EVALUATION OF A FUNGAL ENTOMOPATHOGEN FOR MALARIA MOSQUITO CONTROL, WITH AN EMPHASIS ON TEMPERATURE

A Dissertation in
Entomology

by

Rebecca Heinig

© 2015 Rebecca Heinig

Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

May 2015
The dissertation of Rebecca Heinig was reviewed and approved* by the following:

Matthew B. Thomas  
Professor of Entomology  
Dissertation Advisor  
Chair of Committee

Andrew F. Read  
Professor of Biology and Entomology

Jason L. Rasgon  
Associate Professor of Entomology and Disease Epidemiology

Tracy Langkilde  
Associate Professor of Biology

Gary W. Felton  
Professor of Entomology  
Head of the Department of Entomology

*Signatures are on file in the Graduate School
ABSTRACT

Malaria remains one of the most significant vector-borne diseases worldwide. One of the cheapest, most effective ways of controlling malaria is by targeting the anopheline mosquitoes which transmit it. This is primarily accomplished via the use of chemical insecticides, which may be integrated into bed nets or applied as spray treatments to indoor walls. However, wild mosquitoes are rapidly evolving resistance to all four classes of chemicals currently approved for indoor use, spurring research to identify new control technologies.

Fungal entomopathogens are one such tool. Fungal spores act as contact pesticides and may be deployed in a manner similar to chemicals. While chemicals are selected to kill or knock down mosquitoes within hours of exposure, fungal entomopathogens may require a week or more to kill exposed mosquitoes. However, the parasite which causes malaria also requires at least nine days to mature within the vector before it can be transmitted to a new host, and both this extrinsic incubation period and mosquito survival time following fungal exposure are strongly dependent on environmental temperature. Because little was known about the relationship between temperature and fungal virulence (defined here as rate of kill), we evaluated virulence of the candidate fungal biopesticide *Beauveria bassiana* in anopheline mosquitoes across the range of temperatures relevant for malaria transmission. We found that the fungus consistently killed the vast majority of mosquitoes before they would have been expected to begin transmitting malaria (i.e. in a shorter period of time than the malarial extrinsic incubation period). This result was robust across different diets (mosquitoes fed exclusively on sugar vs. blood fed), species (*Anopheles stephensi* and *Anopheles gambiae*) and levels of diurnal temperature variation (±0°C, ±6°C). We also evaluated the fungus in mosquitoes infected with malaria but found no evidence of an effect of fungal co- or superinfection on malaria infection parameters or on mosquito mortality rate. Theoretical modeling predicted that realistic levels of fungal coverage could deliver significant reductions in infectious adult mosquito population density. When combined with mean temperature data from across Africa, our results suggested that, if fungus was deployed at sufficiently high coverage levels, temperature would have a limited impact on the ability of this biopesticide to suppress malaria-infectious mosquito populations.

We parameterized our model based on data collected at constant temperatures. However, temperatures in the field tend to vary even over the course of a single day. Because mosquitoes are small-bodied ectotherms, their body temperature and the temperature of the pathogens they
carry will vary along with the temperature in their environment. Because organismal performance
tends to be nonlinerly related to temperature, it has long been recognized that performance rates
under constant temperatures may not be equivalent to those under fluctuating conditions, a
phenomenon known as the Kaufmann effect. While this is often accounted for by summing
incremental rates over the range of temperatures experienced, there are indications that this rate
summation technique does not always accurately predict the impacts of realistic levels of diurnal
temperature variation. We tested the accuracy of rate summation by measuring in vitro radial
growth rate of B. bassiana at constant temperatures to create a thermal performance norm curve.
We then used rate summation to predict the impacts of diurnal temperature variation and tested
these predictions empirically. We found that the accuracy of rate summation depended both on
mean temperature and on the patterns of diurnal temperature variation. At moderate levels of
variation, rate summation was accurate, but at high levels, rate summation overestimated
performance at the warm end of the curve. We also found that initial growth conditions
significantly affected estimates of the critical thermal maximum, or the temperature above which
no growth should occur. Acute exposure to temperatures above the critical thermal maximum
paused growth in a time- and temperature-dependent manner, but once growth resumed, growth
rate was not significantly different from controls in the majority of treatments.

Based on these results, we concluded that fungal biopesticides have the potential to
significantly reduce infectious mosquito density and that, based on the results of our rate
summation experiment, our modeling predictions regarding malaria mosquito control likely
underestimate fungal impact under more realistic fluctuating temperature conditions.
# TABLE OF CONTENTS

List of Figures ........................................................................................................ v

List of Tables ........................................................................................................ vii

Acknowledgements ................................................................................................ xi

Chapter 1 Introduction ............................................................................................. 1

Chapter 2 Evaluating the potential for biocontrol of malaria mosquitoes under diverse environmental conditions ......................................................... 7

  Abstract ............................................................................................................... 7
  Introduction ......................................................................................................... 8
  Methods ............................................................................................................ 10
  Results ............................................................................................................ 13
  Discussion ....................................................................................................... 15
  Notes ............................................................................................................... 19

Chapter 3 Prospective biocontrol of malaria vectors using fungal entomopathogens ........... 27

  Introduction ..................................................................................................... 27
  Eaves tube design and proof of concept .......................................................... 27
  Predicted efficacy of fungus delivered via eave tubes under different environmental conditions ................................................................. 28
  Results and Discussion .................................................................................. 30
  Notes ............................................................................................................... 32

Chapter 4 Interactions between a fungal entomopathogen and malaria parasites within a mosquito vector ...................................................................................... 40

  Abstract ......................................................................................................... 40
  Introduction .................................................................................................... 41
  Methods ......................................................................................................... 44
  Results ......................................................................................................... 48
  Discussion .................................................................................................... 49
  Conclusion ................................................................................................... 52
  Notes ............................................................................................................ 52

Chapter 5 Evaluating rate summation for predicting the influence of daily temperature variation ........................................................................................................ 59

  Abstract ......................................................................................................... 59
  Introduction ................................................................................................... 60
  Methods ....................................................................................................... 61
LIST OF FIGURES

Figure 1.1. Predictions of Jensen’s inequality for thermal performance curves ......................5

Figure 2.1. Cumulative proportional survival of adult An. stephensi ..................................20

Figure 2.2. Fungal virulence against An. stephensi across a range of temperatures ..............21

Figure 2.3. Virulence of the fungal biopesticide against An. gambiae .................................22

Figure 2.4. Virulence of the fungal biopesticide against blood fed An. stephensi ...............23

Figure 2.5. Cumulative proportional survival of adult An. stephensi mosquitoes exposed to different fungal AR, mean temperatures and diurnal temperature ranges........24

Figure 2.6. Influence of diurnal temperature variation on fungal virulence against An. stephensi .................................................................................................................25

Figure 2.7. Predicted impact of fungal biopesticide on malaria transmission potential at different mean temperatures .................................................................26

Figure 3.1. Eave tubes installed in a house ........................................................................33

Figure 3.2. Impacts of transient exposure to eave tubes on A. stephensi mortality rates ......34

Figure 3.3. Impact of eave tubes on mosquito mortality rates under semi-field conditions...35

Figure 3.4. Predicted reduction in infectious mosquito density at various temperatures for two daily probabilities of fungal infection (c) ........................................36

Figure 3.5. Predicted impact of fungal biopesticides assuming a daily likelihood of infection (c) of 0.16 ..............................................................37

Figure 3.6. Predicted impact of fungal biopesticides assuming a daily likelihood of infection (c) of 0.025 ..............................................................38

Figure 3.7. Predicted impact of fungus-treated eave tubes on malaria transmission parameters. ....................................................................................................................39

Figure 4.1. Cumulative proportional survival of adult mosquitoes in the Plasmodium yoelii experiment .................................................................53

Figure 4.2. Plasmodium yoelii oocyst intensity (number of oocysts per midgut) ...............54

Figure 4.3. Number of Plasmodium yoelii sporozoites per oocyst (10^3) by treatment .......55

Figure 4.4. Cumulative proportional survival of adult mosquitoes in the Plasmodium falciparum experiment .................................................................56
Figure 5.1. Example thermal performance curve ................................................................. 71
Figure 5.2. Candidate fitted thermal performance curves .................................................. 72
Figure 5.3. Observed and predicted radial growth rates under constant and fluctuating conditions .................................................................................................................. 74
Figure 5.4. Impact of temperature on conidial germination rate ..................................... 75
Figure 5.5. Impact of temporary exposure to 26°C on radial growth rate at high temperatures ......................................................................................................................... 76
Figure 5.6. Impact of acute exposure to 34 or 36°C on radial growth ............................. 77
Figure 5.7. Impact of acute exposure to 34 or 38°C on radial growth .............................. 78
Figure 5.8. Impact of acute exposure to 40 or 42°C on radial growth ............................. 79
Figure 5.9. Experimental thermal regimes in which temperatures exceeded the estimated CT\text{max} ........................................................................................................................................ 81
Figure A.1. Example temperature readouts from fluctuating temperature treatments ...... 93
Figure A.2. Schematic of SEI model .................................................................................... 94
Figure A.3. Graphical evaluation of Weibull parameter fits of high application rate survival curves .................................................................................................................................... 97
Figure A.4. Graphical evaluation of Weibull parameter fits of low application rate survival curves ....................................................................................................................................... 98
Figure A.5. Cumulative proportional survival of An. gambiae .......................................... 100
Figure A.6. Cumulative proportional survival of blood fed adult An. stephensi .......... 102
Figure A.7. Predicted impact of fungal biopesticide on infectious mosquito density .......... 105
Figure A.8. Impact of variation in parameter estimates on proportion reduction in infectious mosquito density at the high fungal application rate .............................................. 106
Figure A.9. Impact of variation in parameter estimates on proportion reduction in infectious mosquito density at the low fungal application rate ........................................ 107
Figure A.10. Impact of parameter variation on equilibrium total infectious mosquito density .............................................................................................................................. 108
Figure A.11. Impact of EIP (above) and background mortality (μ, below) variation on equilibrium total infectious mosquito density ........................................................................ 109
Figure A.12. Predicted proportion reduction in infectious mosquito density at different levels of variation in extrinsic incubation period ................................................................. 110

