved with *M. robertsii* having a higher abundance of protein bands in the control treatment compared to *M. acridum* (U = 3251, z = -3.005, p = 0.0027; Table 5.2, Figures 5.4A and 5.5A). Similarly there was a higher abundance of proteins were present in *M. acridum* when grown on locust media, compared to *M. robertsii* (U = 5151, z = 4.1408, p = >0.0001, Table 5.2, Figures 5.4B and 5.5B). There was no significant differences between the two strains grown on with *Galleria* or *Tenebrio* media (U = 4249.5, z = 0.6787, p = 0.4973 and U = 4068, z = 0.059, p = 0.9524 respectively, Table 5.2, Figures 5.4A-B and 5.5A-B). Treatment effects within species were significantly different, with *M. robertsii* producing fewer proteins when grown of locust ($\chi^2(3)$ = 15.320, p = 0.002) compared to SDA (p = 0.002), *Galleria* (p = 0.036) or *Tenebrio* (p = 0.013). In contrast, *M. acridum* produced fewer proteins on SDA media ($\chi^2(3)$ = 12.536, p = 0.006) when compared to *Galleria* (p = 0.034), or locust (p = 0.007), there was no difference between SDA and *Tenebrio* treatment (p = 0.096).

Table 5.2: Number of bands per treatment

Treatment	Species	
	M. acridum	***M. robertsii***
SDA	4	11
GAL	10	12
LOC	12	3
TEN	11	9
Total	37	33

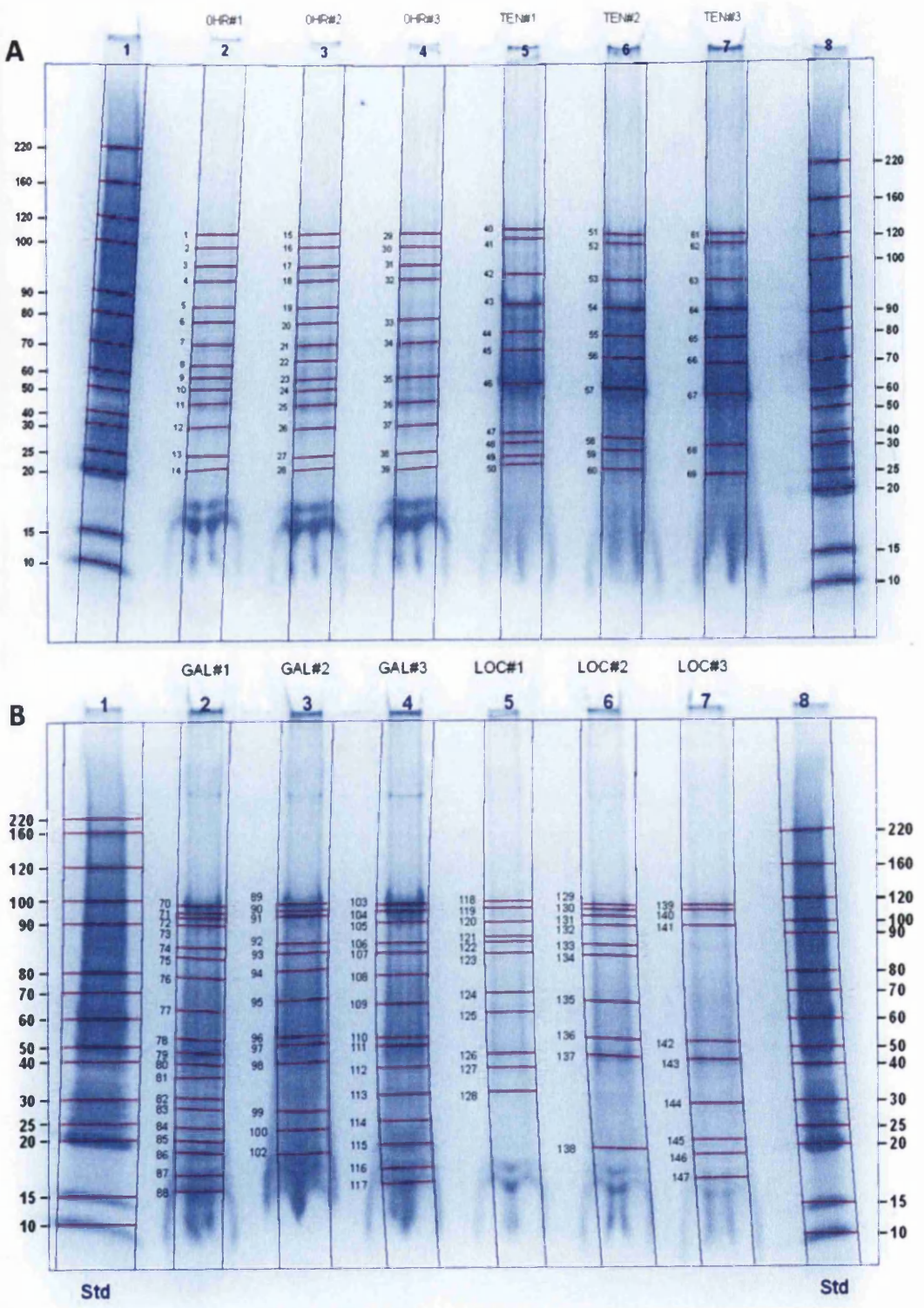


Figure 5.4: 1-DE of *M. robertsii* grown on SDA, *Tenebrio* (A), *Galleria* or Locust (B).

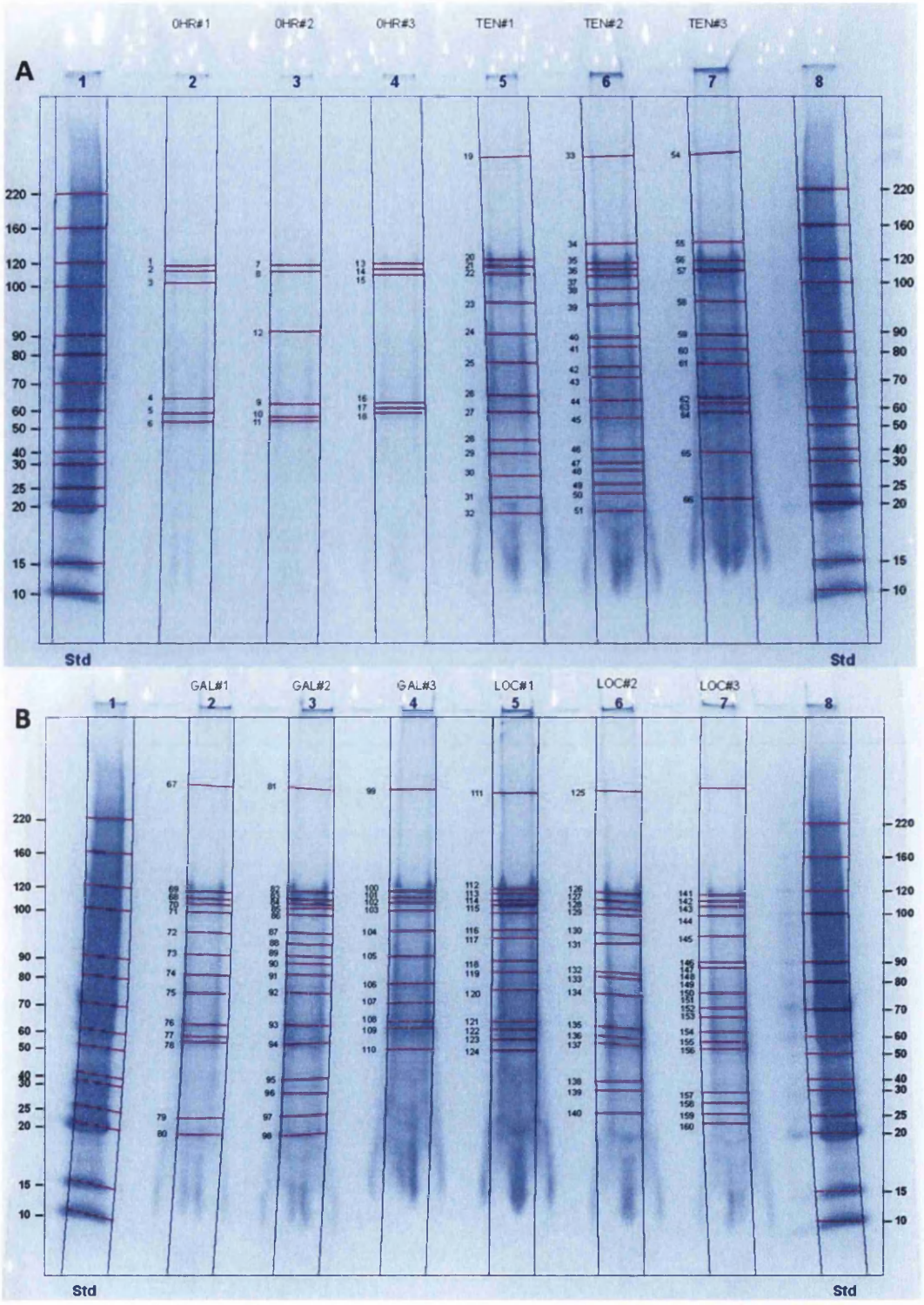


Figure 5.5: 1-DE of *M. acridum* grown on SDA, *Tenebrio* (A), *Galleria* or Locust (B)

Comparison of band distribution across the four media, between the two species is summarised in Table 5.3. In addition, band distribution between media within species was also compared and summarised in Table 5.4.

5.5.5 Protein Identification

A total of 30 distinct protein bands, consisting of 13 unique to *M. robertsii* and 8 unique to *M. acridum* (Figure 5.6), were chosen as representative proteins and analysed using MALDI-ToF MS. 29 proteins were successfully identified through homologues in other fungal species, before aligning candidate peptide sequences to specific *Metarhizium* sequences. 27 bands were matched to functionally characterised proteins, two proteins matched to hypothetical proteins without a predicted domain (Table 5.5). Several ribosomal and nucleic proteins were isolated including translation initiation factors and ribosome biogenesis proteins (Table 5.5) Among the *M. acridum* identified proteins, two secreted proteins were identified to be solely present on locust and *Galleria* media (Tables 5.3-5.5 and Figure 5.6). Two were found to be present on all treatments (Figure 5.6), including Molybdenum cofactor synthesis protein 2B, an important component of nitrate reductase and xanthine dehydrogenase. Aldehyde dehydrogenase, Geranylgeranyl pyrophosphate synthase were present in *M. robertsii* grown on *Galleria* cuticle, in addition *M. robertsii* also produced Histone Lysine Methyltransferase on both SDA and *Tenebrio* media (Tables 5.3-5.4 and Figure 5.6). Lipoxygenase was found to be present on *Galleria* and *Tenebrio* media in both *M. robertsii* and *M. acridum* with the latter also producing lipoxygenase on locust media (Table 5.3 and Figure 5.6). Multiple proteins involved in reactive oxygen resistance were found in both species, on insect cuticle treatments (Table 5.5 and Figure 5.6). In addition, a G-protein linked to signalling processes was identified in both species; however, only *M. acridum* produced this protein in response to an insect host, whereas in *M. robertsii* it was only present on the control treatment.

3-hydroxyanthranilate 3,4-dioxygenase appeared twice as distinct protein bands possibly as a result of differential posttranslational modifications.

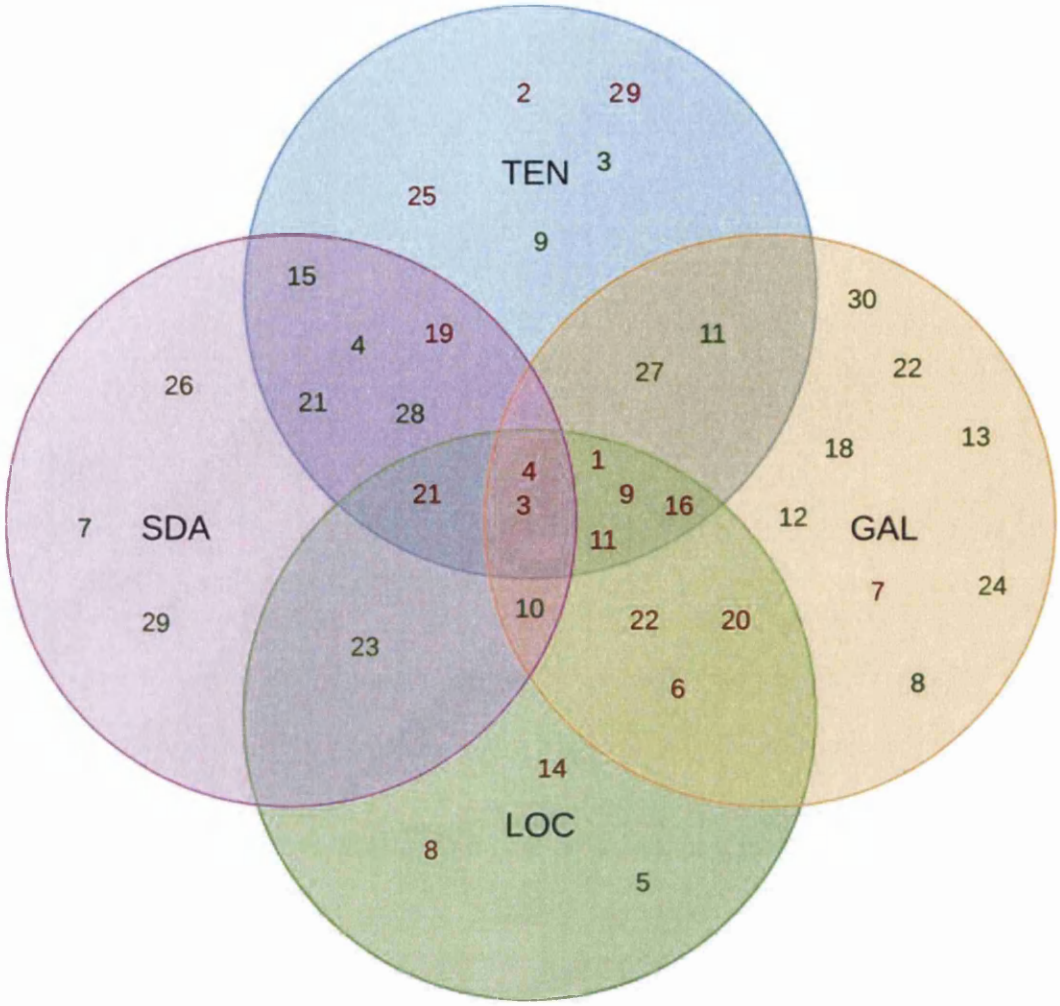


Figure 5.6: Peptides identified unique or common to treatment. Numbers represent proteins by band number corresponding to table 5.3. Red text indicates proteins present in *M. acridum*, green text indicates proteins present in *M. robertsii*. SDA = Control, GAL = *Galleria*, TEN = *Tenebrio*, LOC = Locust cuticle treatments.