Figure B.1. Parasite rate (PR) in the absence of fungus (“no fungus”) or in the presence of fungus with $c=0.16$ estimated based on the relationship described by Smith et al. (2005) ........................................................................................................................................ 112

Figure C.1. Summary of mean ($\pm$ standard error) daily percent mortality rate ......................... 117

Figure D.1. Impact of time interval variation on rate summation estimates ................................. 118
LIST OF TABLES

Table 4.1. Summary of *P. yoelii* malaria infection parameters........................................57

Table 4.2. Summary of *P. falciparum* sporozoite prevalence........................................58

Table 5.1. Thermal performance models and fit results..................................................73

Table 5.2. Radial growth rate following acute exposure to 40 or 42°C...............................80

Table A.1. Model parameters and values.............................................................................95

Table A.2. Summary of Weibull rate ($\mu_f$) and shape ($\beta$) parameter estimates ..........96

Table A.3. Summary of *An. stephensi* survival following fungal exposure. ....................99

Table A.4. Summary of *An. gambiae* survival following fungal exposure.......................101

Table A.5. Summary of blood fed *An. stephensi* survival ............................................103

Table A.6. Summary of the impact of diurnal temperature variation on *An. stephensi* survival ......................................................................................................................104

Table C.1. Detectable effect size estimates for *Plasmodium yoelii* oocyst prevalence and *Plasmodium falciparum* sporozoite prevalence analyses ........................................115

Table C.2. Power estimates for analyses of *Plasmodium yoelii* oocyst intensity and number of sporozoites per oocyst.................................................................116
ACKNOWLEDGEMENTS

Graduate school has been an incredible journey, and there are a number of people who have helped me along the path. I’d like to thank my supervisor, Matt Thomas, for giving me the opportunity to work on such an incredible project and for helping me see the forest whenever I got lost in the trees. I’d also like to thank the other members of my committee: Andrew Read, who told me to stop asking for permission; Jason Rasgon, who always asked insightful questions; and Tracy Langkilde, whose enthusiasm reminded me why I chose to pursue this research. I couldn’t have chosen a better group of traveling companions than the members of the Thomas and Read labs, who kept my spirits up on cloudy days. In particular, I’d like to thank our wonderful technicians Mark Kennedy, Derek Sim and Matt Jones; our fungus wranglers Nina Jenkins and Giovanni Bellicata; and our amazing insectary manager, Janet Teeple, who raised tens (hundreds?) of thousands of mosquitoes for these experiments and still came to work with a smile every day. In addition, I’d like to thank Rob Anderson and Krijn Paaijmans, who served as mentors early in my graduate career.

I’ve been fortunate to be a member of two wonderfully supportive communities at Penn State, the Department of Entomology and the Center for Infectious Disease Dynamics. I’d like to thank the members of the EGSA and the CGSA (particularly Amalie McKee and Lauren Quevillon) for keeping me sane; office staff members Monica Arismendi, Luanne Weatherholtz, Ellen Johnson and Marcia Kerschner for keeping things running behind the scenes; and the members of my writing club for keeping me on target as I wrote this dissertation. I’d also like to thank Ottar Bjørnstad and his students Megan Greischar and Lindsay Beck-Johnson for dragging me kicking and screaming into disease modeling.

Finally, I’d like to thank my family and friends (particularly Alicia Kim, Jade Pong, Alan Chen, Alene Onion and Gary Ogata) for reminding me that there’s a whole big world outside my office.
Chapter 1

Introduction

Malaria continues to kill an estimated 1.2 million people per year and is considered one of the most important vector-borne diseases worldwide (Murray et al. 2012). The Plasmodium parasites which cause human malaria are vectored exclusively by female anopheline mosquitoes, which ingest infective sporozoites while feeding on infectious vertebrate hosts. The majority of current control methods rely on targeting vector mosquitoes using chemical insecticides, which are usually applied to bed nets or to interior walls where mosquitoes may rest following a bloodmeal. Because these insecticides are used in close proximity to humans, vertebrate toxicity is a concern. Only four classes of chemicals are currently approved for indoor use, of which only one is recommended for use in bed nets (World Health Organization 2013a; b). However, insecticide-resistant mosquitoes have been detected in 64 countries (World Health Organization 2013b), and multi-insecticide resistance is becoming increasingly common (Yewhalaw et al. 2011; Mitchell et al. 2012). To maintain or improve malaria control, novel intervention strategies are urgently needed.

Fungal entomopathogens: a promising technology (Chapters 2 and 3)

There have been a number of recent studies investigating the use of entomopathogenic fungi for malaria mosquito control (Blanford et al. 2005, 2011; Farenhorst et al. 2010; Mnyone et al. 2012). The most common fungi used in insect pest control are generalist members of the Metarhizium and Beauveria genera (Charnley & Collins 2007), which, in addition to being successfully deployed to control locusts in Australia (Lomer et al. 2001; Thomas & Kooyman 2004), are effective against a variety of nuisance insects, including bed bugs (Barbarin et al. 2012), house flies (Acharya et al. 2015) and biting midges (Ansari et al. 2011). Like common chemical insecticides, fungal conidiospores act as contact pesticides, attaching to female mosquitoes when they land on treated surfaces. The spores then germinate and utilize a combination of proteolytic enzymes and mechanical pressure to force a penetration peg through the insect cuticle and to invade the body cavity (the hemocoel) (Charnley & Collins 2007). The
host dies several days later as a result of toxic metabolites released during fungal proliferation (Charnley & Collins 2007) and/or nutrient depletion (Clarkson & Charnley 1996). Fungal spores’ mode of action means that they can be deployed in a variety of ways: as residual sprays (Blanford et al. 2005, 2011, 2012a), coatings for resting containers (Farenhorst et al. 2008) or treatments for eave curtains, panels or baffles (Mnyone et al. 2012). Fungi are also effective against insecticide-resistant mosquito strains (Farenhorst et al. 2009; Blanford et al. 2011) and act synergistically when combined with chemical insecticides (Farenhorst et al. 2010; Paula et al. 2011a).

However, while chemicals are generally selected to kill or knock down mosquitoes within hours of contact, fungi may require up to a week or more to kill exposed individuals (Blanford et al. 2005; Scholte et al. 2005). While this slow action has spurred research on genetic modifications to increase virulence (defined here as kill rate) (Fang, Azimzadeh & St Leger 2012), it is an attractive feature from the standpoint of resistance evolution. Chemical insecticides’ rapid action exerts tremendous selective pressure on mosquito populations, but since mosquitoes infected with fungus realize a portion of their potential lifetime fecundity (Mouatcho et al. 2011; Blanford et al. 2011; Darbro et al. 2012), resistance to fungi would be expected to evolve more slowly, if at all (Thomas & Read 2007; Read, Lynch & Thomas 2009; Koella et al. 2009; Lynch et al. 2012). Fungal infection is also associated with a variety of sublethal effects such as reduced feeding propensity (Blanford et al. 2005, 2011; Scholte, Knols & Takken 2006; Howard et al. 2010; George et al. 2011), decreased sensitivity to host odors (George et al. 2011), increased metabolic rates (Blanford et al. 2011), decreased flight ability (Blanford et al. 2011) and reduced predator evasion behavior (Arthurs & Thomas 2001). Taken together, these side effects would be expected to increase background mortality risk while simultaneously decreasing the likelihood that a mosquito would feed on (and potentially infect) a vertebrate host.

It is also possible to block malaria transmission even if mosquitoes are not killed immediately following an infectious feed. After malaria parasites (sexual gametocytes) are ingested by a female mosquito, they travel to her midgut, mate and form oocysts in the midgut wall. Following an extended period of asexual replication, the oocysts burst, releasing infective sporozoites into the hemocoel. The infective sporozoites then travel to the mosquito’s salivary glands to be injected into a new host when the mosquito feeds. This process takes at least nine days (Detinova 1962; Paaaijmans, Read & Thomas 2009; Mordecai et al. 2013), providing slow-acting fungal entomopathogens with a window of opportunity. As long as the mosquito is dead (or incapacitated) before the end of this malarial extrinsic incubation period (EIP), transmission will not occur.
There is one problem: fungal virulence is sensitive to both mean temperatures and diurnal temperature variation (Thomas & Blanford 2003; Kikankie et al. 2010), and this thermal sensitivity can have significant impacts on efficacy. For example, Blanford and Thomas (2001) found that *Metarhizium anisopliae* fungus killed 90% of exposed locusts within 10 days at 30°C. But if locusts were allowed to behaviorally fever at higher temperatures (up to 46°C), their mortality rate was equivalent to that of untreated controls. While there is currently no evidence for behavioral fevering in mosquitoes (Blanford, Read & Thomas 2009), mean temperatures in malarious regions range from 15 to more than 30°C. In addition, these temperatures can fluctuate by upwards of 20°C within the course of a single day (Blanford et al. 2013). However, malaria parasite development rate also varies with temperature (Detinova 1962; Paaijmans, Read & Thomas 2009; Mordecai et al. 2013). The relationships among malaria development rate, fungal virulence and ambient temperature could therefore have significant impacts on the ability of a fungal entomopathogen to control disease transmission.

Very little was known about the relationship between temperature and fungal performance in anopheline mosquitoes across the thermal spectrum relevant for malaria transmission, so in Chapter 2, we evaluated the candidate fungal biopesticide, *Beauveria bassiana* (Bals.-Criv.) Vuill., in the Asian malaria mosquito, *Anopheles stephensi*. We then utilized an age-structured SEI model (Hancock, Thomas & Godfray 2009) to simulate more realistic scenarios in which mosquitoes did not always become infected with the fungus and the malaria at the same time.

There are a variety of models of malaria infection dynamics in mosquitoes, most of which are based on the assumptions of the model developed by Ronald Ross in the early 1900s (Ross 1908, 1911a; b) and improved upon by George MacDonald in the 1950s (MacDonald 1952, 1957). This model tracks malaria infection dynamics in susceptible, infected and infectious classes in both the human and mosquito populations. It also assumes homogeneous mixing between all human and mosquito classes but does not consider mosquito immature stages because they do not transmit malaria.

We chose to use the modeling framework described by Hancock et al. (2009). Like the Ross-MacDonald models, it does not consider mosquito immature stages. It also assumes that malaria infection rates are constant in the human population, which would be a reasonable assumption in the short-term due to the differing timescales of the human and mosquito infections. We selected this modeling framework in part because it allowed us to use a fixed delay to describe the malarial EIP (vs. allowing “instantaneous” maturation of some parasites) and because, by ignoring the larval stages, it required us to make fewer assumptions about mosquito development.
under different temperatures. It also allowed us to model mortality due to fungal infection based on a two-parameter Weibull distribution. Although more computationally complex than the standard exponential distribution, the Weibull more accurately describes the patterns of mortality we observed in our experimental assays. In Chapter 3, we expand on these results by using a GIS to explore spatial and temporal variation in expected fungal efficacy in Africa.

**Interactions between fungus and malaria within the vector (Chapter 4)**

The majority of experiments informing Chapters 2 and 3 were performed in mosquitoes that were not infected with malaria. However, malaria parasites cause significant damage during their invasion of the midgut (Ferguson & Read 2002a). Mosquitoes respond by upregulating a suite of immune factors, including the Toll or IMD pathway (Dong et al. 2006; Garver, Dong & Dimopoulos 2009; Garver et al. 2012) and melanization (Blandin et al. 2004). During the oocyst stage, there is evidence that the malaria parasite acquires host resources to support rapid sporozoite replication (Beier et al. 1990), and once the sporozoites enter the hemocoel, they are again attacked by the immune system (Hillyer, Barreau & Vernick 2007). Fungal infections also trigger a number of immune factors, including cellular responses (Hung & Boucias 1992; Hajek & St. Leger 1994), humoral melanization (Hajek & St. Leger 1994; Yassine, Kamareddine & Osta 2012) and the Toll and JAK-STAT pathways (Dong et al. 2012). The competing demands of these two infections could have downstream effects on fungal proliferation, malaria development or both.

Co-infecting pathogens often interact in unpredictable ways. For example, *B. bassiana* suppresses dengue virus in vector mosquitoes (Dong et al. 2012; Garza-Hernández et al. 2013), while *Wolbachia* bacterial infections can either enhance (Hughes et al. 2012; Murdock et al. 2014) or suppress (Kambris et al. 2010; Hughes et al. 2011; Bian et al. 2013) malaria infections. In our study system, previous work suggested that mosquitoes co-infected with *B. bassiana* fungus and a different rodent malaria strain (*Plasmodium chabaudi*) might experience increased mortality late in infection (Blanford et al. 2005). We followed up on this observation by evaluating interactions between *B. bassiana* co- and superinfections and malaria parasites at either in the early (oocyst) stages of infection or in the later (sporozoite) stages.
Impacts of thermal variation on fungal radial growth rate (Chapter 5)

In Chapters 2 and 3, we did not account for realistic diurnal temperature variation in our modeling framework, even though both malaria (Paaijmans et al. 2010; LaPointe, Goff & Atkinson 2010) and mosquito (Paaijmans et al. 2010, 2013) traits are sensitive to diurnal temperature variation. We based our choice in part on Jensen’s inequality, a property of nonlinear relationships (Ruel & Ayres 1999). In terms of thermal performance, Jensen’s inequality states that, for equivalent mean temperatures, variation over a concave curve will reduce performance, variation over a convex curve will increase performance and variation where the relationship is linear will have no impact (Figure 1.1). Within the entomological literature, this is also known as the Kaufmann or rate summation effect (Worner 1992). Predictions can be made about quantitative impacts of fluctuating temperatures on performance by summing incremental rates over time to generate a composite rate for the thermal regime of interest, a technique known as rate summation (Worner 1992). Based on rate summation across our fungal virulence curve and Mordecai et al.’s (2013) malaria EIP curve, we predicted that, under fluctuating temperature conditions, malaria development rate would decrease more quickly than fungal virulence.

![Rate vs Temperature](image)

Figure 1.1. Predictions of Jensen’s inequality for thermal performance curves. The blue curve represents the thermal reaction norms (e.g. performance at constant temperatures), and black arrows represent predicted impacts of fluctuating temperatures.

Rate summation assumes there are no carryover effects between time points, i.e. the rate at a given time point is determined exclusively by temperature during that time point. While this may be true for certain portions of the curve, there is growing evidence that it may not be a valid assumption when temperatures approach or exceed the thermal maximum (i.e. the x-intercept at
the warm end of the curve) (Klass, Blanford & Thomas 2007a; Niehaus et al. 2012; Arrighi et al. 2013; Paaijmans et al. 2013). In Chapter 5, we test the assumptions of rate summation by evaluating in vitro radial growth rate of *B. bassiana* under a variety of different thermal regimes.

Finally, in Chapter 6, we summarize the results of the previous chapters and discuss possible avenues for future research.
Chapter 2

Evaluating the potential for biocontrol of malaria mosquitoes under diverse environmental conditions

Abstract

The effectiveness of conventional malaria vector control is being threatened by the spread of insecticide resistance. Biopesticides utilizing naturally-occurring insect-killing fungi could provide biological alternatives to chemical insecticides. Numerous lab studies have shown that isolates of fungal pathogens such as Beauveria bassiana can infect and kill adult mosquitoes, including those resistant to chemical insecticides.

Unlike chemical insecticides, fungi may take up to a week or more to kill mosquitoes following exposure. This slow speed of kill can still reduce malaria transmission because the malaria parasite itself takes several days to complete its development within the mosquito. However, both fungal virulence and parasite development rate are strongly temperature dependent so it is possible that efficacy of a biopesticide could vary across different transmission environments.

To evaluate this issue we examined virulence of a candidate fungal isolate against two key malaria vectors across temperatures from 10-34°C. Regardless of temperature, the fungus killed more than 90% of exposed mosquitoes before the predicted end of the malarial extrinsic incubation period, a result that was robust to realistic diurnal temperature variation.

To further explore this result we incorporated temperature sensitivities of a suite of mosquito, parasite and fungus life history traits that combine to determine malaria transmission intensity into a stage-structured malaria transmission model. The model predicted that at realistic levels of coverage, fungal biopesticides have the potential to deliver substantial reductions in the density of malaria infectious mosquitoes across all temperatures representative of malaria transmission environments.
Synthesis and Applications. Our study provides a prospective evaluation of the potential for biocontrol of adult malaria vectors, with a combined empirical and modeling approach that provides a bridge from lab to field conditions. Our results suggest that \textit{B. bassiana} could be a potent tool for malaria control and support further development of fungal biopesticides to manage infectious disease vectors.

Introduction

Despite substantial control efforts, malaria remains one of the most important vector-borne diseases of humans worldwide (World Health Organization 2013b). Recent years have seen a number of studies exploring the potential use of insect-killing fungal pathogens to target malaria’s adult mosquito vectors (Blanford \textit{et al.} 2005, 2011; Farenhorst \textit{et al.} 2010; Mnyone \textit{et al.} 2012). Entomopathogenic fungi have a number of features which make them particularly well-suited for malaria control. They infect mosquitoes on contact and so lend themselves to a variety of deployment strategies already in use for conventional insecticides, including treatments for cloth or netting (Scholte \textit{et al.} 2005; Farenhorst \textit{et al.} 2011; Mnyone \textit{et al.} 2012) and indoor residual sprays (Blanford \textit{et al.} 2011, 2012a). Fungal pathogens are also effective against mosquito strains that are resistant to available chemical insecticides (Farenhorst \textit{et al.} 2009, 2010; Blanford \textit{et al.} 2011), making them a viable alternative in areas where insecticide resistance is undermining vector control efforts (Yewhalaw \textit{et al.} 2011; Mitchell \textit{et al.} 2012). Finally, in contrast to chemical insecticides, which generally knock down or kill susceptible mosquitoes within 24 h of exposure (e.g. World Health Organization 2006), fungal biopesticides can take more than a week to kill exposed mosquitoes (Blanford \textit{et al.} 2005; Scholte \textit{et al.} 2005). Because these individuals continue laying eggs in the days following fungal exposure (Mouatcho \textit{et al.} 2011; Blanford \textit{et al.} 2011; Darbro \textit{et al.} 2012), fungal biopesticides are expected to reduce selection for new resistance phenotypes (Thomas & Read 2007; Read, Lynch & Thomas 2009). Thus, fungal pathogens could create opportunities for controlling resistant mosquitoes and/or developing novel insecticide resistance management strategies.

The extent to which such a slow-acting product like a fungal biopesticide can provide effective malaria control heavily depends on two factors (Hancock, Thomas & Godfray
2009; Read, Lynch & Thomas 2009; Koella et al. 2009; Lynch et al. 2012). First, it depends on the relationship between fungal virulence, defined here as the time required for the fungus to kill the insect, and the parasite extrinsic incubation period (EIP), which is the amount of time required for malaria to mature within its mosquito vector. *Plasmodium* parasites require an EIP of at least nine days before they can be transmitted to new hosts (Detinova 1962; Paaijmans, Read & Thomas 2009; Mordecai et al. 2013). This provides a window of opportunity for fungal action; as long as the mosquito dies before the malaria parasite completes its EIP, there will be no disease transmission.