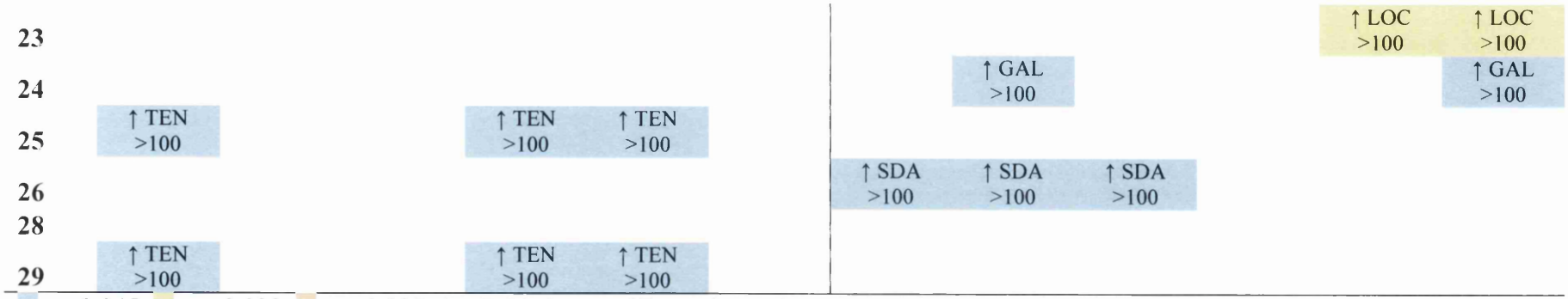
Table 5.3: Comparison of protein distribution between species

Protein Band	*M. acridum* vs *M. robertsii*: Species comparison			
	SDA	GAL	TEN	LOC
1		↑ Mac >100	↑ Mac >100	↑ Mac >100
2			↑ Mac >100	
3	↑ Mac >100	↑ Mac >100		↑ Mac >100
4	↑ Mac 4.28	↑ Mac >100		↑ Mac >100
5				↑ Mrob >100
6		↑ Mac >100		↑ Mac >100
7	↑ Mrob >100	↑ Mac >100		
8		↑ Mrob >100		↑ Mac >100
9	↑ Mrob >100	↑ Mac >100		↑ Mac >100
10	↑ Mrob >100	↑ Mrob >100		↑ Mrob >100
11			↑ Mrob >100	↑ Mac >100
12		↑ Mrob >100		
13		↑ Mrob >100		
14				↑ Mac >100
15	↑ Mrob >100		↑ Mrob >100	
16		↑ Mac >100	↑ Mac >100	↑ Mac >100
17	↑ Mrob >100		↑ Mrob >100	
18		↑ Mac >100		
19	↑ Mac >100		↑ Mac >100	
20		↑ Mac >100		↑ Mac >100
21	↑ Mac 4.94		↑ Mrob 2.27	↑ Mac >100
22				↑ Mac >100
23	↑ Mrob >100			↑ Mrob >100
24		↑ Mrob >100		
25			↑ Mac >100	
26	↑ Mrob >100			
27		↑ Mrob >100	↑ Mrob >100	
28	↑ Mrob >100		↑ Mrob >100	
29	↑ Mrob >100		↑ Mac >100	
30		↑ Mrob >100		

$p = 0.05$, $p = 0.037$, ↑ = Greater Fold change, SDA = Control, GAL = *Galleria*, LOC = Locust, TEN = *Tenebrio*

Table 5.4: Comparison of protein distribution between media.

Prot. Band	*M. acridum*: media comparison						*M. robertsii*: media comparison					
	SDA v TEN	SDA v GAL	SDA v LOC	TEN v GAL	TEN v LOC	GAL v LOC	SDA v TEN	SDA v GAL	SDA v LOC	TEN v GAL	TEN v LOC	GAL v LOC
2	↑ TEN >100			↑ TEN >100	↑ TEN >100							
3							↑ TEN >100			↑ TEN >100	↑ TEN >100	
4										↑ TEN >100	↑ TEN >100	
5									↑ LOC >100		↑ LOC >100	↑ LOC >100
7		↑ GAL >100		↑ GAL >100		↑ GAL >100	↑ SDA >100	↑ SDA >100	↑ SDA >100			
8			↑ LOC >100		↑ LOC >100	↑ LOC >100		↑ GAL >100		↑ GAL >100		↑ GAL >100
11	↑ TEN >100						↑ TEN >100				↑ TEN >100	
12								↑ GAL >100		↑ GAL >100		↑ GAL >100
13								↑ GAL >100				
14			↑ LOC >100		↑ LOC >100							
15										↑ TEN >100		
17								↑ SDA >100	↑ SDA >100			
18								↑ GAL >100				
21										↑ TEN >100		
22								↑ GAL >100				



$p = 0.045$, $p = 0.039$, $p = 0.037$, ↑ = Fold change, SDA = Control, GAL = *Galleria*, LOC = Locust, TEN = *Tenebrio*, >100 = fold change greater than 100

Table 5.5: Identification of protein differentially expressed in *M. robertsii* and *M. acridum*

Band #	ID	Biological/molecular function	Overall Biological Process	Cellular component
3	Molybdenum cofactor synthesis protein 2B	Transferase	Molybdenum cofactor biosynthesis	Cytoplasm
8	Eukaryotic translation initiation factor 3	Nucleotide binding/translation initiation factor activity	Protein biosynthesis	Cytoplasm
9	3-hydroxyanthranilate 3,4-dioxygenase	Dioxygenase/Oxidoreductase/Iron ion binding	Pyridine nucleotide biosynthesis	Cytoplasm
13	Aldehyde dehydrogenase	Oxidoreductase	Cellular aldehyde metabolic process	Cytoplasm
18	Geranylgeranyl pyrophosphate synthase	Transferase	Protein Transport/Carotenoid Biosynthesis	Cytoplasm
24	3-hydroxyanthranilate 3,4-dioxygenase	Dioxygenase/Oxidoreductase/Iron ion binding	Pyridine nucleotide biosynthesis	Cytoplasm
25	Adenylyl-sulphate kinase	Kinase/Transferase	Amino acid biosynthesis/cysteine biosynthesis	Cytoplasm
19	Orotidine 5'-phosphate decarboxylase	Decarboxylase Lyase/orotidine-5'-phosphate decarboxylase activity	de novo' pyrimidine nucleobase biosynthetic process	Cytoplasm
7	G-protein alpha subunit	GTP binding/GTPase activity/signal transducer activity	G-protein coupled receptor signalling pathway	Membrane
23	MFS transporter, putative	Transporter activity	transmembrane transport	Membrane
22	NADH-cytochrome b5 reductase	Oxidoreductase activity	Cellular response to oxidative stress/ergosterol biosynthesis	Membrane/Mitochondrion
10	Mitochondrial co-chaperone GrpE	adenyl-nucleotide exchange factor activity	Protein folding	Mitochondrion
21	NADH dehydrogenase subunit 4L	NADH dehydrogenase (ubiquinone) activity	ATP synthesis coupled electron transport	Mitochondrion
27	Phosphoglycerate kinase	Phosphoglycerate kinase activity/ATP	Glycolysis	Mitochondrion

		binding		
2	Pyridoxine biosynthesis protein PDX1	Active oxygen resistance/Catalytic activity	pyridoxal phosphate biosynthetic process	N/A
11	Lipoxygenase	Oxidoreductase	Fatty acid biosynthesis	N/A
14	Pyridoxal-dependent decarboxylase domain protein	Carboxylase activity/pyridoxal phosphate binding / Active oxygen resistance	Carboxylic acid metabolic process	N/A
16	Hypothetical protein MAC_03959	N/A	N/A	N/A
29	hypothetical protein MAA_09721	N/A	N/A	N/A
5	Ribosome biogenesis protein nsa2	Ribonucleoprotein complex binding	Ribosome biogenesis	Nucleus
28	Ribosome biogenesis protein YTM1	Ribonucleoprotein complex binding	Ribosome biogenesis/rRNA processing	Nucleus
1	C6 transcription factor, putative	DNA binding/zinc ion binding	Transcription/Transcription regulation	Nucleus
15	Histone Lysine Methyltransferase	Methyltransferase	Transcription	Nucleus
30	Fungal Zn binuclear cluster domain containing protein	DNA binding/zinc ion binding	Transcription/Transcription regulation	Nucleus
4	Proteasome regulatory particle, non-ATPase-like protein	Genetic information processing, Folding, sorting and degradation	Proteolysis	Proteasome
26	26S proteasome regulatory subunit rpn1	Enzyme regulator activity	regulation of protein catabolic process	Proteasome
12	60S ribosomal protein L3	Structural constituent of ribosome	Translation	Ribosome
6	Putative serine esterase family protein	Hydrolase/Serine esterase	protein demethylation	Secreted
20	Subtilisin-like protease	Serine-type endopeptidase activity	Proteolysis	Secreted

5.6 DISCUSSION

Both *M. robertsii* and *M. acridum* produce a vast array of peptides on both insect cuticles and artificial culture media. Examination of a portion of these proteins provides a snapshot of those proteins which play a role in utilising the disparate substrates provided. In this study, of the proteins identified, 28% were unique to *M. acridum*, 41% were unique to *M. robertsii* and 31% were common to both species (Table 5.2).

Although presence of different insect cuticles did not present different transcript expression profiles, to that of the SDA control, it is interesting to note that *M. robertsii* had a greater expression of those genes associated with virulence, compared with *M. acridum*, which could be reflective of *M. robertsii* generalist habit. Down regulation of *MaPKA* in response to *Tenebrio* suggests that *M. robertsii* is not receiving adequate cues from this host to initiate signal transduction via the cAMP-PKA signal pathway, crucial for determining virulence. Furthermore, no upregulation of *Pr1* could re-emphasise this point, equally low levels could be attributed to low fungal biomass of cells producing *Pr1*.

The comparison between fungal species and host cuticle treatments yielded several differentially produced proteins; those identified are involved in numerous biological and molecular functions (Table 5.5). *M. robertsii* represented a greater abundance of proteins on an individual medium, suggesting *M. robertsii* has much plasticity and the ability to regulate genes and protein production according to cues from the substrate. This plasticity is also reflected in its geographic distribution and generalist behaviour of *M. robertsii*. In contrast, *M. acridum* protein distribution was more concentrated in overlapping zones, perhaps indicating tighter regulation of genes, which will in turn limit the ability of this fungus to adjust to different substrates or hosts.

Stark differences were noted between insect cuticle media, with *Galleria* medium containing a greater cumulative abundance of proteins. This is certainly reflective of the hosts natural susceptibility to a number of different pathogens, including *Metarhizium anisopliae, Beauveria bassiana* and *Isaria fumosoroseus* (Zimmermann 1986; Lionakis 2011). Although other fungal strains can kill locusts, they are generally less susceptible to infection as with the locust specific *M. acridum* being

most effective (Lomer *et al.* 2001), the susceptibility of this host is inferred by the abundance few proteins produced by *M. robertsii* on this substrate. Furthermore, *M. robertsii* specific proteins on locust medium were primarily involved in general house-keeping roles, rather than host recognition or pathogenesis.

The insect cuticle itself can be a hostile environment, interspersed with numerous toxic lipids, phenols and other defensive compounds (Andersen, Hojrup & Roepstorff 1995). The fungus must overcome these for successful pathogenesis to occur. The presence of several proteins linked with reactive oxygen resistance may be an indication of the fungi's attempt to combat insect defences. Furthermore, degradation of hydrocarbons within this layer enables differentiation of the infection structures and penetration of the cuticle (St. Leger *et al.* 1989; James, Buckner & Freeman 2003; Jarrold *et al.* 2007). Degradation of the cuticular layer is facilitated by the fungal mediated hydrocarbon oxidation and assimilation pathway (Pedrini *et al.* 2013), which is largely governed via the activity of cytochrome P450 mono-oxygenases and aldehyde dehydrogenases. Recently aldehyde dehydrogenases have been implicated in the oxidation of fatty aldehydes to fatty acids for use in membranes and storage lipids (Pedrini, Crespo & Juárez 2007; Pedrini *et al.* 2013; Zhang *et al.* 2012). The fact that this enzyme was identified in presence of *Galleria* demonstrates recognition and degradation of the insect cuticular components.

The presence of lipoxygenase in both *M. acridum* and *M. robertsii* on insect cuticle is interesting as they are known to function in oxylipin biosynthesis. Production of oxylipins in fungi is widespread and involved in numerous functions, including the regulation of asexual spore development and secondary metabolite production (Tsitsigiannis & Keller 2007; Brodhun *et al.* 2013). In addition oxylipins have been implicated in pathogenesis, in *Candida albicans* due to their role in both signalling and regulation of morphogenesis of infection structures (Tsitsigiannis & Keller 2007). Several proteins involved in metabolism were isolated, Molybdenum cofactor synthesis protein 2B is of particular interest, due to its role in in purine catabolism and nitrogen metabolic regulation, which is known to influence pathogenesis in several fungal species including *Aspergillus fumigatus*, *Magnaporthe grisea*, *C. albicans* and *Cladosporidium fulvum* (Marzluf 1997; Cultrone *et al.* 2005; Schwarz, Mendel & Ribbe 2009), it is therefore assumed to have a similar function in *M. acridum*.

Identification of histone lysine Methyltransferase is important in directing epigenetic DNA modification, an important factor in regulating gene expression. It is therefore assumed its presence in *M. robertsii* grown on *Tenebrio* medium, could be an indicator of changes in development or metabolic production, a sign of environmental adaptation or even virulence (Lachner & Jenuwein 2002; Butt *et al.* 2006; Brosch, Loidl & Graessle 2008)

In conclusion, proteomic screening has elucidated potential candidates for transcript analysis that may be novel in pathogenesis of *Metarhizium*. Although, certain strains of *Metarhizium* show differences in virulence and specificity, it is proposed that those proteins identified conserved between *M. robertsii* and *M. acridum* will make good candidates for further investigation in their role in pathogenesis. In addition, the comparison of transcripts of these genes in mosquito larvae will further enhance the knowledge base of the host-pathogen interaction.