Second, biopesticide coverage is important since this determines the probability, and hence timing, of fungal infection. Infecting a mosquito with fungus at or even before the point at which it acquires malaria parasites provides more time for the fungus to work, thus increasing the likelihood that mortality will occur before the end of the EIP.

Environmental temperature significantly affects fungal virulence (Thomas & Blanford 2003; Kikankie et al. 2010), and this can have a substantial impact on biocontrol efficacy. Studies on the biocontrol of locusts and grasshoppers, for example, showed that a fungal biopesticide could vary in performance from highly virulent (100% host mortality in about 7-10 days) to almost completely avirulent (negligible fungal-induced mortality after several weeks) depending on prevailing environmental conditions (Blanford & Thomas 2001; Klass, Blanford & Thomas 2007a; b). Similarly, the EIP of the malaria parasite is highly sensitive to environmental temperature, ranging from about nine to >50 days as temperatures deviate from the optimum towards the upper and lower thermal extremes (Detinova 1962; Paaijmans, Read & Thomas 2009; Mordecai et al. 2013). Given that the malaria transmission-blocking potential of a fungal pathogen is highly dependent on speed of kill relative to the EIP, these thermal sensitivities could significantly impact the effectiveness of this novel control tool. This is especially so given that malaria can be transmitted across a broad range of mean temperatures from around 18-34°C (Paaijmans, Read & Thomas 2009; Blanford et al. 2013). Additionally, daily fluctuations in temperature frequently exceed 10°C, and these short-term variations in temperature are further expected to influence mosquito, parasite and pathogen traits (Paaijmans et al. 2010, 2013; Murdock et al. 2012b; Murdock, Moller-Jacobs & Thomas 2013).

Although fungal virulence in mosquitoes has been evaluated in a small subset of temperatures, it is not yet known how virulence varies across the full thermal range
relevant for malaria transmission. There has also been no assessment of the implications of this variation for malaria control. To address this knowledge gap, we examined how temperature affected survival rates of adult *Anopheles stephensi* Liston and *Anopheles gambiae* Giles mosquitoes exposed to the candidate fungal biopesticide *Beauveria bassiana* (Bals.) Vuill. In combination with published estimates of temperature dependence in EIP, background mortality rate, biting rate and vector competence, we then utilized these novel empirical data in an age-structured model describing mosquito-malaria interactions (Hancock, Thomas & Godfray 2009) to explore how both probability of fungal infection (i.e. effective coverage) and environmental temperature might impact the potential of fungal treatments to reduce malaria transmission. We found that fungal biopesticides dramatically reduce the density of malaria-infectious mosquitoes irrespective of temperature due to differences in the thermal sensitivities between fungal virulence and the EIP of the malaria parasite. The empirical data together with the theoretical predictions provide strong support for the further development of this prospective biocontrol technology.

**Methods**

**Preparation of exposure substrates.** We simulated spray treatments on clay/mud walls typical of traditional village hut dwellings by using an airbrush to apply fungal suspensions to white earthenware clay tiles (World Health Organization 2006; Blanford *et al.* 2011, 2012a). *Beauveria bassiana* (isolate I93-825) conidia were suspended in oil (80% Ondina:20% Isopar M) at concentrations of either $10^7$ or $10^9$ conidia ml$^{-1}$ as described previously (Blanford *et al.* 2011). We applied the suspensions to tiles at a rate of 80 ml m$^{-2}$, resulting in application rates (AR) of approximately $8 \times 10^{10}$ conidia m$^{-2}$ for the high AR tiles and $8 \times 10^8$ conidia m$^{-2}$ for the low AR tiles. Control tiles were sprayed with blank oil mixture, and all of the tiles were air-dried overnight.

**Mosquito exposure to fungus.** Exposure assays were performed on 3-5 day-old female mosquitoes raised under standard insectary conditions at 26°C and followed standard WHO protocols for testing efficacy of residual insecticide applications (World Health Organization 2006). Groups of approximately 30 mosquitoes were aspirated into a
WHO cone in the center of each tile. After 30 min, the mosquitoes were aspirated into a nylon-covered cup with a cotton ball saturated with a 10% glucose-0.05% para-aminobenzoic acid (PABA) solution providing nutrition. Dead mosquitoes were removed daily, dried overnight at low humidity then placed in sealed tubes with wet cotton balls in a 26°C incubator. Sporulation was visually assessed approximately four days later to confirm cause of death. Unless stated otherwise, there were four replicate cups for each treatment. All exposures took place at 26°C.

We performed four sets of experiments. First, we evaluated the effect of different constant temperatures on fungal virulence. Following fungal exposure, An. stephensi mosquitoes were moved to incubators held at temperatures 10-34°C. Mosquitoes were evaluated at eleven temperatures in two separate mosquito cohorts. Group 1 included 20, 22, 24, 26, 28, 30 and 34°C temperature treatments, while group 2 included 10, 14, 18, 26 and 32°C treatments. The repeated 26°C treatments acted as internal controls.

Second, we tested whether the interaction between temperature and fungal virulence was consistent across mosquito species by performing the same assays in An. gambiae mosquitoes at 20, 26 and 32°C (representing cool, nominally optimum, and warm conditions).

Third, we repeated the assays using blood fed An. stephensi. Mosquitoes were fed on anaesthetized rats for 30 minutes just prior to fungal exposure. Fully engorged females were then exposed to tiles as described above and held at temperatures between 10-32°C. As an internal control, we also exposed a set of non-blood fed mosquitoes from the same cohort; this group was held at 26°C. There were three replicate cups per treatment.

Finally, we investigated the impact of realistic diurnal temperature variation on fungal virulence. Following fungal exposure, An. stephensi mosquitoes were placed in incubators either at constant temperatures (20, 26 or 32°C) or programmed to mimic realistic daily temperature patterns with a diurnal temperature range (DTR) of 12°C (e.g. 20±6°C) (Parton & Logan 1981).

**Analysis.** Survival rates within each experiment were compared using Kaplan-Meier log-rank tests in IBM SPSS for Windows (v. 20). We then calculated the mean time required for 90% mortality (LT90) as a metric for overall virulence and compared the results to the inverse of the temperature-dependent *P. falciparum* parasite development rate (PDR) curve described by Mordecai *et al.* (2013). In the fluctuating temperature
assay, we used rate summation (Liu, Zhang & Zhu 1995; Paaijmans, Read & Thomas 2009) to estimate the impact of the experimental DTR on malarial extrinsic incubation period.

**Modeling.** In the field, mosquitoes would be expected to encounter the fungus at different points in the EIP. To investigate how the daily probability of becoming infected with the fungus could impact the ability of fungal biopesticides to reduce malaria transmission, we utilized the age-structured model developed by Hancock et al. (2009) (Figure A.2). The model describes mosquito malaria infection dynamics as a susceptible-exposed-infected (SEI) process. Each class is further divided into mosquitoes that have been exposed to fungus and those that have not. Adult mosquitoes enter the susceptible class at a constant rate. They then enter the malaria exposed class at a rate equal to the product of the human bite rate, malaria transmission efficiency, and proportion of humans with transmissible malaria. Following a fixed delay equal to the length of the malarial EIP, the mosquitoes enter the infectious class. Background mortality occurs at a constant rate in all classes. In addition to becoming infected with the malaria parasite, the mosquitoes become infected by fungus at a rate determined by the daily probability of fungal infection. Malaria infection progress in fungus-infected mosquitoes is tracked in a parallel set of classes which experience additional mortality due to fungal infection modeled by the Weibull function:

\[
M_F(u) = \beta \mu_F (\mu_F u)^{\beta-1}
\]

where \(\mu_F\) and \(\beta\) are constants and \(u\) is the time since fungal infection.

We parameterized \(\mu_F\) and \(\beta\) for each temperature and fungal AR combination based on the results of the non-blood fed *An. stephensi* assays because they were the most comprehensive in terms of temperature coverage (Table A.2). The *P. falciparum* EIP, bite rate, transmission efficiency, and background mortality rate were parameterized based on the curves fit by Mordecai et al. (2013). We adjusted the daily probability of fungal infection \((c)\) to examine the impact of different levels of effective fungal coverage. Fungal efficacy was assessed based on the predicted proportional reduction in equilibrium total density of infectious mosquitoes. In addition, we performed a local sensitivity analysis (Hamby 1994) to evaluate how uncertainty in the parameter estimates affected the model predictions.
For this analysis, we ignored all prelethal effects of fungal infection on mosquito bite rate (see Discussion). Consistent with malaria vector control models, the proportion of humans with transmissible malaria was held constant (Killeen et al. 2007; Hancock, Thomas & Godfray 2009; Koella et al. 2009). We also held the adult recruitment rate constant because, while it affected the absolute densities in each class, it did not affect the relative densities reported here (Hancock, Thomas & Godfray 2009).

For additional methods, see Appendix A.

Results

Effects of mean temperature on fungal virulence and malaria parasite development. Fungal virulence in the non-blood fed An. stephensi varied dramatically with temperature (P<0.001, Figure 2.1). In the high application rate (AR) treatments, median survival times (MSTs) ranged from 4-14 days, with the most rapid mortality occurring at temperatures between 20-32°C. Mosquitoes exposed to the low AR survived roughly 1-2 days longer within each temperature treatment (group 1: χ²=592.622, P<0.001; group 2: χ²=631.402, P<0.001), but the relationship between temperature and survival time was qualitatively similar to that of the high AR treatments.

Temperature also significantly affected An. stephensi mortality rates in the control treatments (group 1: χ²=322.102, df=6, P<0.001; group 2: χ²=233.710, df=4, P<0.001), but here the pattern was quite different. Although mortality rates increased at temperatures >30°C, they remained quite low (<30%) at cooler temperatures.

The time required for the fungus-exposed An. stephensi to reach 90% mortality (LT₉₀) was shorter than the predicted P. falciparum extrinsic incubation period (EIP) across all temperatures at both ARs (Figure 2.2). The LT₉₀ curve was also broader than the EIP curve, indicating that fungal virulence was more robust to changes in mean temperature than malaria parasite development.

Patterns of survival in An. gambiae exposed to fungus were similar to those observed in An. stephensi, and the LT₉₀ remained consistently shorter than the estimated EIP for P. falciparum (Figure 2.3). However, control survival rates were lower in An. gambiae. By day 19, 100% of mosquitoes had died in the 26 and 32°C controls (vs. ≤20% and 66% for An. stephensi in the 26 and 32°C treatments, respectively), and 66% had died in the 20°C conditions.
controls (vs. 7% in \textit{An. stephensi}). The cause of this higher mortality is unclear but is comparable to the rates observed by others (Mnyone \textit{et al.} 2009, 2011) (but see Bayoh 2001).

The results of the blood fed experiment were similar to the previous experiments. Blood feeding was also associated with slightly higher mortality rates in the fungal treatment groups; at 26°C, MSTs were one day longer in the non-blood fed groups (high AR $\chi^2=8.57$, \(p=0.003\); low AR $\chi^2=12.14$, \(p<0.001\)). Mortality in the blood fed controls was also higher, particularly at cooler temperatures. However, the estimated LT$_{90}$ values were in line with those from the non-blood fed experiments (Figure 2.4).

\textbf{Additional influence of diurnal temperature variation.} Diurnal temperature variation had very little impact on fungal virulence, increasing MSTs by \(\leq 1\) day across the three temperatures (Figure 2.5). In contrast, diurnal temperature variation dramatically increased mortality in the control treatment groups at 32°C (92% vs. 21% survival on day 15). Rate summation predicted that a DTR of 12°C would slow parasite development at temperatures above 22°C leading to increases in EIP of up to five days. These predicted effects of temperature variation on malaria parasite growth exceeded the effects we observed on fungal virulence such that the addition of temperature variation increased the relative differences between EIP and LT$_{90}$ (Figure 2.6).

\textbf{Modeling control potential across different environments.} Because we predicted that temperature variation would affect malaria parasite development rate more than fungal virulence, we took the conservative approach of using mean temperatures alone to evaluate the control potential of the fungus in the transmission model. This assumption likely underestimates fungal impact but simplified use of temperature in the model for the other temperature-dependent traits for which the effects of temperature variation are unclear and rate summation is not necessarily appropriate.

In the absence of fungus, the predicted baseline infectious mosquito densities were consistent with the entomological inoculation rate data summarized in Mordecai \textit{et al.} (2013); both peaked at approximately 24°C. When fungus was included in the model, mosquito densities decreased at all temperatures. The predicted infectious mosquito density peak also shifted toward warmer temperatures, occurring at 26°C at low daily probabilities of fungal infection (\(c=0.04\)) and shifting further as infection probability
increased (e.g. 28°C when c=0.16). This peak shift would not have been predicted based on fungal virulence alone, which varied little between 26-30°C.

As expected, the relative reduction in density of infectious mosquitoes was sensitive to the daily probability of fungal infection (Figure 2.7). At the high AR, when c was 0.16, reduction in infectious mosquito density was >88% irrespective of temperature. At lower values of c, control was reduced and the influence of temperature became apparent. Control was greatest under cool conditions (18°C), declining to a minimum at 32°C and then rising again as temperature increased. For the low AR, the impact of fungus was marginally reduced but the qualitative relationships between temperature and infection probability remained similar.

Our sensitivity analysis indicated that the proportion reduction in infectious mosquito density was most sensitive to changes in EIP. However, even at unrealistically low EIP estimates (i.e. 40% reduction in EIP across all temperatures, which, at 30°C, resulted in an EIP estimate of 5.6 days), the qualitative relationship between temperature and fungal efficacy remained the same and fungal application was still predicted to reduce infectious mosquito density by >75% at c=0.16.

**Discussion**

This study demonstrates that, following realistic exposures, the fungal pathogen *B. bassiana* has the ability to kill two of the major malaria vectors from Asia and Africa (*An. stephensi* and *An. gambiae*, respectively) well within the estimated extrinsic incubation period (EIP) for *P. falciparum* malaria. These results are consistent across the range of mean temperatures associated with malaria transmission and are robust to additional diurnal temperature variation. Modeling suggests that the fungus could dramatically reduce transmission potential irrespective of environment, as long as the probability of fungal infection is sufficiently high (see later Discussion). This apparent robustness is not because the fungus is unaffected by temperature, but rather because fungal virulence is less sensitive to changes in temperature than malaria parasite development rate. As probability of fungal infection falls, the impact of temperature on control potential becomes more marked, with the exact patterns dependent on the combined thermal sensitivities of mosquito, parasite and pathogen. These insights support
previous work highlighting the importance of thermal ecology for understanding insect-
pathogen/parasite interactions and predicting the success of microbial biocontrol agents
(Thomas & Blanford 2003; Klass, Blanford & Thomas 2007a; b; Lambrechts et al. 2011;
Murdock et al. 2012b).

We made a number of simplifying assumptions in our analysis, many of which are
likely to underestimate fungal efficacy. For example, entomopathogenic fungi cause a
number of sub-lethal effects in infected hosts, which could further reduce malaria
transmission. Fungal infection inhibits mosquito feeding by decreasing both sensitivity to
host odors (George et al. 2011) and feeding propensity (Blanford et al. 2005, 2011;
Scholte, Knols & Takken 2006; Howard et al. 2010; George et al. 2011), and mosquitoes
that do not feed cannot transmit malaria. Fungi have been shown also to increase insect
metabolic rates while simultaneously decreasing flight ability (Blanford et al. 2011) and
predator evasion behavior (Arthurs & Thomas 2001), factors which could significantly
increase mosquito mortality rates under natural field conditions. One study also suggested
that, near the end of the EIP, daily mortality rates were higher in mosquitoes infected
with both fungus and malaria relative to those infected with fungus alone (Blanford et al.
2005) (but see Heinig & Thomas 2015), which would further reduce disease transmission
potential. We also assumed that human malaria prevalence remained constant. While this
would likely be true in the short term, long-term reductions in infectious mosquito
density would be expected to further decrease the human infection rate, which would
further reduce the probability that mosquitoes would become infected.

Our simplifying treatment of diurnal temperature variation was also conservative. We
demonstrated that addition of a realistic DTR slightly reduced fungal virulence, which is
consistent with the fact that the thermal performance curve for the fungus is broad and
relatively flat, indicating that fungal virulence is relatively insensitive to temperature
across this operative range. While insufficient empirical data exist to fully characterize
the effects of temperature variation on malaria parasite growth, previous studies have
shown that even a moderate level of diurnal temperature variation can affect both malaria
(Paaijmans et al. 2010; LaPointe, Goff & Atkinson 2010) and mosquito traits (Paaijmans
et al. 2010, 2013). Approximating the effects of DTR using rate summation indicated that
temperature variation should slow peak parasite growth more than fungal virulence
because the temperature dependence of EIP is more strongly non-linear (i.e. it has a
steeper, narrower thermal performance curve) (Ruel & Ayres 1999). Given that our
sensitivity analysis demonstrated that the model was most sensitive to variation in EIP, model outputs based on mean temperatures likely underestimate the potential impact of fungus on malaria transmission potential. This is especially so as rate summation itself tends to underestimate the impact of thermal variation on rate processes when temperature fluctuations exceed the critical maximum temperatures (Worner 1992; Klass, Blanford & Thomas 2007a; Paaijmans et al. 2013).

A number of other unknowns could also influence our predictions. For example, the impact of blood feeding on fungal virulence remains unresolved. We observed similar mortality patterns in fungus-exposed mosquitoes regardless of whether or not they fed on rodent blood (see also Blanford et al. 2011), but Mnyone et al. (2011) found that mosquitoes fed on human blood prior to B. bassiana infection survived longer than non-blood fed individuals. However, this effect was minimal during the periods when the mosquitoes would have been expected to be seeking hosts; blood fed mosquitoes exposed to fungus on the same day as the bloodmeal survived a median of one day longer than non-blood fed mosquitoes, and there was no significant survival difference in those exposed three days later. While this will be an important consideration in the context of traps not associated with blood feeding (e.g. Lwetoijera et al. 2010), it should have little impact on delivery methods which target host-seeking females.

An additional uncertainty is whether the standard relationships we used to describe the various temperature dependencies are appropriate for all locations. A recent paper exploring potential effects of local adaptation on vector-borne disease transmission noted that complex ‘genotype × genotype × environment’ interactions were possible for systems in which both a parasite and vector evolved in response to local conditions (Sternberg & Thomas 2014). However, few data are available on malaria and mosquito local adaptation, making it difficult to explore the possible significance for transmission or control.