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CHAPTER 6

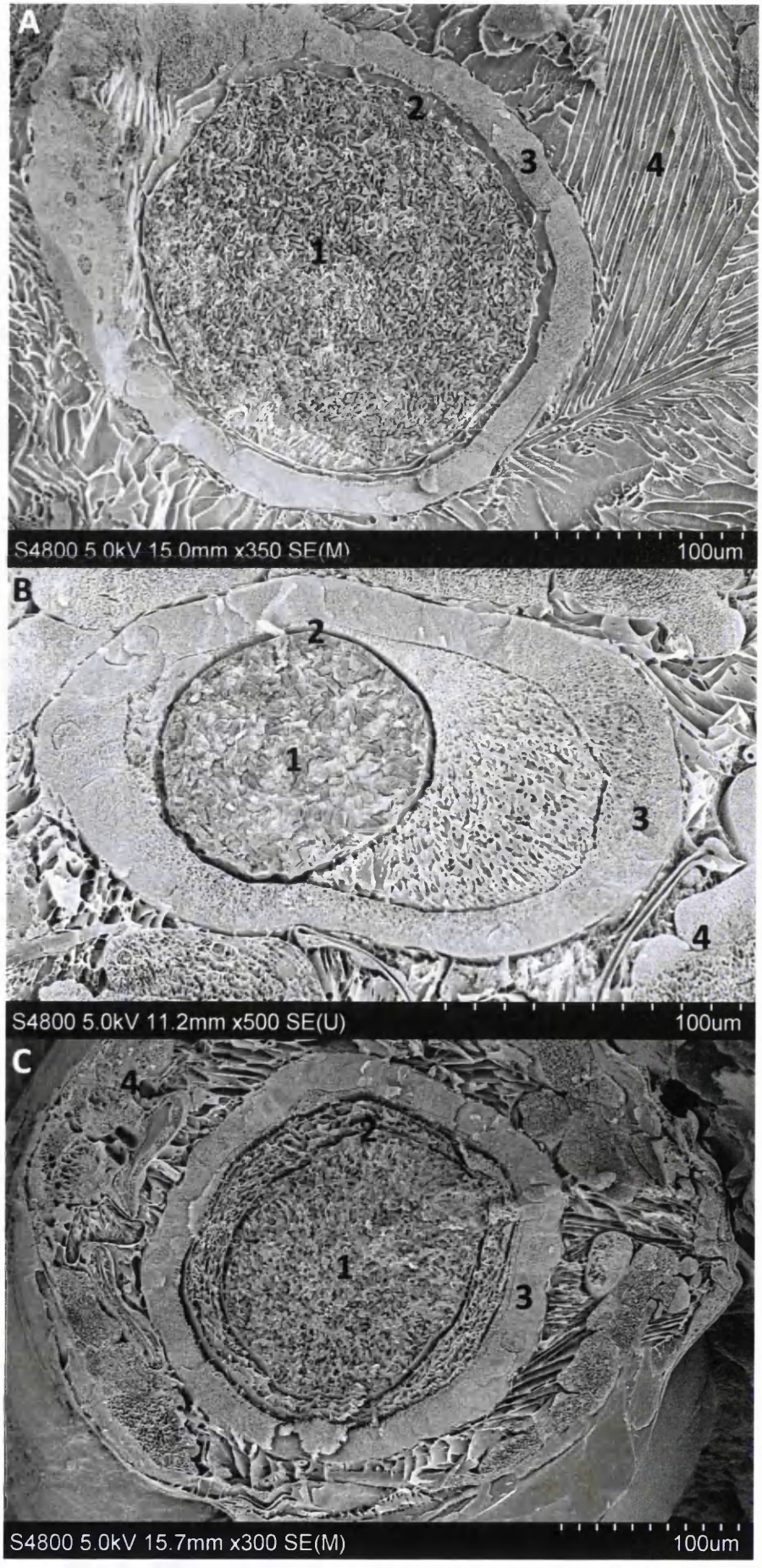
FINAL DISCUSSION

FINAL DISCUSSION

6.1 KEY FINDINGS

This thesis aimed to investigate the underlying mechanisms conveying pathogenesis, virulence and specificity of *Metarhizium* sp. for use in mosquito larvae control. A multidisciplinary approach was taken to provide an overarching view of the host-pathogen interactions. The studies within this thesis provide new insights into the mode of pathogenesis in mosquito larvae. Notably that pathogenesis in a terrestrial environment cannot be extrapolated to the aquatic environment.

The mode of infection and pathogenesis in mosquito larvae was previously thought to be similar to that of a terrestrial host. With previous workers observing adhesion to the siphon and perispiracular valves, followed by germination and extensive mycelial growth and colonisation (Lacey, Lacey & Roberts 1988). Microscopy analysis conclusively showed that *Metarhizium* was not able to colonise the haemocoel of *Ae. aegypti*, like it would in a terrestrial environment. This, however, was not peculiar to *Aedes,* lack of colonisation was also observed in *Anopheles* and *Culex* larvae (Figure 6.1). Throughout the course of this thesis, germination in larvae in water was not recorded. If, however, the larvae was removed from the aquatic environment and incubated, colonisation and re-emergence of the fungus was witnessed. Further analysis with fluorescent microscopy confirmed that conidia were still viable even after the host death (Figure 6.2). In light of these observations it was becoming apparent that the behaviour of *Metarhizium* in response to mosquito larvae was not like that of other terrestrial insect hosts.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C
1
2
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4
S4800 5.0kV 15.7mm x300 SE(M)
100um

Figure 6.1. (overleaf) Comparison of mosquito gut.
Aedes aegypti (A), *Anopheles stephensi* (B) and *Culex quinquefasciatus* (C) guts 48hr post inoculation with *Metarhizium*. There is no colonisation of the haemocoel. Gut lumen packed with conidia (1), Peritrophic membrane (2), Midgut epithelium (3) and Haemocoel (4)

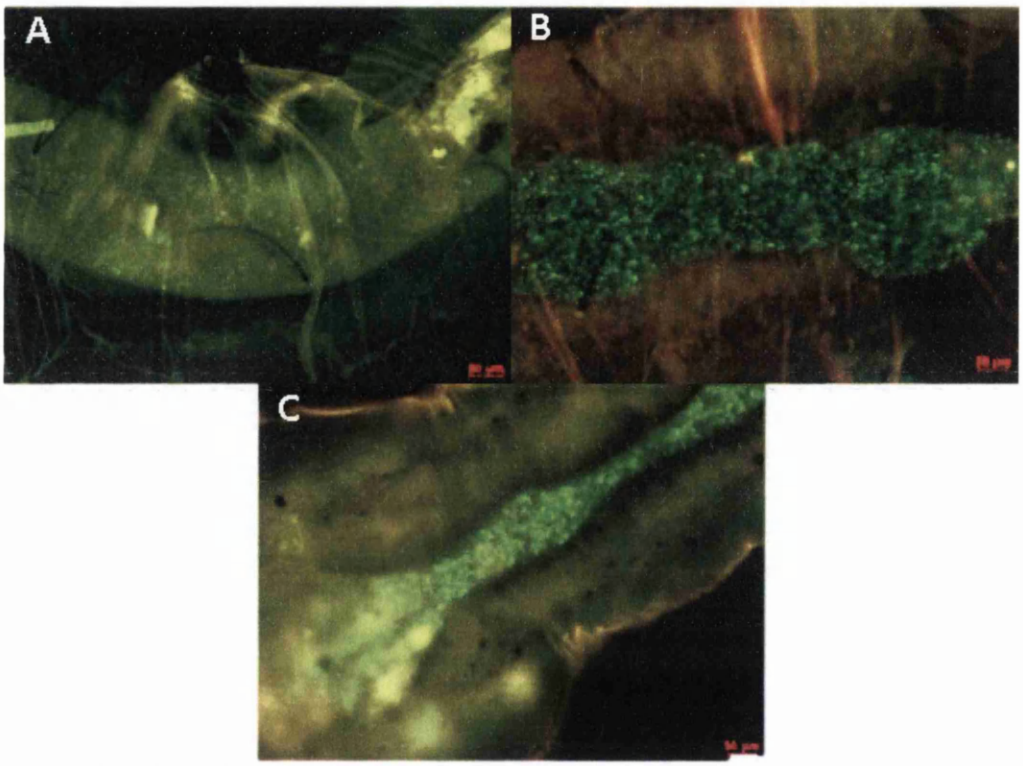

Figure 6.2. Conidia viable in gut lumen even after host death.
Ae. aegypti larvae were inoculated with $1x10^7$ conidia mL^{-1} of *Metarhizium* EAMa 01/58 Su, images were taken 48hr post inoculation. Non infected (A), infected living (B) and infected dead (C) *Ae. aegypti* larvae.

In the second chapter *Metarhizium* was found to be highly effective in the control of all three species of mosquito larvae, with ARSEF 4556 being the most virulent. The mode of pathogenesis of all three species of mosquito appears to be similar, whereby the fungus was able to kill without germinating and colonising the mosquito body. Subtle differences were noted between 'wet' and 'dry' formulations, it is therefore suggested that spore bound proteases are removed by surfactants used in creating a spore suspension. Differential susceptibility of the mosquito species has been attributed to feeding behaviour of these larvae, with *Anopheles* and *Culex* preferentially adopting a filter feeding method, filtering particulate matter within the water column (Merritt, Dadd & Walker 1992; Merritt *et al.* 1996), whereas *Aedes*

feed through browsing, scrapping food matter from the surrounding substrate (Eisenberg, Washburn & Schreiber 2000). In addition, their respiratory behaviour may contribute to their susceptibility, with Anopheline larvae lying parallel to the water surface allowing for more contact with spore, particularly those that have been applied as a 'dry' powder. These observations are important to consider when developing a larval control strategy, as it would be beneficial to utilise a more targeted approach. Furthermore, the fact that *Metarhizium* sp. was highly efficacious against all three species means that potentially all three species of mosquito larvae can be killed in the same body of water, with the same strain of fungus. As a consequence, this will make commercialisation and control more viable, reducing both the cost of production and deployment as well as pest load.

Examination of the gene expression during infection of *Ae. aegypti* larvae and *Tenebrio* adults, sought to observe whether the pattern of host-recognition and infection was similar for aquatic and terrestrial hosts, and as a consequence, whether the same virulence determinants are implicated in mosquito mortality. In addition, analysis of the insect stress management and defence system provided the first look at host-pathogen interactions, in this system, from both sides. It was shown that the insect detects the presence of the pathogen, indicated by elevated expression of selected antimicrobial peptide and stress management genes. The down-regulation of *Hsp70* and upregulation of *TPX10* genes correlate well with the protease induced apoptosis. Caspase activity, indicative of an apoptotic response, increased with time, concomitant with larval mortality, further corroborating the idea of accidental death. The mosquito larval defence response to *Metarhizium* is not robust, and it is proposed that this is because mosquito larvae would not normally encounter *M. anisopliae* and subsequently they have not evolved an appropriate defence response. From the pathogen point of view, *M. anisopliae* is not adapted for the aquatic environment and is unable to adhere to the surface of the insect and establish an infection court via the normal route, therefore killing the mosquito host inadvertently when ingested. *M. anisopliae* retains pre-formed pathogenic determinants which mediate host mortality, indicated by the expression of pathogenicity genes in the mosquito gut lumen but unlike true aquatic fungal pathogens, it does not attach to the gut and colonise the larval host. Since this is the case, it is concluded that the ensuing mortality is inadvertent hence the verdict of accidental death.

The use of Atomic force microscopy, gene expression analysis and lipidomic analysis has provided a more detailed study of the processes and mechanisms influencing host-pathogen interactions of *M. anisopliae* to both mosquito larvae and a typical terrestrial host *Tenebrio molitor*. It was demonstrated that *M. anisopliae* is able to form firm attachment to a terrestrial host cuticle, under water, indicating the ability of this fungus to adapt and form associations in an aquatic environment. Furthermore, it has been shown for the first time that *M. anisopliae* recognizes *Aedes* larvae as potential host, forming weaker interactions, which are likely to be weak receptor-ligand bindings. The fungus, however, is unable to maintain and consolidate the initial attachment with a firm adhesion. It can only be postulated that the mosquito larvae cuticle is not a conducive environment for development, this could be attributed to the lack of long chain hydrocarbons in the larvae cuticle, required for germination. As a consequence, it is hypothesised that the fungus is able to regulate and functionalise its cell surface properties, preventing attachment. Despite the advances made in investigating the underlying mechanisms of attachment in bacteria to substrata, the processes involved in fungal attachment and host-pathogen interactions remain largely unknown. The use of spore functionalised AFM tips together with force mapping would aid to elucidate specific recognition sites on the terrestrial host cuticle and the underlying molecular and chemical properties that enable attachment.

By investigating the mechanisms regulating host range and specificity in *Metarhizium* species, studying proteome, transcript levels and enzyme activity during infections and combining this with functional studies of key candidate genes, through the use of gene tagging and knock out (see Appendix 2) it was intended to provide a better understanding of the mechanisms underlying the mode of pathogenesis in mosquito larvae. Great progress was made in developing a methodology for protein extractions and future experiments will include investigating whether transcripts of those proteins elucidated are present and or upregulated in *Metarhizium* in response to mosquito larvae. Additional studies will also undertake a comparative study of *M. robertsii* and *M. acridum* secretome in response to host and non-host media.

6.2 FUTURE DIRECTIONS

6.2.1 Practical implications *in situ*

It is important to understand how the host and pathogen interact in order to better develop current control strategies and in light of these new observations a re-evaluation of current control strategies is required in order to fully maximise the use of these entomopathogenic fungi. Observing that the mode of infection is different to that of a terrestrial system, whereby inoculum is ingested - rather than entering via direct penetration, future control strategies may seek to combine inoculum with a food source to ensure a higher percentage of conidia is ingested.

This thesis has provided the foundations for development of a more targeted control strategy. Further experimentation would seek to combine the fungus with a lure and kill strategy to actively attract the mosquito females to a water source inoculated with *Metarhizium*.

6.2.2 *in vivo* studies

Members of the genus *Metarhizium* are model fungal pathogens of insects, ticks and mites with potential for controlling pests of crops and those of animal and medical importance. Different species in the genus show varying host range, from generalist with a broad host range to specialists which infect specific hosts. While certain genes involved in general pathology have been characterised and investigated, namely *Pr1* and *Mad* genes (chapters 3 and 4), the genetic mechanism which define host range and therefore, aggressiveness of the fungal pathogens are still not understood.

The genome sequences of *M. robertsii* and *M. acridum* have been published recently and genetic tools for transformation and heterologous gene express have been developed. This system therefore provides an ideal candidate for the study of host-pathogen interactions with the potential for practical application in the control of insect pests.

Further work is required to identify key genetic determinants of host range and aggressiveness in *Metarhizium* spp. through the characterisation of candidate proteins identified (chapter 5) through green fluorescent protein (GFP) tagging, targeted gene disruption and heterologous gene transfer. Through employing a

comparative approach to investigate the mechanisms regulating host range and specificity in *Metarhizium* species, we are able to study transcript levels of the proteins identified alongside functional studies of candidate genes by gene tagging with GFP and targeted gene knock outs. This approach will provide a complete and deep understanding of the mechanisms underlying why and how certain species can parasitize different insect hosts, what the main mechanisms controlling virulence are, and what are the main factors driving adaptation and evolution in animal pathogenesis.

Other benefits of the work will include the development of tools enabling *in vivo* studies of this model system, identifying targets for developing improved insect biocontrol and the provision of resources (e.g. genetically manipulated strains) that can be used in studies in addition to mosquito control. In addition, it is envisaged that a *Metarhizium* strain - highly virulent and specific to mosquito larvae, will be created and commercialised for use in biocontrol schemes.

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APPENDIX 1

APPENDIX 1

A.1 INTRODUCTION

Attempts were made to create a vector-mediated *M. robertsii* transformant; allowing for the expression of Prl-GFP fusion proteins, with a selectable marker i.e. Hygromycin B Phosphotransferase gene (hph), under the control of a constitutive promoter. Thus further contributing to the understanding of genes involved in pathogenesis. Transformants contain GFP from two separate fusion plasmids, pUNIV–EGFP and pEiLov-N1 using both the full gene and the first exon of Prla gene. pUNIV-EGFP is known to express well in all organism, pEiLov-N1 utilizes a fluorescent tag derived from the light oxygen or voltage-sensing (LOV) domain of phototropin. In plants it is known to be more effective than GFP (Chapman *et al.* 2008), however, it's efficacy in a fungal system has not been explored. This strategy aims to exploit iLOV performance, with the inclusion of GFP. Both regions of gene insert include a promoter region inducing synthesis of fusion. Due to a large final product size an exon based alternative was developed. The smaller product translated by the exon is more readily incorporated into the fusion, allowing the visualisation of variance in gene regulation within the fungus on various hosts, while a full gene strategy was developed to allow a fully operational protein fusion to be synthesized, allowing spatiotemporal analysis of the gene expression within infection of the insect.

A.2 CLONING STRATEGY

A. Clone An_trpC terminator into pBluescript II KS

An_trpC Terminator derived from pDCE-*asl*001 (pBlue GMGT5, pBlue::AbGDP::msc001::Gusspacer::mcs002::cTERM5, 25/2/03)

Primers required:
An_trpC_Spe1_F: GCTTCTACTAGTGATCCACTTAACGTTACTGAA

An_trpC_Xma1_R: GCTTCTACCCGGGTGGAGATGTGGAGTGG
*Restriction sites for Spe1 and Xma1

1) Digest pBluescript II KS with XmaI + SpeI = 2951bp
2) Digest An_trpC with XmaI + SpeI = 776bp
    i. Gel purify insert
    ii. Gel purify vector
3) Ligate 1+2 together → pBluescriptIIKS::trpC – 3727bp

B. Clone An_trpC terminator into pGRhph001 (Figure A.1)

4) Digest pGRhph001 with NotI + EcoRV = 5365bp
5) Digest 3 with NotI and EcoRV = 809bp
    i. Gel purify trpC insert
    ii. Gel purify pGRhph001 vector
6) Ligate 4+5 → pGRhph001::trpC = 6170bp

C. Clone Pr1a gene into pEiLOV and pUNIV to create gene fusions (Figure A.2)

Forward primer constructed using 850-1150bp of sequence [Pr1a + 3000bp each side] and [Pr1a first exon] with a *Xho1* and *Not1* tail

Xho1-Not1-Pr1-A-F:
GCTTCTCTCGAGCGGCCGCTTACCTCTTGCCTATCTCGTC
*Restriction sites for Xho1 and Not1, Pr1a

Reverse primers were designed using *Xho1* to allow for directional cloning of the first exon + fluorescent tag

Pr1Aex1+1iLov_R: GCTTCTCTCGAGTATCTTCAGCTCCTCCTTGGT

Pr1Aex1+0UNIV_R: GCTTCTCTCGAGATCTTCAGCTCCTCCTTGGT

Reverse primers were also designed using *Xho1* to allow directional cloning for the whole gene + fluorescent tag

Pr1Aend1UNIV_R: GCTTCTCT CGAGGCACCGTTGTAGGC

Pr1Aend2iLOV_R: GCTTCTCT CG AGTAAGGCACCGTTGTAGGC

7) Digest pUNIV with XhoI Vector fragment = 5433bp
8) Digest GOI e.g. Pr1a Exon with XhoI
    i. Gel purify Pr1a Exon insert
    ii. Gel purify pUNIV vector
9) Ligate 7+ 8 → pUNIV::Pr1aExon – 8734bp

D. Clone gene fusion into pGRhph001::trpC ready for transformation into *Metarhizium* (Figure A.3)

10) Digest 9 with Not I
11) Digest 6 with Not I
12) Ligate 12 + 13 → pGRhph001::trpC::Pr1aExon::EGFP – 14,905bp

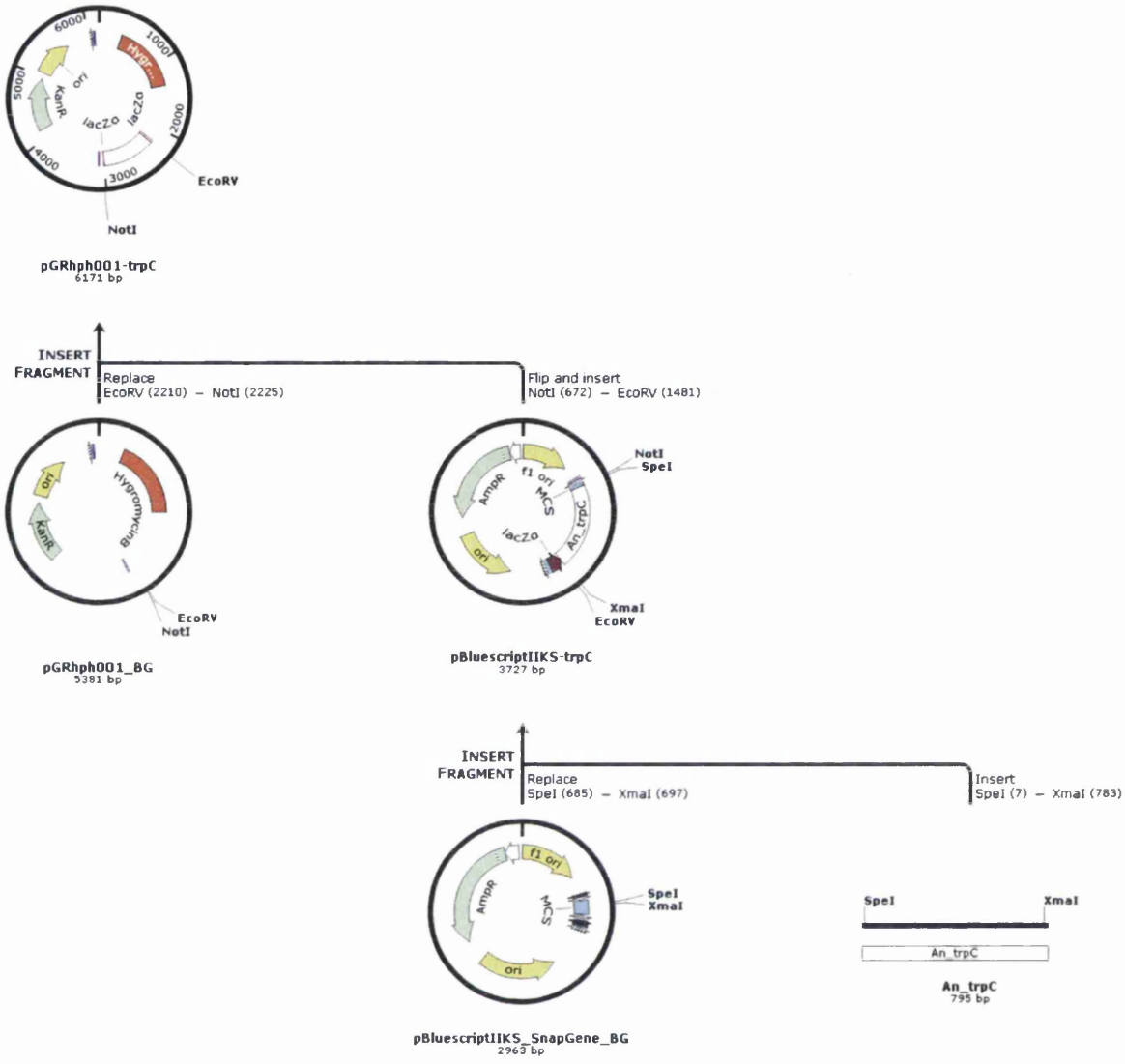

Figure A1: Strategy for construction of Pr1-GFP fusion vector part II.
Aspergillus nidulans trpC gene terminator cloned into pBluescriptKSII, prior to inserting and cloning into pGRhph001 vector

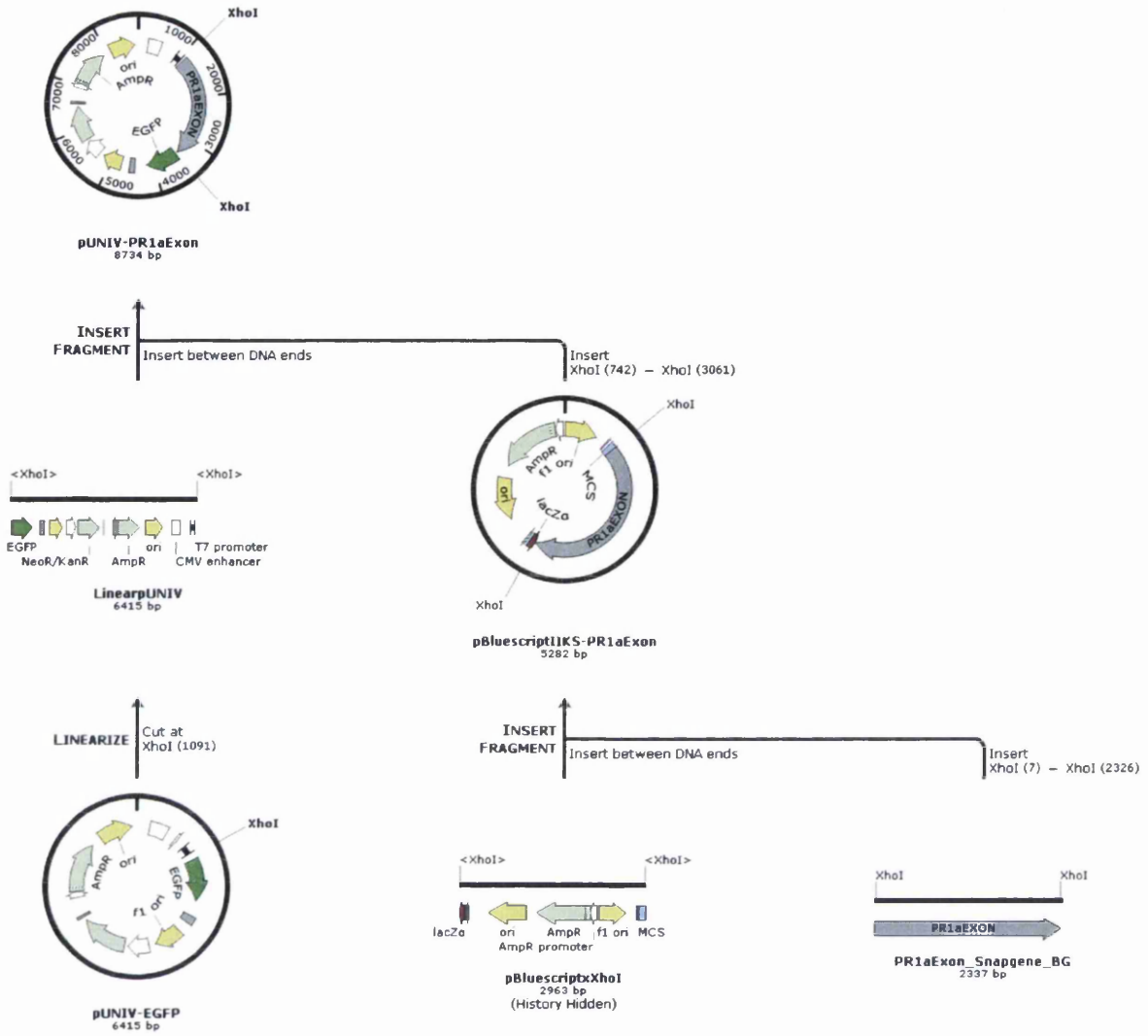

Figure A2: Strategy for construction of Pr1-GFP fusion vector. pBluescript KSII vector with *Metarhizium anisopliae Pr1* exon, cloned cut and inserted into pUNIV-EGFP vector

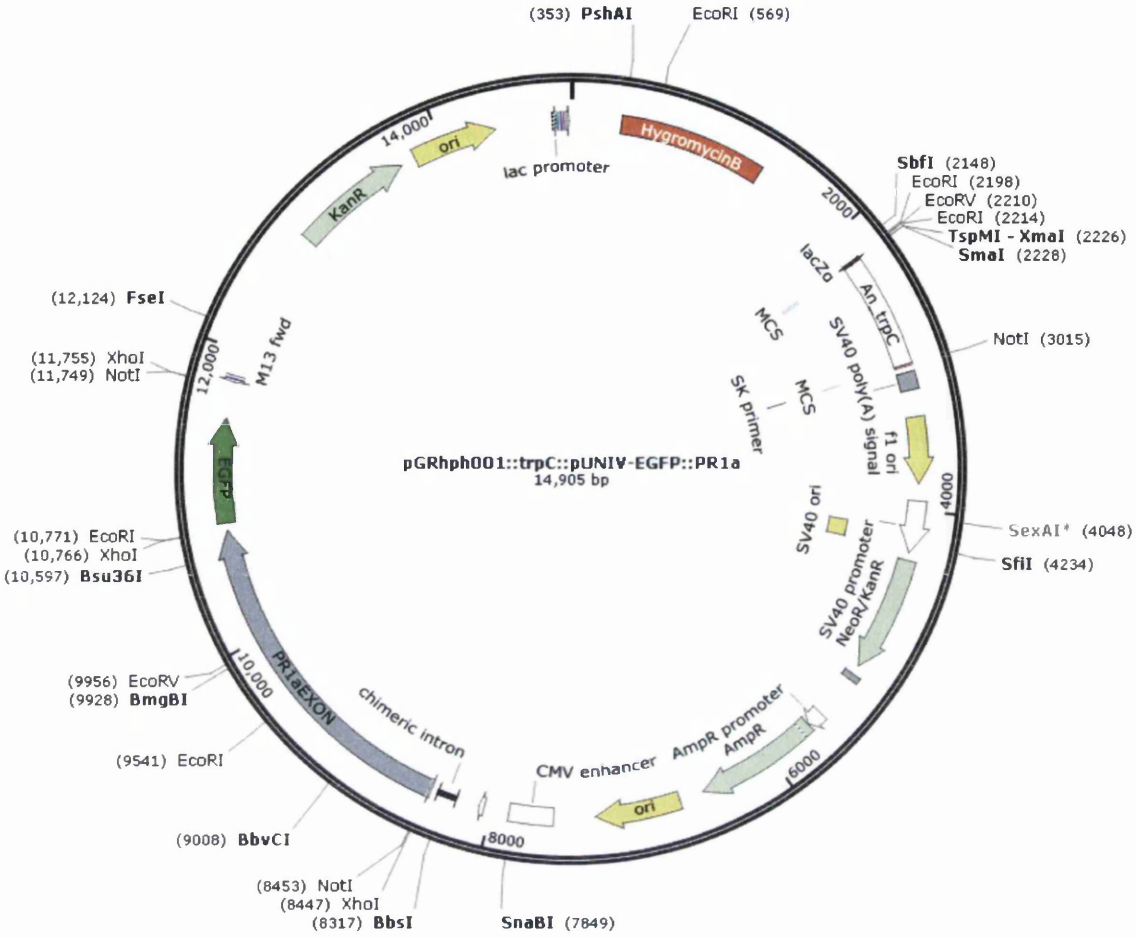

Figure A3: Strategy for construction of Pr1-GFP fusion vector part III.
Final fusion vector of pGRhph001 with trpC terminator and pUNIV-EGFP containing Pr1a exon.