One key question that remains to be addressed is how the daily probability of infection (c) in our model relates to potential delivery of a fungal product in the field. Infection probability depends on several key components, the first of which is the probability of contact per house. Semi-field studies have demonstrated probabilities of fungal infection per night (i.e. per house visit) ranging from 23% to 75% depending on delivery strategy (Scholte et al. 2005; Mnyone et al. 2012). The fungal dose acquired by mosquitoes in these studies does not seem to be as high as generally reported from the
lab, but research currently underway suggests that this problem can be resolved (MBT and collaborators, unpublished data). The second component is the proportion of houses receiving the intervention (population coverage). In certain areas, use of bed nets or IRS can exceed 80%, although adoption rates can be much lower (World Health Organization 2013b). If we assume that 80% of houses are treated with fungus and that mosquitoes are entering houses approximately 1/3 of the time (corresponding to the gonotrophic cycle), we would need about 60% infection probability per house to achieve an effective coverage \(c\) of 0.16. Our work suggests this coverage level could significantly reduce the density of malaria-infectious mosquitoes, even under the least favorable conditions for the fungus. Encouragingly, much lower levels of effective coverage could still have substantial impacts on infectious mosquito density. These arguments are similar to other modeling studies that suggest effective coverage of insecticide-based interventions is generally much lower than expected from estimates of population coverage, and that levels of effective coverage well below 50% can deliver good control (Koella, Saddler & Karacs 2012). Moreover, even partially effective tools can provide a valuable contribution within integrated vector management strategies (Hancock 2009; Thomas et al. 2012).

A number of studies have combined empirical and theoretical approaches to evaluate and improve biocontrol (e.g. Godfray & Waage 1991; Thomas, Wood & Lomer 1995; Fenton et al. 2002; Klass, Blanford & Thomas 2007a; Hancock, Sinkins & Godfray 2011; Lynch et al. 2012; Reilly & Elderd 2014). The current study emphasises environmental temperature as a possible determinant of success. We find that, rather than acting as a simple scaling phenomenon, the influence of temperature depends on net effects across parasite, fungus and mosquito traits that differ in their thermal sensitivities. The importance of temperature is likely to extend beyond fungi to other candidate vector control agents, including Wolbachia (McGraw & O’Neill 2013; Bian et al. 2013; Murdock et al. 2014) and microsporidia (Koella, Lorenz & Bargielowski 2009), and to the majority of bacterial, viral and filarial pathogens vectored by mosquitoes. The empirical and theoretical approaches we present here provide a framework for evaluating temperature effects in these other systems.
Notes

The research in this chapter is awaiting resubmission at the Journal of Applied Ecology with Krijn Paaijmans, Penelope Hancock and Matthew Thomas as coauthors. The authors would like to thank Sigma Xi for funding, Janet Teeple and Dannielle Kroczynski for insectary support, Nina Jenkins for fungal culture support, Simon Blanford and Eleanore Sternberg for experimental assistance and members of the Thomas and Read laboratories for helpful discussion. Additional material may be found in Appendix A.
Figure 2.1. Cumulative proportional survival of adult *An. stephensi* maintained at a range of temperatures (indicated by line color) following exposure to clay substrates treated with fungus at high or low AR or with blank oil (‘control’). Lines represent mean daily survival (±SE) from four replicates of approximately 30 mosquitoes in the fungal treatments and 2-4 replicates in the controls (n=2 for 10°C and 26°C group 2; n=3 for 14°C, 18°C and 28°C; n=4 for all others). The 26°C treatments were repeated twice.
Figure 2.2. Fungal virulence against *An. stephensi* across a range of temperatures. Points represent the mean number of days (±SE) required to achieve 90% mortality (LT90) in four replicates of n=30 mosquitoes exposed to fungus at high or low AR. The 26°C treatments were repeated twice with the duplicate points indicating the means for each run. Malarial EIP is based on the *P. falciparum* curve fit by Mordecai *et al.* (2013).
Figure 2.3. Virulence of the fungal biopesticide against *An. gambiae* across three temperatures with *An. stephensi* results from the first experiment included for reference. Points represent the mean number of days (±SE) required to achieve 90% mortality ($LT_{90}$) in four replicates of n=30 mosquitoes exposed to fungus at high or low AR. The malarial EIP is based on the *P. falciparum* curve fit by Mordecai et al. (2013).
Figure 2.4. Virulence of the fungal biopesticide against blood fed *An. stephensi* (‘blood’). Non-blood fed results at 26°C (‘no blood’) are included for reference. Data points represent the mean number of days (±SE) required to achieve 90% mortality (LT$_{90}$) in three replicates of n=30 mosquitoes exposed to fungus at high or low AR. EIP is based on the *P. falciparum* curve fit by Mordecai *et al.* (2013).
Figure 2.5. Cumulative proportional survival of adult An. stephensi mosquitoes exposed to different fungal AR, mean temperatures and diurnal temperature ranges (DTR). Following exposure, mosquitoes were maintained at mean temperatures of 20°C (top), 26°C (middle) or 32°C (bottom), at either constant temperatures (DTR=0) or a DTR of 12°C (DTR=12). Data were collected until all mosquitoes exposed to fungus were dead (23 days in the 20°C treatments; graph truncated for consistency).
Figure 2.6. Influence of diurnal temperature variation on fungal virulence against *An. stephensi*. Data points represent the mean number of days (±SE) to achieve 90% mortality (LT90) for mosquitoes exposed to clay substrates treated at high or low fungal AR and subsequently held under constant (DTR=0) or fluctuating temperature conditions (DTR=12). There were n=4 cups of approximately 30 mosquitoes per cup for all treatments except one (low application rate, 20°C, DTR=0), where n=3. The constant-temperature P. falciparum EIP curve is from Mordecai *et al.* (2013), and the EIP under fluctuating conditions (DTR=12) was predicted using rate summation.
Figure 2.7. Predicted impact of fungal biopesticide on malaria transmission potential at different mean temperatures. The lines show proportional reduction in the density of malaria infectious mosquitoes relative to no intervention for various daily probabilities of fungal infection (c) at the high and low fungal application rates.
Chapter 3

Prospective biocontrol of malaria vectors using fungal entomopathogens

Note: It is intended that the results from this chapter will be published as part of a larger study on the viability of fungal biopesticides for malaria control under realistic field conditions. The proposed paper discusses the control potential of the eaves tube, a novel candidate delivery system for fungal spores (or other powdered contaminants) which was developed as part of a large collaborative research project in which I have participated (see www.mcdproject.eu). The proposed paper will be a multi-author study for which the work included in this chapter will form one part. Accordingly, I have limited the introduction to a basic description of the eaves tube concept and focus the Methods, Results and Discussion on the sections of the larger paper for which I am responsible.

Introduction

Over the last 10 years there have been numerous studies exploring the potential for use of fungal pathogens for control of adult mosquito vectors. While many studies suggest that entomopathogenic fungi could provide a useful addition to conventional chemical active ingredients, they still have not been thoroughly evaluated in field settings. This is due in part to the fact that, until recently, there has not been a compelling delivery system. Here, we discuss the feasibility of using eaves tubes with spore-treated electrostatic netting as a practical and cost-effective deployment strategy for the candidate fungal entomopathogen *Beauveria bassiana*.

Eaves tube design and proof of concept

Eaves tubes are plastic cylinders designed to fit in the ventilation holes in the eaves of African houses (Figure 3.1), which are a primary entry point of malaria vector species (Lindsay &
Snow 1988; Lindsay et al. 2003; Kirby et al. 2009; Njie, Dilger & Lindsay 2009). A piece of electrostatic netting is stretched over one side of each eaves tube, blocking mosquito entry into the house, and the netting can be coated with powdered fungal spores or other contaminants (e.g., chemical insecticides or silica powder). Actively host-searching mosquitoes become contaminated when they enter the tubes and contact the netting as they follow odor plumes of the household occupants at night. Laboratory tests with B. bassiana fungus have shown that even transient exposure to fungus-treated netting can result in high mosquito mortality rates (Figure 3.2). These patterns of mortality are consistent with those reported using residual treatments of oil formulated spores (Chapters 2 and 4; Blanford et al. 2005, 2011, 2012). Fungal spores remain viable in eave tubes fitted to real village houses for around two months (MBT and collaborators, unpublished data). Retreatment is extremely easy and cost effective as it simply requires old netting to be switched out with newly treated netting (and the old netting can then be washed and reused). Trials in semi-field screen houses indicate that eaves tubes infect a large proportion (60-70%) of host-seeking mosquitoes per night (Figure 3.3). Taken together, this work demonstrates that eaves tubes offer a viable and cost-effective approach for delivering fungal biopesticides for control of adult malaria mosquitoes.

**Predicted efficacy of fungus delivered via eave tubes under different environmental conditions**

**Methods**

**Modeling fungal impact.** We used the age-structured model developed by Hancock et al. (2009) to predict how the candidate fungal biopesticide B. bassiana (strain I93-825) would impact infectious mosquito densities at different effective coverage rates and temperatures. The model describes malaria infection dynamics in the mosquito population as a susceptible-exposed-infectious (SEI) process. Adult mosquitoes enter the susceptible class at a constant rate. They become infected with malaria and move to the exposed class at a rate dependent upon the rate at which they feed on humans (the human bite rate), the proportion of humans with transmissible malaria, and the probability that the malaria parasite is successfully transmitted from the human to the mosquito (transmission efficiency). Following a fixed delay corresponding to the extrinsic incubation period (EIP) associated with malaria parasite development, the mosquitoes enter the infectious class and are potentially able to transmit disease. Background mortality occurs in all
classes at a constant rate. In addition to becoming infected with malaria, the mosquitoes are also exposed to the fungal biopesticide at rate determined by the daily probability of fungal infection ($c$). Malaria infection progress in these mosquitoes is tracked in a parallel set of classes, and the additional mortality due to fungal infection in these classes is modeled as a Weibull function of time since fungal exposure. Fungal efficacy was assessed based on the proportion reduction in the equilibrium total density of infectious mosquitoes in the presence of fungus (i.e. with a daily probability of fungal infection, $c, >0$) relative to the predicted density in the absence of fungus ($c=0$). For additional model details, see Hancock et al. (2009) and Appendix A.