A.2 SUMMARY

Attempts to create a Pr1-GFP fusion vector were unsuccessful. The constructions outlined in figure A1 and A2 were successfully completed; however, the fusion of these two vectors was not. A new cloning strategy is currently under development.

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APPENDIX 2

OPEN ACCESS Freely available online

PLOS | ONE

Metarhizium anisopliae Pathogenesis of Mosquito Larvae: A Verdict of Accidental Death

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Abstract

Metarhizium anisopliae, a fungal pathogen of terrestrial arthropods, kills the aquatic larvae of *Aedes aegypti*, the vector of dengue and yellow fever. The fungus kills without adhering to the host cuticle. Ingested conidia also fail to germinate and are expelled in fecal pellets. This study investigates the mechanism by which this fungus adapted to terrestrial hosts kills aquatic mosquito larvae. Genes associated with the *M. anisopliae* early pathogenic response (proteinases *Pr1* and *Pr2*, and adhesins, *Mad1* and *Mad2*) are upregulated in the presence of larvae, but the established infection process observed in terrestrial hosts does not progress and insecticidal destruxins were not detected. Protease inhibitors reduce larval mortality indicating the importance of proteases in the host interaction. The *Ae. aegypti* immune response to *M. anisopliae* appears limited, whilst the oxidative stress response gene encoding for thiol peroxidase is upregulated. *Cecropin* and *Hsp70* genes are downregulated as larval death occurs, and insect mortality appears to be linked to autolysis through caspase activity regulated by *Hsp70* and inhibited, in infected larvae, by protease inhibitors. Evidence is presented that a traditional host-pathogen response does not occur as the species have not evolved to interact. *M. anisopliae* retains pre-formed pathogenic determinants which mediate host mortality, but unlike true aquatic fungal pathogens, does not recognise and colonise the larval host.

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Introduction

Mosquitoes vector a wide range of diseases (e.g. dengue, yellow fever and malaria) which can have a devastating impact on human health. Almost half the world's population is at risk to mosquito-transmitted diseases and the range has expanded due to climate change and increased trade [1]. Many chemical pesticides have been withdrawn due to the risks they pose to humans and the environment, and development of resistance in pest populations. Recent studies show that *Metarhizium anisopliae*, a soil borne fungal pathogen of terrestrial insects, offers an environmentally friendly alternative to chemicals for the control of mosquitoes. *M. anisopliae* will kill adult and larval stages of *Aedes*, *Anopheles* and *Culex* mosquitoes [2,3] yet the mechanism of how this terrestrial pathogen kills the aquatic larval stage is unclear. Strains of *M. anisopliae* have been developed to control a wide range of terrestrial arthropods including pests of agro-forests crops and vectors of human and animal diseases [4,5]. Infection of terrestrial arthropod hosts by *M. anisopliae*, like that of most other entomopathogenic fungi, follows a consistent pattern. Firstly, spores adhere to the surface of the host cuticle followed by germination and differentiation of an appressorium from which a narrow penetration peg is produced which penetrates the cuticle using a combination of enzymes and mechanical force [6,7]. Following colonization of the hemocoel, the fungus erupts through the intersegmental membranes and differentiates conidiophores and conidia. The key pathogenicity determinants of *M. anisopliae* include cuticle degrading enzymes like *Pr1* (subtilisin protease) and toxic cyclic peptides like destruxins [8]. Fungal virulence appears to be correlated with *Pr1* and destruxin production; with hyper producers being more virulent [8]. Equally important are the adhesins, *Mad1* and *Mad2*, which play an important role in adhesion of *M. anisopliae* to the insect cuticle [9]. Disruption of the *Mad1* gene reduces virulence by reducing adhesion of conidia to the host surface [10]. It is assumed that the pattern of infection outlined above occurs in aquatic mosquito larvae [2].This paper demonstrates for the first time that *M. anisopliae* kills the mosquito larvae *via* a mechanism which does not entail the traditional infection processes.

Based on the limited number of observational studies conducted on *M. anisopliae* infection of mosquito larvae, possible routes of invasion have been reported including entry *via* penetration of the cuticle, the respiratory siphon or alimentary canal, however, the precise mechanism remains elusive. Lacey

et al., [11] noted that when larvae of *Culex quinquefasciatus* broke the water tension with their perispiracular valves for air intake, floating conidia of *M. anisopliae* adhered to the inside surface of the valves, germinated and invaded the siphon tip tissue, then extended into and blocked the trachea resulting in suffocation and death. Lacey *et al.*, [11] also noted that conidia suspended in the water were ingested and occluded the larval gut, initiating mortality within 6 to 24 hr after ingestion. In contrast, Riba *et al.*, [12] reported that *M. anisopliae* conidia killed *Ae. aegypti* within 1.1 days before intra-hemocoelic invasion. Some workers suggest that death is due to colonization of the hemocoel by the fungus [2,12], others suggest it is due to toxins released by ingested conidia without colonisation of the hemocoel [11,13].

Materials and Methods

Fungal strains and production

Metarhizium anisopliae isolate ARSEF 4556, identified as highly pathogenic to mosquitoes and midges [4], was maintained on Sabouraud dextrose agar (SDA) or broken Basmati rice [14]. Conidia used in assays had over 95% viability. A green fluorescence protein (GFP) transformed strain of *Metarhizium brunneum* EAMa 01/58 Su was maintained on SDA.

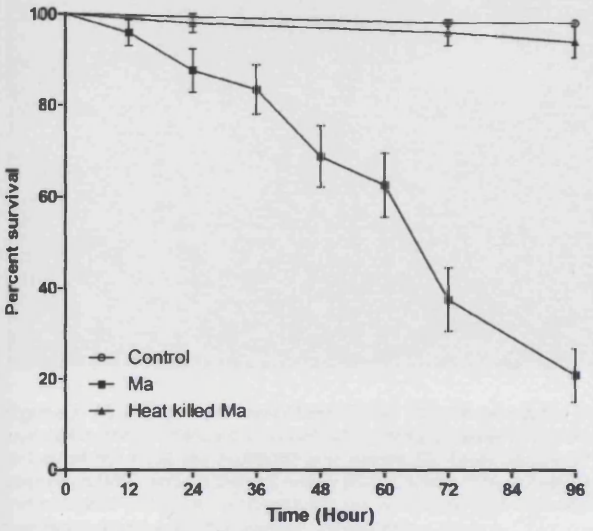

Figure 1. Heat killed treatment increased the survival of *Ae. aegypti* larvae. Late $3^{rd}-4^{th}$ instar *Ae. aegypti* larvae (n = 72) were inoculated with live and heat killed conidia of *M. anisopliae*. Kaplan-Meier method was used to plot cumulative survival curves of larvae after inoculation, log-rank test was used to assess difference in survival between treatments. The curves of non-exposed and heat killed treatment show no statistical difference. Live conidia significantly decreased survival compared to heat killed conidia ($p<0.001$). Larvae with no fungal treatment were used as a negative control.The aim of this study was to establish the cause of death as this could have profound implications on the formulation and deployment of *Metarhizium* and other terrestrial entomopathogenic fungi to control mosquito larvae. This study, utilising a combination of microscopy, molecular and biochemical methods, shows that *Metarhizium* invasive strategies, evolved for terrestrial hosts including adult mosquitoes, have not evolved for killing aquatic insect hosts with resultant mortality in mosquito larvae being multi-factorial.
doi:10.1371/journal.pone.0081686.g001

Mosquito source and maintenance

Aedes aegypti (strain AeAe) eggs, obtained from the London School of Hygiene and Tropical Medicine, were hatched in distilled water and the larvae fed on Tetramin® fish food, at room temperature (22°C±2°C).

Inoculation of *Aedes* larvae with *Metarhizium* conidia

Assays were performed using 24 well plates (Nunc, Roskilde, Denmark) with one larva per well. *M. anisopliae* ARSEF 4556 was assayed at 10^7 conidia ml^{-1} against L_{3-4} larvae. Additional assays were done using heat killed conidia to determine the role of extracellular enzymes in pathogenesis. Extracellular enzymes were denatured by wrapping the conidia in aluminium foil and autoclaving for 15 min at 121°C. Conidial viability was assessed using the plate count technique [15]. Control larvae were exposed to either 1ml 0.03% Aq Tween 80 or distilled water. Larval mortality was recorded daily up to 7 days. All assays were performed at room temperature with a 16L:8D photoperiod. There were 24 larvae per assay which was repeated three times. This format was used in subsequent assays to study host-pathogen interactions, in particular, insect defense responses and regulation of *M. anisopliae* pathogenicity determinants.

Larvae (n = 20) were inoculated with conidia of ARSEF 4556 as outlined above and examined at 0, 24, 48 and 72 hr post inoculation (pi). Healthy and infected larvae were examined by light microscopy (LM) to determine if there were preferential sites for spore adhesion and to monitor passage of the fungal conidia through the gut. Larvae (n = 20) were also examined by cryo scanning electron microscopy (SEM) using a Hitachi S4800 field emission microscope equipped with a Quorum PPT2000 cryo-genic stage and preparation chamber. Full details on the cryo-SEM are provided in Text S1 in File S1. Additional studies were done using a GFP-transformed strain of *M. brunneum*. The surface and gut contents of infected *Ae. aegypti* larvae (n = 10)as well as fecal pellets were examined by fluorescence microscopy (FM) using a Zeiss fluorescence microscope.

Mass spectrometry analysis of destruxins

Assays were performed using 24-multi-well plates with ten *Ae. aegypti* larvae (L_{3-4}) per well containing 1 ml aqueous suspension of 1×10^7 conidia ARSEF 4556 or 1 ml of distilled water (control). After 24 hr incubation, the larvae were removed and prepared for destruxin extraction and analysis by mass spectroscopy as described by Butt *et al.*, [16]. The effect of destruxins on *Ae. aegypti* larvae was also tested by introducing larvae to 1 ml of distilled water spiked with 1nmole of destruxin A (Sigma-Aldrich) and determining larval survival. Briefly, destruxin extracts were analyzed by nano-reverse phase liquid chromatography (Ultimate Pump, LC-Packing, Dionex, The Netherlands) using an electro-spray ion trap MS (LCQ Deca XP, ThermoElectron, Hemel Hempstead, UK). Matrix Assisted Laser Deionisation Mass Spectroscopy (MALDI MS) was used to confirm the levels of detection below which any destruxins might be exhibited within the larvae themselves. For this purpose, larval extract was re-dissolved in a matrix solution and 1 µl of the resulting mixture was spotted onto the MALDI plate and allowed to dry at room temperature. A 10 mg/mL alpha cyanocinnaminic acid (CHCA) in 50:50 0.1% trifluoroacetic acid (TFA): acetonitrile (ACN) matrix solution was used and a Voyager DE-STR instrument (Applied Biosystems, UK) was utilized in reflectron mode. An acceleration voltage of 20,000V and a grid voltage of 70% was utilized in order to study any peptides present and a 1pmole standard of destruxin A applied to the plate as a control and as a method of determining the level below which any destruxins are

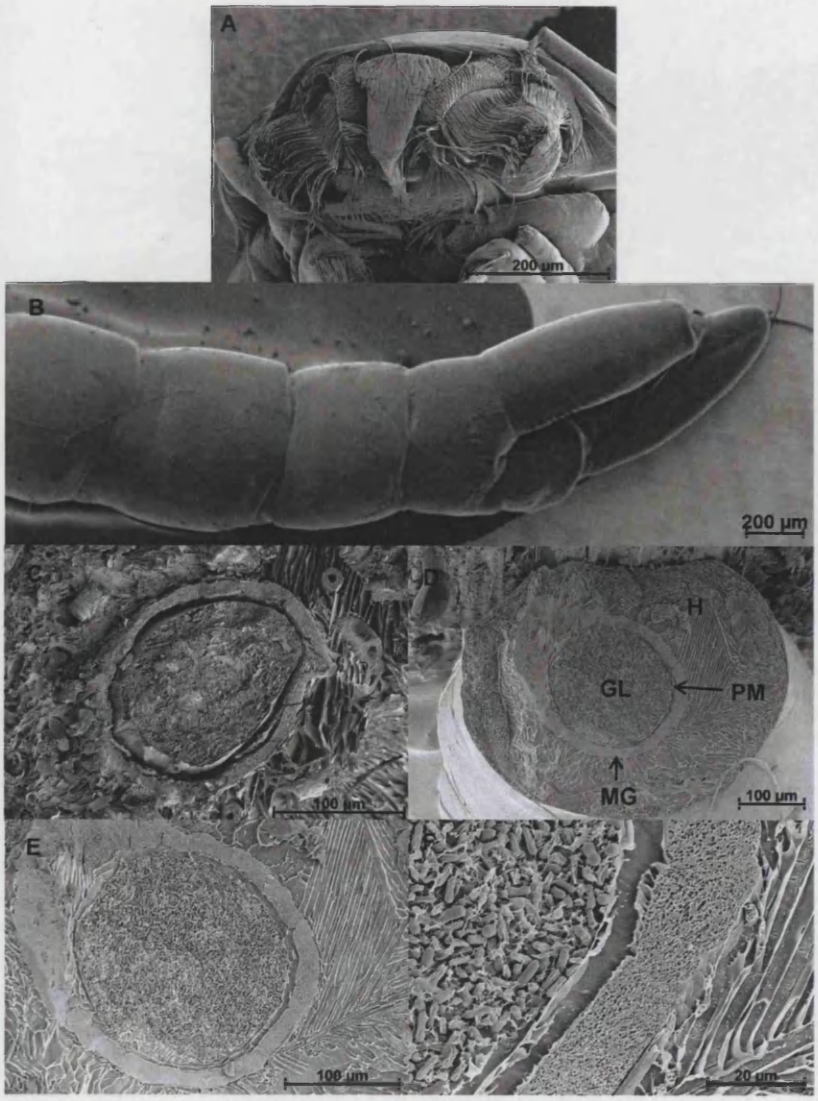

Figure 2. *M. anisopliae* do not attach to or invade mosquito hemocoel. Larvae inoculated with 1×10^7 conidia ml^{-1} *M. anisopliae*, 48 hr post-inoculation, were subjected to Cryo-SEM to establish areas of attachment and penetration of the fungus. No conidia were observed attached to the surface of the head (A), abdomen and siphon (B). Cross section of control larva (C) and infected larva (D) showing the gut lumen (GL), midgut epithelium (MG), and peritrophic matrix (PM). Conidia of *M. anisopliae* were restricted to and appeared to occlude the gut lumen. (D–E). Both swollen and collapsed conidia were observed in the gut lumen with no evidence of conidia penetrating the gut wall (F) and invading the hemocoel (H).Light, fluorescence and scanning electron microscopy
doi:10.1371/journal.pone.0081686.g002

present if signal intensity were below the standard. Full details are provided in Text S2 in File S1.

Enzymology

(i) Protease inhibition assays. To determine if the extracellular proteases were responsible for larval mortality. Larvae (n = 24) were exposed to *M. anisopliae* ARSEF 4556 conidia containing either chicken egg white (0.1 mg/ml), EDTA (1 mM) or α2-macroglobulin (1 µg/ml) which were inhibitors specific for *Pr1*, metalloprotease and global (serine, cysteine, metallo-) proteases, respectively. All the inhibitors were purchased from Sigma-Aldrich. Controls consisted of buffer and buffered inhibitor. Mortality was recorded at 0, 12, 24, 36, 48 and 72 hr pi. Assays were also done using heat killed conidia at 10^7 conidia ml^{-1}.

(ii) Caspase assays. Activity of caspases 2, 3, 7 and 8 was assayed using luminometric kits in accordance with the manufacturer's guidelines (Promega). Six larvae were examined per treatment with the endpoint luminescence being measured after 1 hr, in four replicate wells for each larvae. Full details are given in Text S3 in File S1.

Transcript quantification of insect and fungus-derived genes

(i) Samples, RNA extraction and cDNA synthesis. Full details of the transcript quantification are given in Text S4 in

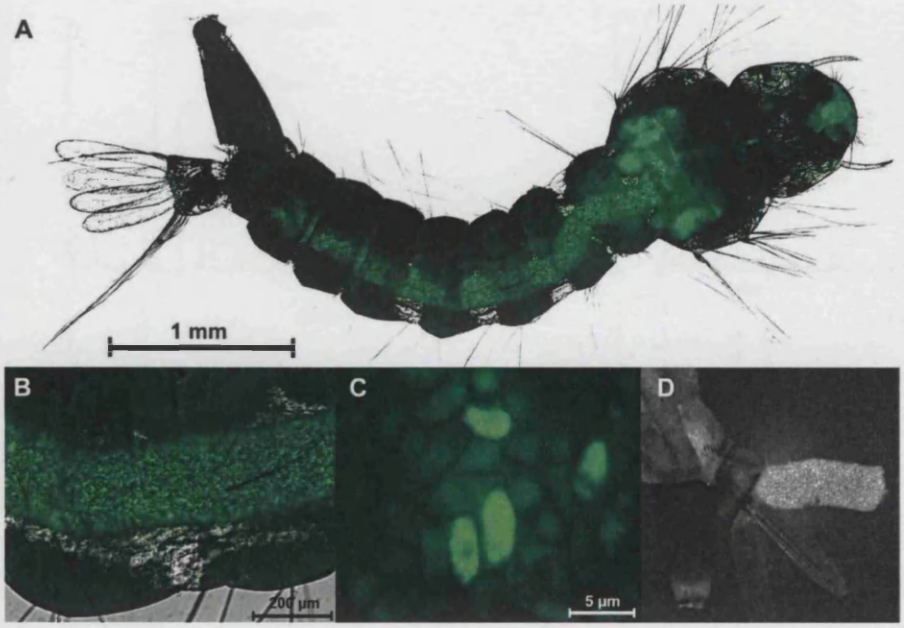

Figure 3. *Metarhizium* conidia expressing GFP in the gut and fecal pellets demonstrating activity and viability. Larvae inoculated with conidia of a GFP transformed strain of *Metarhizium brunneum* were examined 48 hr pi to assess viability and activity of the conidia. (A) Conidia occlude most of the gut lumen, some autofluorescence is seen in the head and thorax region. (B) Numerous conidia are active and expressing the GFP within the gut lumen. (C) High magnification of conidia expressing GFP, the non-fluorescing conidia may be inactive or dead, (D) Fecal pellet being expelled from an infected larva showing many active conidia.
doi:10.1371/journal.pone.0081686.g003

File S1. Briefly, *Ae. aegypti* larvae (L_{3-4}) (n = 3, 10 larvae per replicate) were exposed to *M. anisopliae* ARSEF 4556, controls included larvae not exposed to fungus and a terrestrial insect, *Tenebrio molitor*. Samples were frozen under liquid nitrogen and stored at −80°C until required. All samples were ground with a micropestle and total RNA extractions carried out using the RNeasy Micro kit (Qiagen) following the manufacturer's instructions. RNA concentration and purity was assessed at 260 and 280 nm absorbance using a Nanophotometer (Implen). Total RNA (1 µg) was either RQ1 RNase-free DNAse (Promega) treated and reverse transcribed using the qScript cDNA synthesis kit (Quanta Biosciences), or reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen) with gDNA elimination reaction, for the experiment to quantify insect-derived transcripts and fungus-derived transcripts, respectively. Relative cDNA quantity was analyzed by PCR using two reference genes for insect or fungal cDNA samples to ensure consistency between values: *Ae. aegypti* ribosomal S7 (accession number: AAEL009496) and ribosomal protein 49/L32 (AAEL003396) and *M. anisopliae* 18S rRNA and elongation factor tEF (Table S1 in File S1).

(ii) Quantitative PCR (qPCR). Transcript levels were determined using the Rotor-Gene 6000 (Corbett Research) or CFX96™ Real-Time PCR detection system (Biorad) for *Ae. aegypti* and *M. anisopliae* gene targets respectively. Primers were designed to amplify key *Ae. aegypti* response genes and *M. anisopliae* pathology-related genes (Table S1 in File S1).

The accompanying software for each qPCR instrument was used to analyze the raw data and carry out quality control for each sample. The cycle threshold (Ct) value was determined for each reaction and normalized to the geometric mean of the appropriate endogenous reference genes. Relative gene expression was calculated using the comparative C_t method ($2\text{-}\Delta\Delta C_t$) following established methodology [17].

ROS production, lipid peroxidation and antioxidant system activity

Three insects per time point were sample were homogenized in 100 µl of ice-cold phosphate buffered saline (10 mM phosphate buffer, 150 mM NaCl, pH 7.2) containing phenylthiourea (1 mg/ml). The homogenate was centrifuged for 5 min, 10,000 g at 4°C and activities determined as described by Dubovskiy *et al.*[18], in 12 replicates for ROS production and Lipid peroxidation and 9 replicates for Super Oxide Dismutase (SOD), Glutathione-S-transferase (GST) and Catalase activity. Full details are provided in Text S5 in File S1.

Statistical Analysis

Differences in mosquito larvae survival between live and heat killed conidia and protease inhibited conidia were analysed using Kaplan-Meier survival analysis to plot cumulative survival functions by treatment with pairwise comparison over log-rank test [4]. Biochemical and molecular data sets were analyzed using two-way Analysis of Variance (ANOVA) with Bonferroni's post-test. Prior to analysis gene expression data was logarithm (base 10) transformed, conforming to ANOVA assumption of homogeneity of variance [19]. All statistical analyses were carried out using SPSS v21.0 [20] and GraphPad Prism v5.0 (GraphPad Software, USA).

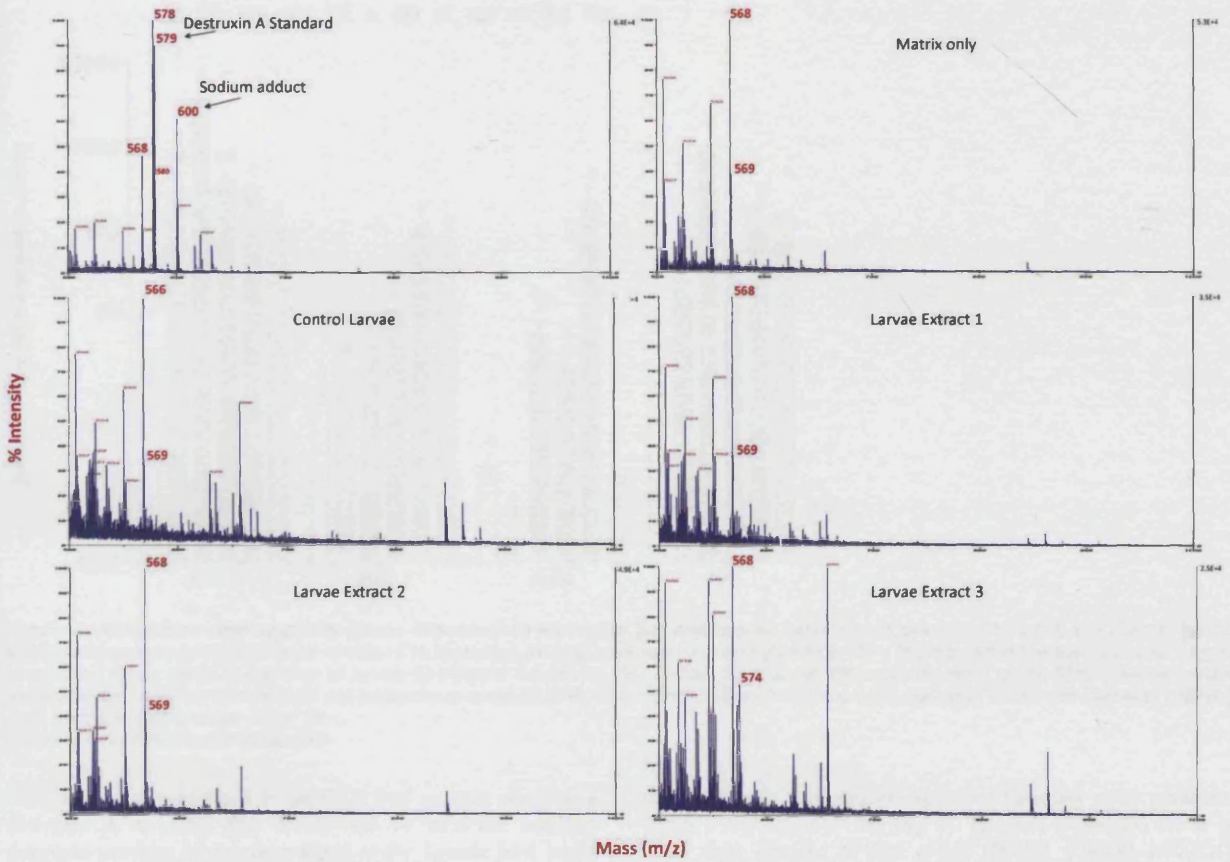

Figure 4. Destruxins not detected in larval extracts. Larval extracts were analysed by MALDI ToF alongside a destruxin A standard. No destruxin signals were detected with both larval extract and control larvae containing only those ions formed due to the MALDI matrix itself.
doi:10.1371/journal.pone.0081686.g004

Results

Virulence of *Metarhizium anisopliae* ARSEF 4556 for *Aedes aegypti* larvae

Ae. aegypti larvae were highly susceptible to *M. anisopliae* 4556 with the earliest mortalities being observed 24 hr pi and percentage mortality reaching 60%−90% between 72 and 96 hr pi (Fig. 1). Heat killed conidia were used to determine whether conidia caused blockage within the larvae or were required to actively bring about mortality. Mortality was significantly higher in larvae exposed to live conidia than those exposed to heat killed conidia ($p<0.001$) and there was no significant difference in mortality between the heat killed conidia ($p<0.153$) and the untreated control (Fig. 1).

Metarhizium conidia fail to infect *Aedes* larvae

Microscopy studies clearly showed little or no attachment of conidia to the surface of the mosquito body (Figs. 2A and B) with conidia being concentrated in the gut lumen (Figs. 2D-F). In some larvae, the conidia occluded the gut lumen while in others gaps were observed between the conidial clumps. None of the conidia in the gut had produced a germ tube and whereas some conidia were hydrated and swollen others appeared collapsed (Fig. 2F). Conidia had a prominent hydrophobin rodlet layer with little evidence of mucilage secretion. FM showed that conidia in the gut and those present in fecal pellets expressed the GFP and were clearly viable and active (Fig. 3). Most non-fluorescing GFP conidia were probably quiescent.

SEM examination of cross sections of abdomen showed no obvious differences in the appearance of the gut epithelial cells, peritrophic matrix and other structures of treated and untreated (control) larvae (Figs. 2C and D–F). There was no visible evidence of damage to the internal organs. Compact fecal pellets were produced by control and infected larvae (Fig. 3) suggesting the peritrophic matrix, peristalsis and other gut functions were intact at least until the time of death. The fungus never crossed the gut; it did not colonize the hemocoel as it would terrestrial arthropod hosts.