We parameterized the model for different temperatures based on data from a number of previous studies. The relationships between temperature and the anopheline life history traits (human bite rate and malaria transmission efficiency) were estimated based on the curves described by Mordecai et al. (2013). The malarial EIP was estimated based on the Plasmodium falciparum malaria parasite development rate curve described in the same study. The additional mortality due to fungal infection was estimated based on assays conducted in Anopheles stephensi mosquitoes (Chapter 2). The model was evaluated at 2°C intervals from 18-34°C at two different daily probabilities of fungal infection (Figure 3.4).

**Creating prediction surfaces.** To explore the implications of these predictions for spatial and temporal variation in malaria vector control, we fit a first-degree polynomial model to the predicted reductions in infectious mosquito density associated with each of the two daily infection probabilities using the *lm* procedure in R (R Development Core Team 2013), then added higher-order terms until there was no significant improvement in model fit as indicated by ANOVA. We then used these curves in combination with monthly mean temperature surfaces extrapolated from weather station records across Africa as described by Paaijmans et al. (2010) to create maps of predicted reductions in infectious mosquito density across the African continent for four different months (January, April, July and December).

**Estimation of EIR and PR.** To explore how these reductions in infectious mosquito density could potentially affect disease transmission, we explored how the predicted changes in infectious mosquito density might affect entomological inoculation rate (EIR), the number of infectious bites received per person per day. It is equal to the product of the human biting rate, which is the number of bites received per person per day, and the sporozoite rate, which is the proportion of
mosquitoes which are infectious (Smith & McKenzie 2004). The EIR in the presence of eaves tubes \((E^*)\) was calculated as follows:

\[
E^* = E(1 - \rho)
\]

\[\text{eqn 3.1}\]

where \(E\) is the initial entomological inoculation rate in the absence of fungus and \(\rho\) is the proportion reduction in infectious mosquito density in the presence of fungus predicted by the model described above.

The proportion of the population infected with malaria (the parasite rate) is often described as being log-linearly related to EIR (Beier, Killeen & Githure 1999; Hay et al. 2005; but see Smith et al. 2005). Here, we estimated the parasite rate in the absence of fungus \((P)\) and parasite rate in the presence of fungus \((P^*)\) based on the general relationship described by Hay et al. (2005):

\[
P = (0.1694)\log(E) + 0.3635
\]

\[\text{eqn 3.2}\]

\[
P^* = (0.1694)\log(E^*) + 0.3635
\]

\[\text{eqn 3.3}\]

but the models described by Beier et al. (1999) and Smith et al. (2005) generated qualitatively similar results (Figure B.1).

**Results and Discussion**

If we assume that the probability of fungal infection per house per night is 60%, that mosquitoes are seeking a blood meal 1/3 of the time (corresponding to the gonotrophic cycle), and that eave tubes are installed in 80% of houses, the corresponding daily probability of infection, \(c\), would be approximately 0.16. At this level of effective coverage, the fungal biopesticide was predicted to reduce infectious mosquito density by \(\geq 90\%\) year-round in the majority of areas (Figure 3.5). These estimates may be conservative as they do not account for the impact of sublethal effects on mosquito mortality rates and host-finding ability which would be expected to further reduce infectious mosquito densities (Hancock, Thomas & Godfray 2009; Hancock 2009). Temperature would be predicted to impact fungal efficacy only at much lower levels of effective coverage (e.g. \(c=0.025\), Figure 3.6). But even at these low coverage levels, fungus would still be predicted to have a significant impact on infectious mosquito density, particularly when combined with other interventions as part of an integrated vector management strategy (World Health Organization 2004).
The analysis above demonstrates the fungus to have potential to dramatically reduce the number of infectious malaria mosquitoes over more or less all transmission settings. However, the impact this reduction has on malaria prevalence within a particular setting will depend on the baseline entomological inoculation rate (EIR, the number of infectious bites per person per day). The EIR can be estimated by multiplying the total number of vector bites per human per day (the human biting rate) by the proportion of mosquitoes which are infectious (the sporozoite rate) (Smith & McKenzie 2004). All else being equal and assuming host and vector population homogeneity, reducing the infectious mosquito density by 90% will therefore reduce the EIR by 90%. Thus the modeling results suggest that, at a daily probability of infection on 0.16, fungus has the potential to dramatically reduce EIR (Figure 3.7a).

The relationship between EIR and parasite rate (PR, the proportion of people infected with malaria) generally described as log-linear (Beier, Killeen & Githure 1999; Hay et al. 2005; but see Smith et al. 2005). Thus, when initial EIR is low, small reductions are predicted to have large impacts on PR. In contrast, when initial EIR is high, much larger changes can have minimal impacts on PR because very few infectious bites are required to maintain high levels of malaria infection; reducing EIR from 10 and one infectious bite per year has a higher proportional impact in PR than reducing EIR from 100 to ten. Reduced PR impact at high EIRs is a problem common to all vector control technologies (e.g. Curtis et al. 1998), and fungal biopesticides are no exception. Based on the overall EIR-PR relationship described by Hay et al. (2005), fungal treatment could potentially reduce PR to very low levels if initial EIR is low, but if initial EIR is high, the maximum impact of the fungus may depend in part on prevailing mean temperature (Figure 3.7b). At the local level, the relationship between EIR and PR is also affected by a number of other factors, including the degree of urbanization (Hay et al. 2005), host heterogeneity (Smith et al. 2005) and vector characteristics. In addition, long-term reductions in PR will further reduce the proportion of mosquitoes which become infected with malaria, which will in turn further reduce EIR. Predicting these dynamic effects will require more complex and assumption-rich theoretical frameworks than the ones used here (e.g. Reiner Jr. et al. 2013; Beck-Johnson et al. 2013), but the general trends described above suggest that fungus-associated reductions in infectious mosquito density could lead to significant reductions in disease burden under certain conditions.

It is not yet known how eaves tubes will interact with existing vector control technologies. Both long-lasting insecticide-treated nets and indoor residual sprays target mosquitoes which
have already entered dwellings, so if eaves tubes prevent mosquitoes from ever coming inside, there may be little to gain by combining these interventions. Under real-world conditions, the benefits of using multiple technologies will likely depend on a variety of location-specific factors, including insecticide resistance levels, excito-repellency properties of existing chemical treatments (Quinones et al. 1998; Killeen & Smith 2007), mosquito behavioral characteristics (Njie, Dilger & Lindsay 2009; Killeen et al. 2014) and coverage levels of existing technologies.

Eave tubes create, for the first time, the genuine possibility of operationalizing biocontrol of adult malaria mosquitoes. This approach is predicted to be robust for transmission settings across the African endemic zone. Although spore persistence is not comparable to that of DDT-based IRS, which remains effective for more than six months (World Health Organization 2013a), the small treatment area and ease of application associated with eave tubes make retreatment every 2-3 months a potentially viable option. In addition, the amount of product required to treat eave tube netting is relatively small, potentially creating opportunities for in-country production, which may help to increase local buy-in to the technology (Thomas & Read 2007). Eave tubes have already been installed in 1000 houses in Tanzania and are showing promising results. Use of fungus in eave tubes should significantly reduce selection pressure for resistant mosquito phenotypes (Thomas & Read 2007; Read, Lynch & Thomas 2009; Koella et al. 2009; Lynch et al. 2012), making eaves tubes both a potent weapon in the fight against insecticide resistance and an innovative technology for malaria control.

Notes

The author would like to thank members of the MCD Project for the use of figures (where indicated) and Justine Blanford for assistance with GIS mapping portion of the project. Additional material may be found in Appendix B.
Figure 3.1. Eave tubes installed in a house in Ifakara, Tanzania (A), detail of an eave tube from the interior of the house showing treated netting (B) and detail of treated electrostatic netting (C).
Figure 3.2. Impacts of transient exposure to eaves tubes on *A. stephensi* mortality rates. Ten-day-old *A. stephensi* females were gently aspirated into plastic bottles fitted with eave tube netting. Following an exposure period of either five or 30 seconds (indicated by line type), they were removed from the bottles and placed into cups/cages. Lines represent daily mean proportional survival in n=30 mosquitoes. Results are reproduced courtesy of Eleanore Sternberg and Matthew Thomas of the MCD Project (unpublished data).
Figure 3.3. Impact of eaves tubes on mosquito mortality rates under semi-field conditions. Eave tubes were fitted into plywood replicates of traditional African houses inside experimental habitats (netted greenhouses with opaque plastic roofs to exclude rain). During the assays, a volunteer slept inside the house under an untreated bed net to act as a lure. Adult female *Anopheles arabiensis* mosquitoes were released into the experimental habitat at dusk and retrieved the next day, and their daily mortality was recorded. Mortality rate in the fungus-silica treatments was high day 0-4 (at which point cumulative mortality in the treated mosquitoes was approximately 60-70%) but was similar to that of the controls from day 5 onward. Images and results are reproduced courtesy of the MCD Project (unpublished data).
Figure 3.4. Predicted reduction in infectious mosquito density at various temperatures for two daily probabilities of fungal infection (c). The fitted curves describing each daily fungal infection probability (c) were:

\[ f(T, c = 0.025) = 22.19764 - 3.397456T^2 + 0.20045857T^3 - 0.0052662847T^4 + 0.00005162771T^5 \]  
\[ f(T, c = 0.16) = 40.43416 - 10.392557T^2 + 1.131053T^3 - 0.06505317T^4 + 0.002086031T^5 - 0.00035385967T^6 \]  
\[ \text{eqn 3.4} \]  
\[ \text{eqn 3.5} \]
Figure 3.5. Predicted impact of fungal biopesticides assuming a daily likelihood of infection ($c$) of 0.16. Predicted proportion reduction in infectious mosquito density relative to predicted density in the absence of fungus ($c=0$) is indicated by color.
Figure 3.6. Predicted impact of fungal biopesticides assuming a daily likelihood of infection ($c$) of 0.025. Predicted proportion reduction in infectious mosquito density relative to predicted density in the absence of fungus ($c=0$) is indicated by color.
Figure 3.7. Predicted impact of fungus-treated eaves tubes on malaria transmission parameters. Corrected entomological inoculation rate ($EIR_c$, A) and parasite rate ($PR_c$, B) were estimated based on EIR values in the absence of fungus. Estimates were made assuming $c = 0.16$ at three temperatures (indicated by line color) associated with different predicted proportional reductions in infectious mosquito density (0.99, 0.94 and 0.89 at 20, 26 and 30°C, respectively). EIR and estimated PR in the absence of fungus (“no fungus”) were included for comparison.
Chapter 4

Interactions between a fungal entomopathogen and malaria parasites within a mosquito vector

Abstract

**Background.** Mosquitoes are becoming increasingly resistant to the chemical insecticides currently available for malaria vector control, spurring interest in alternative management tools. One promising technology is the use of fungal entomopathogens. Fungi have been shown to impact the potential for mosquitoes to transmit malaria by reducing mosquito longevity and altering behavior associated with flight and host location. Additionally, fungi could impact the development of malaria parasites within the mosquito via competition for resources or effects on the mosquito immune system. This study evaluated whether co-infection or superinfection with the fungal entomopathogen *Beauveria bassiana* affected malaria infection progress in *Anopheles stephensi* mosquitoes.