Destruxins are not the cause of larval death

Destruxins, common virulence determinants of *M. anisopliae*, were not detected by mass spectroscopy in *Ae. aegypti* larvae that had ingested conidia of *M. anisopliae*. The LCMS analysis was utilized to profile for any destruxin signals present using constant neutral loss signals established in our laboratories that highlight low levels of destruxins [16]. No signals were detected which represent destruxins in any of the five replicate larval extractions (See supplementary figure S2) and re-analysing the data for the m/z of specific destruxins also confirmed their absence (data not shown). The LCMS system was tested using a 10fmole cytochrome C digest and 1 pmole destruxin A, however system test data was not routinely recorded and therefore the larval

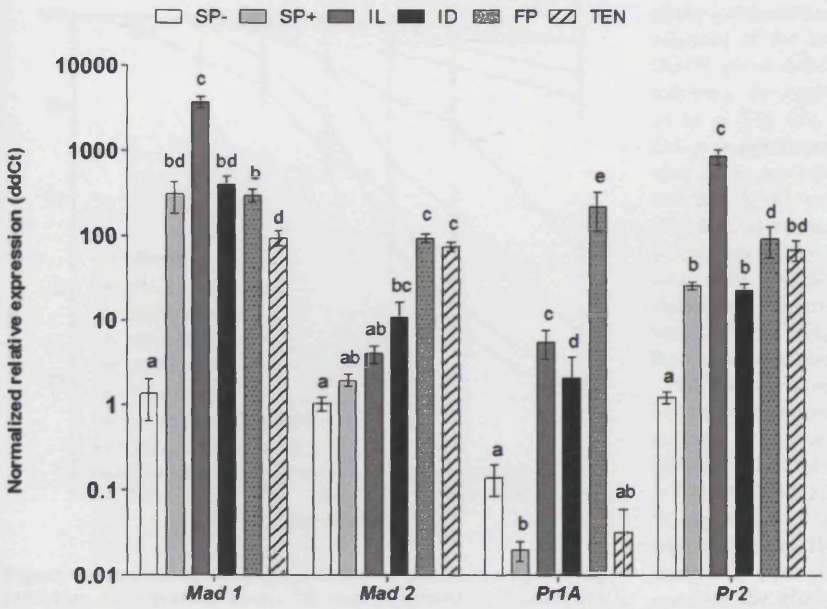

Figure 5. *Metarhizium* pathogenicity genes expressed in mosquito gut and faeces. Expression of protease (*Pr1A, Pr2*) and adhesin (*Mad1, Mad2*) pathogenicity related genes by conidia of *M. anisopliae*, 48 hr pi, analysed by quantitative PCR. SP−: Spore pellet in the absence of *Ae. aegypti* larvae, SP+: Spore pellet in presence of larvae, IL: infected live larvae, ID: infected dead larvae, FP: mosquito fecal pellet, TEN: *Tenebrio molitor* (terrestrial host) positive control. Data was presented as mean (± SEM) means with different letters denoting statistical differences (two-way ANOVA). Data normalized to average dCt of SP−.
doi:10.1371/journal.pone.0081686.g005

extracts were also analysed by MALDI ToF analysis alongside a destruxin A standard (Fig. 4). As can be seen the standard destruxin provides an excellent signal at the 1pmole level whilst the control experiment and larval extracts contain only those ions formed due to the MALDI matrix itself and no destruxin signals can be determined. This allows us to confirm the absence of destruxins, at least up to the 1pmole level per larval experiment.

Metarhizium pathogenicity genes expressed in mosquito gut and faeces

Proteases (*Pr1*, *Pr2*) and adhesins (*Mad1*, *Mad2*) play a key role in fungal pathogenicity, expression of the genes for these components was analyzed and was shown to be generally much higher in the gut lumen and remained high in the fecal pellets (Fig. 5). Expression of *Pr1* was significantly lower in conidia in the presence of *Ae. aegypti* larvae compared with all the other treatments ($F(5,47) = 96.09$, $p<0.01$) but was high in infected living, infected dead and fecal pellet samples ($F(5,47) = 96.09$, $p<0.05$) even when compared with the *T. molitor* treatment ($F(5,47) = 96.09$, $p<0.001$). *Pr1* expression was greatest in fecal pellets ($F(5,47) = 96.09$, $p<0.001$) then infected larvae ($F(5,47) = 96.09$, $p<0.05$) followed by infected dead larvae (Fig. 5). All treatments had a significantly higher expression of *Pr2* than the spore pellets not exposed to larvae with expression being highest in infected living larvae ($F(5,47) = 96.09$, $p<0.001$) then the fecal pellet ($F(5,47) = 96.09$, $p<0.01$; Fig. 5). Spore pellets exposed to larvae and infected dead larvae had lower and significantly similar expression levels. Conidia exposed to *T. molitor* had similar *Pr2* expression to the spore pellet exposed to the larvae, infected dead larvae and the fecal pellets (Fig. 5).

The pattern of expression of *Mad1* was comparable with the expression of *Pr2* with the exception of a slightly lower relative expression in the fecal pellet (Fig. 5). Infected living larvae showed significantly greater expression than any other treatment ($F(5,47) = 96.09$, $p<0.01$; Fig. 5). Greatest expression levels of *Mad2* were detected in fecal pellets ($F(5,47) = 96.09$, $p<0.001$), *T. molitor* ($F(5,47) = 96.09$, $p<0.001$) and infected dead ($F(5,47) = 96.09$, $p<0.01$) when compared with the spores not exposed to larvae (Fig. 5).

Mortality linked to fungal protease-induced apoptosis

One possible mechanism that may eventually lead to mortality of the larvae is the activation of apoptotic pathways in the larvae (involving caspase enzymes) by active agents released by the conidia. As the active agents identified were proteases, the effect of inhibiting these enzymes on larval mortality was investigated and the larval caspase activity also monitored.

Mortality of larvae incubated with the fungus was significantly lower in the presence of protease inhibitors with the exception of EDTA which was not significantly different from the *M. anisopliae* treated larvae (Fig. 6) The inhibition with chicken egg white increased percentage survival after treatment with the fungus from approximately 10% to 30%, whilst inhibition by α2-macroglobulin improved this value to 50% of the untreated larvae. As well as the effect of the proteases produced by the fungus on larval survival, the study of the effect of the inhibition of such bioactive entities on apoptosis was studied. Activity of caspases 2, 3/7 and 8 was significantly higher in *Ae. aegypti* larvae inoculated with live conidia of *M. anisopliae* without protease inhibitors than with inhibitors (Fig. 7). Interestingly, in the *M. anisopliae* treated larvae, activity increased dramatically, concomitant with larval mortality, between 36 and 72 hr pi (Figs. 6, 7). Caspase activity was significantly lower in larvae in the presence of protease inhibitors for the whole period of the assay ($F(5,72) = 661.39$, $F(5,72) = 90.4$, $F(5,72) = 75.42$ (caspase 3/7, 2 and 8 respectively) $p<0.001$; Fig. 7A, B and C). Caspase 2, 3/7 and 8 activity was generally

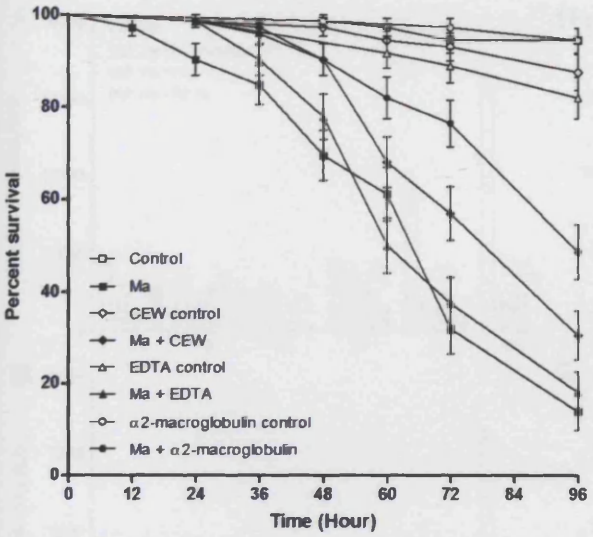

Figure 6. Survival of *Ae. aegypti* larvae in presence of protease inhibitor. *Ae. aegypti* larvae (n = 72) were inoculated by *M. anisopliae* with and without protease inhibitors. CEW: Chicken Egg White a *Pr1* specific inhibitor, α2 mac: α2 macroglobulin a global protease inhibitor and EDTA a metalloprotease inhibitor. Kaplan-Meier method was used to plot cumulative survival curves of larvae after inoculation, log-rank test was used to assess differences in survival between treatments. Uninhibited conidia caused greater mortality than conidia treated with inhibitors with the exception of EDTA ($p<0.001$). Controls consist of either 0.05% Aqueous Tween only, or 0.05% Aqueous Tween with protease inhibitor.Caspase activity, particularly caspases 3/7 and 8, was consistently higher in *Ae. aegypti* larvae exposed to live conidia compared to the heat killed conidia 48–72 hr pi ($F(5,54) = 203.60$, $F(5,54) = 71.15$ (caspase 3/7 and 8 respectively) $p<0.001$; Fig. 7B–D). Caspase activity elicited by heat killed conidia increased over time up to 72 hr pi.
doi:10.1371/journal.pone.0081686.g006

lower 24–72 hr pi in the presence of EDTA than the other inhibitors ($F(5,72) = 1359.03$, $F(5,72) = 486.01$, $F(5,72) = 271.46$ (caspase 3/7, 2 and 8 respectively) $p<0.001$; Fig. 7A, B and C).

Oxidative stress is not an obvious mediator of apoptosis

Oxidative stress within organisms can also be a trigger for the initiation of apoptosis and therefore various indicators of such stress were studied in larvae with and without conidia. Examination of reactive oxygen species generation, lipid peroxidation, catalase, superoxide dismutase and glutathione-S-transferase activity during two days of pathogenesis revealed no major differences between uninfected and *M. anisopliae* infected mosquito larvae except glutathione-S-transferase activity which was higher ($F(2,48) = 12.10$, $p<0.01$) 48 hr pi in infected (Figs. S1A–E). At 72 hr pi when most larvae were dead or dying we found the decrease in ROS generation ($F(2,60) = 5.05$, $p<0.01$); lipid peroxidation ($F(2,59) = 1.66$, $p<0.05$); catalase ($F(2,48) = 6.77$, $p<0.01$); and glutathione-S-transferase ($F(2,48) = 12.10$, $p<0.01$) activity in infected insects (Figs. S1A–E).

Immune and stress management systems fail to protect mosquito larvae

Given the release of active proteases by the fungus and elicitation of a pathogenic response when exposed to the larvae, the study of the larval response to the fungus was also undertaken. The analysis examined the larval defense mechanisms (predominantly antimicrobial peptides) as well as the stress response of the larvae. Expression of the antimicrobial peptide (AMP) genes *AeDA* and *AeDB* was not significantly up regulated following *Ae. aegypti* larval ingestion of live *M. anisopliae* conidia 24 hr pi (Fig. 8A). While expression levels of *Ada-DefD* and *Ada-CcG* was significantly greater in samples exposed to *M. anisopliae* after 24 hr compared with time zero ($F(4,50) = 16.12$, $p<0.05$) it was not significantly different from the 24 hr untreated control (Fig. 8A). However, the gene was significantly down regulated in larvae exposed to *M. anisopliae* 48 hr pi compared with the other treatments ($F(4,50) = 16.12$, $p<0.05$; Fig. 8A). *AeCA2* was significantly down regulated in larvae at 48 hr pi compared with 24 hr pi ($F(4,50) = 16.12$, $p<0.01$) but these did not differ from unexposed larvae at 48 hr and 24 hr, respectively (Fig. 8A).

Of the five stress management genes examined, a significant increase in expression of the *TPX10* was observed in larvae exposed to *M. anisopliae* 24 hr compared with the time zero ($F(4,40) = 7.71$, $p<0.001$) and 24 hr untreated control ($F(4,40) = 7.71$, $p<0.01$; Fig. 8B). While there was a significant increase in expression of *TPX10* at 48 hr in exposed larvae compared with time zero ($F(4,40) = 7.71$, $p<0.05$) this was not significant compared with the unexposed larvae at 48 hr (Fig. 8B). In contrast, the *Hsp70* gene was down-regulated in larvae exposed to conidia 48 hr pi compared with the untreated control ($F(4,40) = 7.71$, $p<0.01$; Fig. 8B). No significant changes were observed for both AMPs and stress genes in the untreated controls at 0, 24 and 48 hr (Fig. 8A and B).

Discussion

This study shows that conidia of *M. anisopliae* do not firmly adhere to the surface of the cuticle of *Ae. aegypti* larvae and do not gain entry by penetrating the host cuticle. Conidia have been reported adhering to the cuticle, particularly the siphon and mouthparts of the fungus [21], thereby infecting the larvae in a similar manner with which it infects terrestrial hosts [2].

Conidia attach to terrestrial hosts initially *via* passive hydrophobic forces followed by secretion of enzymes and adhesion compounds to anchor the spore to the cuticle surface [6,7]. The failure of conidia to adhere to the cuticle of terrestrial hosts has been attributed to the cuticle chemistry, with some compounds altering hydrophobicity or being fungistatic [6,22]. It is feasible that the mosquito larval cuticle is not conducive for adhesion, with any mucilage produced by the fungus being diluted in the water. In contrast, aquatic pathogens of mosquitoes such as *Lagenidium giganteum* (Oomycetes) and *Coelomomyces punctatus* (Chytridiomycetes) produce zoospores that can attach to and penetrate the larval cuticle before colonizing the hemocoel. *Culicinomyces clavisporus*, an aquatic Sordariomycete related to *M. anisopliae*, produces conidia which, following ingestion by the larvae, adhere to and penetrate through the chitinous wall in the fore- and hindgut [23].

In conidia of *M. anisopliae*, *Mad1* expression in the presence of mosquito larvae suggest the fungus had responded to cuticular cues in a similar manner to a terrestrial host despite its failure to adhere through passive hydrophobic forces. *Mad1* was up regulated particularly inside the gut of live insects suggesting that the fungus had perceived additional cues. *Mad2* was not upregulated in the same manner, however, expression of both these genes was significantly higher in the gut of dead insects and fecal pellets possibly due to nutritional stress which would also explain why no germ tubes were produced. Nutrient starvation is known to up regulate *Mad2* [9]. The concomitant upregulation of *Mad1*, *Mad2*, *Pr1* and *Pr2* by the ungerminated conidia of *M.*

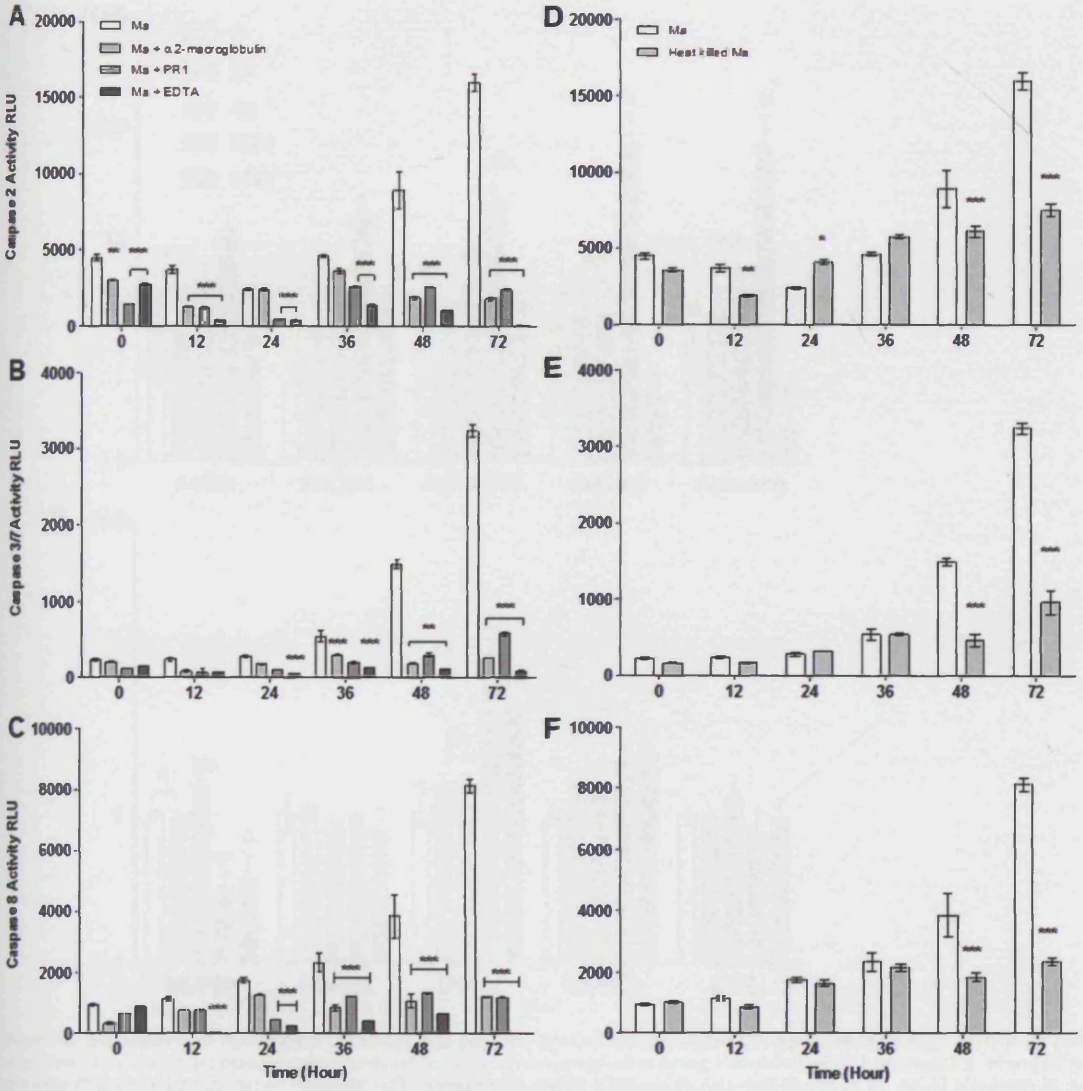

Figure 7. Caspase activity in *Ae. aegypti* exposed to *M. anisopliae* and protease inhibitors. Caspase activity in response to *M. anisopliae* with and without protease inhibitors (A–C) and exposed to live and heat killed conidia (D–F). Data was presented as mean (± SEM) (two-way ANOVA, ***-$p<0.001$, **-$p<0.01$, *-$p<0.05$, compared with *M. anisopliae* uninhibited control).
doi:10.1371/journal.pone.0081686.g007

anisopliae suggest that the fungus is mounting a response to infect but fails to progress due to the lack of stimuli normally present in the terrestrial arthropod host. Conidia of *M. anisopliae* are readily ingested by mosquito larvae with some workers suggesting this to be the main route of infection [3]. Our studies show that the conidia failed to produce germ tubes and penetrate the gut wall, nor do they cause inflammation of the midgut epithelium or interfere with gut function, allowing the insect to remove conidia in compact fecal pellets at least until death. Toxins, particularly destruxins, have been implicated as the cause of mosquito larval death, produced by ungerminated conidia on the cuticle, inside the gut or released following digestion of *M. anisopliae* conidia [13,24,25]. In our study, no destruxins were detected in *Ae. aegypti* larvae that had ingested conidia of *M. anisopliae* 4556, even though this strain is known to produce destruxins, thus discounting these compounds as the cause of death.

Extracellular proteases of *M. anisopliae*, with the exception of metalloproteases, contribute significantly to *Ae. aegypti* larval mortality which appears to be mediated through stress induced apoptosis. *Pr1* and *Pr2* were expressed during passage through the insect gut, in the fecal pellet and recently killed larvae. Chicken egg white, an inhibitor of *Pr1*, significantly improved survival of *Ae. aegypti* larvae but not to the same extent as the global protease inhibitor, α2 macroglobulin, suggesting that several proteases working in concert were contributing to larval mortality. Not all proteases contribute to mortality since inhibition of *Pr2* did not improve survival (unpublished). EDTA treated insects posed an anomaly as these exhibited low caspase activity but high larval mortality. It is possible that EDTA, besides inhibiting metalloproteases, interfered with cation dependent cellular processes such as signalling, homeostasis, and caspase activation [25] which would exacerbate the stress caused by the fungal pathogen. This is clearly an area for further

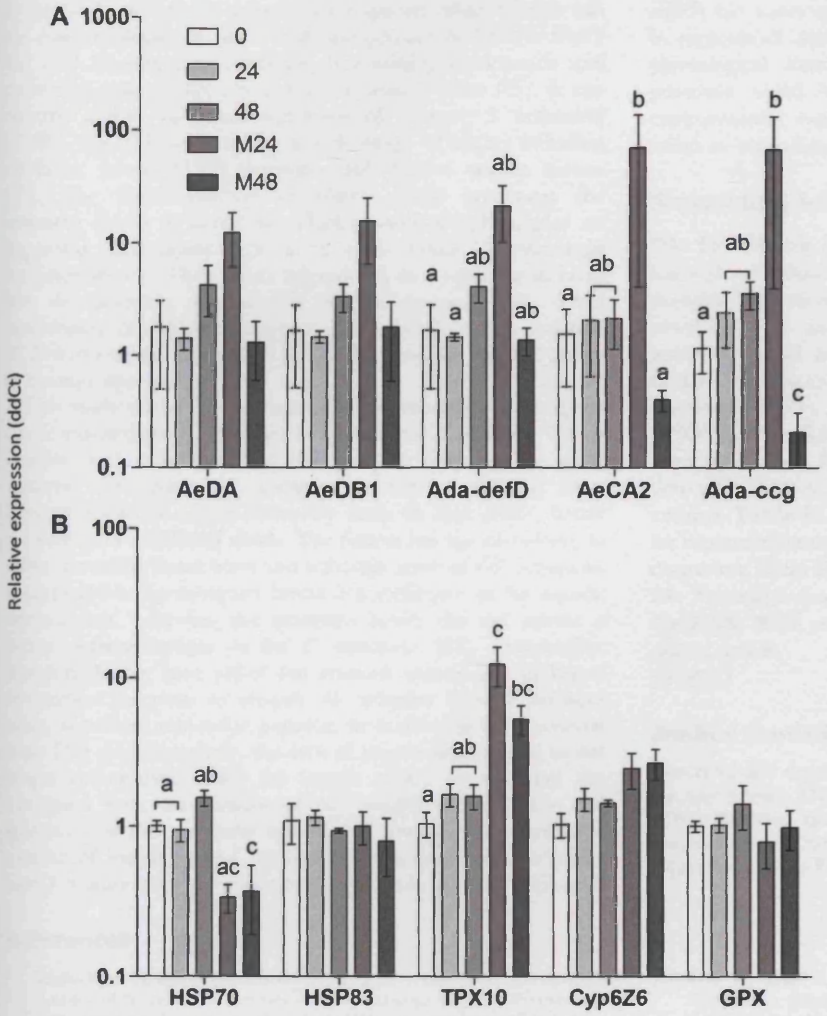

Figure 8. Expression of *Ae. aegypti* antimicrobial peptide and stress management genes during exposure to *M. anisopliae*. Expression of antimicrobial and stress management genes on *Ae. aegypti* were analysed in larvae inoculated with *M. anisopliae* 0, 24 and 48 hr pi by quantitative real time PCR. Antimicrobial genes included; *AeDA* (Defensin A), *AeDB1* (Defensin B), *Ada-defD* (Defensin D), *AeCA2* (Cecropin A), *Ada-ccg* (Cecropin G) and stress management genes; *HSP70* (Heatshock protein 70), *HSP83* (Heatshock protein 83), *TPX10* (Thiol peroxidase 10), *Cyp6Z6* (Cytochrome P450), *GPX* (Glutathione peroxidase). Data was presented as mean (± SEM) means with different letters are statistically different (two-way ANOVA)
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investigation. Mortality in heat killed conidia and untreated control was statistically similar suggesting that extracellular proteases contributed significantly to larval mortality. Proteases will accrue with time as more conidia pass through the gut. The high survival of larvae in the presence of protease inhibitors and heat killed conidia show that death does not arise due to blockage of the mouthparts or breathing apparatus as suggested by some previous studies [11].

Upregulation of *A. aegypti* antimicrobial peptide (AMP) genes, peaking 24 hr pi, is the typical immune response of insects exposed to pathogens, stress or injury [18]. The mosquito larvae did not mount a strong AMP mediated defense response to *M. anisopliae;* the only significant activity was downregulation of cecropins A and G, 48 hr pi, which coincided with a significant increase in caspase activity and larval mortality. Indeed, mortality appeared to be correlated with caspase activity. Activities of initiator (caspases 2 and 8) and effector (caspases 3 and 7) caspases increased with time suggesting an increasing number of cells undergoing apoptosis. Once a threshold of dead cells had been reached the insect would be unable to sustain life functions resulting in death. Apoptosis is known to be induced by oxidative damage either from oxygen free radicals or hydrogen peroxide directly or from their generation in cells by injurious agents [26]. Insects, like many other organisms, actively produce reactive oxygen intermediates as signalling molecules to control processes such as, apoptosis, abiotic stress responses, and pathogen defense [18,26]. Cellular antioxidant mechanisms countering oxidative stress include soluble free radical scavenger molecules such as glutathione and enzymes like superoxide dismutases, catalases and peroxidases. Most of these enzymes were not elevated in *M. anisopliae* infected *Ae. aegypti* larvae, with the exception of glutathione-S-transferase, 48 hr pi but at 72 hr pi they had all fallen possibly due to insects being close to death. Expression of the stress management genes at the critical 48 hr pi was not as extensive as reported in terrestrial insects [26], presumably due to the mosquito larvae never encountering the

fungus and evolving an appropriate response. Most notable was the downregulation of *Hsp70* and upregulation of *TPX10*. *Hsp70* has vital housekeeping functions, maintaining homeostasis and protecting cells against thermal and oxidative stress [27]. It can directly inhibit apoptosis upstream of caspase 3 activation [27,28]. *Hsp70* is activated by a wide range of factors including cytokines, energy (ATP) depletion and reactive oxygen species [27]. The downregulation of *Hsp70* would predispose the mosquito larvae to apoptosis. Thiol peroxidases (TPx) play an important antioxidant role in a wide range of organisms including insects. They utilize thioredoxin as a substrate to carry out detoxification of reactive oxygen species [29]. Thiol peroxidases can inhibit apoptosis [29], therefore, upregulation of *TPX10* may be an attempt by the *M. anisopliae* infected larvae to contain apoptosis.

This study shows for the first time that mortality of mosquito larvae exposed to *M. anisopliae* is multifactorial. It is not due to invasion and colonisation of the host, as proposed by other workers, but entails *M. anisopliae* proteases triggering stress induced apoptosis which ultimately leads to host death, hence the verdict of accidental death. The fungus has the machinery to infect terrestrial insect hosts and although some of this apparatus is expressed in the mosquito larvae it is ineffective in the aquatic environment. Likewise, the mosquito larvae did not mount a strong defenseresponse as for *C. clavisporus* [23]. Presumably, mosquito larvae have either not evolved appropriate pathogen recognition receptors to identify *M. anisopliae* derived pathogenicity associated molecular patterns, as is the case for terrestrial hosts [30] or alternatively, the lack of success with regard to the fungal colonization limits the insects ability to recognise the attempted infection. Failure of *M. anisopliae* to colonize and sporulate on the mosquito host would result in no horizontal transfer of inoculum and for biocontrol management strategies would require regular application unlike the aquatic pathogens which can cause epizootics because of their ability to reproduce in mosquitoes and other aquatic invertebrates [3]. Genetic or physiological manipulation of *M. anisopliae* to over produce proteases could accelerate larval mortality and pose little environmental risks because of the inability of the fungus to infect or reproduce in mosquito larvae.

Supporting Information

File S1 Figure S1-S2, Table S1, Text S1-S5. Figure S1. Limited antioxidant activity in mosquito larvae exposed to *M. anisopliae*. Activity of mosquito larvae exposed and not exposed to conidia of *M. anisopliae*. (A) Reactive oxygen species (ROS) generation, and activity of (B) MDA (lipid peroxidation), (C) catalase, (D) Superoxide dismutase (SOD),and (E) glutathione-S-transferase (GST). Data presented as mean ± (SEM) (Two-way ANOVA, **-$p<0.01$, *-$p<0.05$ compared with uninfected for the same time point). **Figure S2.** LCMS chromatogram showing no detectable *Metarhizium anisopliae* destruxin in *Aedes aegypti* larval extracts. **Table S1.** *Metarhizium anisopliae* and *Aedes aegypti* loci used for expression analysis. **Text S1.** Cryo-SEM. **Text S2.** Analysis of destruxins. **Text S3.** Enzyme and enzyme inhibitor assays. **Text S4.** Transcript quantification of insect and fungus-derived genes. **Text S5.** ROS production, lipid peroxidation and antioxidant system activity.
(DOCX)

Author Contributions

Conceived and designed the experiments: TMB BPJG DCE. Performed the experiments: TMB BPJG CG TGGM JWDT JP ED AA IMD IGJ MWP. Analyzed the data: TMB BPJG ED IMD DCE. Contributed reagents/materials/analysis tools: TMB TGGM ED EQM. Wrote the paper: TMB BPJG ED DCE.

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