**Methods.** The study used two parasite species to examine possible effects of fungal infection at different parasite development stages. First, the rodent malaria model *Plasmodium yoelii* was used to explore interactions at the oocyst stage. *Plasmodium yoelii* produces high oocyst densities in infected mosquitoes and thus was expected to maximize host immunological and resource demands. Second, fungal interactions with mature sporozoites were evaluated by infecting mosquitoes with the human malaria species *Plasmodium falciparum*, which is highly efficient at invading mosquito salivary glands.

**Results.** With *P. yoelii*, there was no evidence that fungal co-infection (on the same day as the blood meal) or superinfection (during a subsequent gonotrophic cycle after parasite infection) affected the proportion of mosquitoes with oocysts, the number of oocysts per infected mosquito or the number of sporozoites per oocyst. Similarly, for *P. falciparum*, there was no evidence that fungal infection affected sporozoite prevalence. Furthermore, there was no impact of infection with either malaria species on fungal virulence as measured by mosquito survival time.
Conclusions. These results suggest that the impact of fungus on malaria control potential is limited to the well-established effects on mosquito survival and transmission behavior. Direct or indirect interactions between fungus and malaria parasites within mosquitoes appear to have little additional influence.

Introduction

Malaria vector control programs are currently being threatened by increasing insecticide resistance in adult mosquito populations (Yewhalaw et al. 2011; Ranson et al. 2011; Badolo et al. 2012; Asidi et al. 2012). This has spurred interest in the development of alternative management tools, including the use of entomopathogenic fungi (Blanford et al. 2005, 2011; Farenhorst et al. 2010; Mnyone et al. 2012). Fungal spores infect mosquitoes on contact and can be integrated into a number of delivery systems, including wall treatments (Mnyone et al. 2010; Blanford et al. 2011, 2012a; b), eave nets and curtains (Mnyone et al. 2012), baited traps (Lwetoijera et al. 2010), and point source targets (Scholte et al. 2005; Farenhorst et al. 2008; Mnyone et al. 2012). Conidial storage and persistence characteristics are competitive with those of commonly-used chemical insecticides (Blanford et al. 2012a). Importantly, fungi are effective against existing insecticide-resistant mosquito populations (Farenhorst et al. 2009, 2010; Blanford et al. 2011) and are expected to impose reduced selection for new resistance traits relative to conventional chemicals (Thomas & Read 2007; Read, Lynch & Thomas 2009; Koella et al. 2009; Lynch et al. 2012).

Chemical insecticides generally kill or knock down susceptible mosquitoes within hours of exposure (World Health Organization 2006). In contrast, fungal biopesticides are relatively slow-acting, taking up to a week or more to kill exposed mosquitoes (Blanford et al. 2005; Scholte et al. 2005). This slower action can still be sufficient to block malaria transmission, since mosquitoes do not become infective until the end of the malarial extrinsic incubation period (EIP) of approximately two weeks. Fungal infection also causes a number of sublethal effects (e.g., decreased host location ability (George et al. 2011) and feeding propensity (Scholte, Knols & Takken 2006; Howard et al. 2010; Blanford et al. 2011, 2012b) that further reduce the probability of disease transmission. One early study found that the combination of high mortality and low sporozoite rates associated with Beauveria bassiana fungal infection significantly reduced the number of Anopheles stephensi mosquitoes which both survived to the end of the EIP and were
potentially able to transmit *Plasmodium chabaudi* malaria (Blanford et al. 2005). The authors also noted that mosquitoes co-infected with fungus and malaria exhibited an upward trend in daily mortality rate toward the end of the EIP which was not evident in mosquitoes infected with either malaria or fungus alone (Blanford et al. 2005). However, there has been negligible follow-up work to validate this preliminary observation. If either co-infection (i.e., mosquitoes infected with fungus and malaria parasites at more or less the same time during the same gonotrophic cycle) or superinfection (i.e., mosquitoes encountering the fungus at a later gonotrophic cycle when the malaria parasite is already established) alter the development of either the fungus or malaria parasite, there could be important implications for malaria control. For example, if fungal infection directly or indirectly inhibited sporozoite invasion of the salivary glands, control programs could potentially use fungal strains that were less virulent to mosquitoes, which would in turn reduce selection for resistance in vector populations (Thomas & Read 2007; Read, Lynch & Thomas 2009; Koella et al. 2009; Lynch et al. 2012).

The effects of co- and superinfection are highly variable in other mosquito-pathogen systems. The fungal entomopathogens *Metarhizium anisopliae* and *Beauveria bassiana* can inhibit dengue virus replication and dissemination in co-infected *Aedes aegypti* mosquitoes (Dong et al. 2012; Garza-Hernández et al. 2013). Infection with certain species of *Wolbachia* bacteria also can inhibit establishment of other bacterial (Kambris et al. 2009), nematode (Kambris et al. 2009) and viral (Moreira et al. 2009; Glaser & Meola 2010; Bian et al. 2010; Walker et al. 2011; van den Hurk et al. 2012; Blagrove et al. 2012) superinfections. The impacts of *Wolbachia* infection on malaria parasites have been mixed, with reductions in oocyst densities occurring under some conditions (Kambris et al. 2010; Hughes et al. 2011; Bian et al. 2013) and enhancement observed under others (Hughes et al. 2012; Murdock et al. 2014). The mechanisms underlying these phenotypes remain unresolved but appear to be mediated by resource competition (Cook & McGraw 2010) and/or upregulation of immune factors (Moreira et al. 2009; Kambris et al. 2010; Bian et al. 2010; Hughes et al. 2011; Pan et al. 2012; Dong et al. 2012; Rancès et al. 2012).

Similar mechanisms could affect interactions between fungal pathogens and malaria parasites. The mosquito responds to the early ookinete stages of malaria infection by upregulating immune responses including melanization (Blandin et al. 2004) and the Toll pathway or, in the case of human malarials, the IMD pathway (Dong et al. 2006; Garver, Dong & Dimopoulos 2009; Garver et al. 2012). There is evidence that malaria parasites utilize host resources as sporozoites replicate within the oocysts (Beier 1998), and resource depletion might increase host susceptibility to secondary infection. During the final stage of infection, sporozoites are actively
degraded in the hemocoel (Hillyer, Barreau & Vernick 2007), potentially either reducing (via depletion) or enhancing (via upregulation) the availability of hemocytes to combat additional infectious agents. Fungal infection itself triggers a number of similar immune responses in insect hosts. Early fungal invasion of the hemocoel is countered by cellular immune responses (Hajek & St. Leger 1994), which can result in granulocyte depletion as the infection progresses (Hung & Boucias 1992). Later in the infection, immune factors involved in the humoral melanization response (Hajek & St. Leger 1994; Yassine, Kamareddine & Osta 2012) and the Toll and JAK-STAT pathways (Dong et al. 2012) are involved in countering fungal proliferation. Thus, depending on the timing of the malaria and fungal exposures, there might be extensive overlap in immune and resource demands on the mosquito host.

This study explored whether co- or superinfection with a candidate strain of the fungal entomopathogen \textit{B. bassiana} affected a number of malaria infection parameters. Two different malaria species were used to evaluate potential interactions at different stages of the malaria life cycle. To examine impacts at the oocyst stage, \textit{Anopheles stephensi} mosquitoes were infected with \textit{Plasmodium yoelii}, a rodent malaria species which produces high oocyst densities in infected mosquitoes (Vaughan, Hensley & Beier 1994). High oocyst intensities have been found to increase vector mortality rates (Ferguson & Read 2002b; Dawes \textit{et al.} 2009; Pollitt \textit{et al.} 2013) (but see Ferguson & Read 2002b), so it was expected that any mortality costs associated with malaria-fungus co-infection would be maximized in high-intensity infections. The mosquitoes were exposed to fungus either immediately following the blood meal (to simulate co-infection) or three days later to simulate superinfection during the next gonotrophic cycle following oocyst establishment. There were no significant effects of co- or superinfection on oocyst prevalence, oocyst intensity, sporozoite replication (represented by the number of sporozoites per oocyst), or mosquito mortality rate among the various treatment groups.

To determine whether there were late-stage interactions between fungal superinfection and malaria sporozoites, mosquitoes were infected with \textit{Plasmodium falciparum}, a human malaria species which produces lower oocyst densities but is much more efficient at invading mosquito salivary glands than \textit{P. yoelii} (Vaughan, Noden & Beier 1994; Vaughan 2007). Late fungal infection was simulated by exposing mosquitoes to fungus either eight or 11 days after the infectious blood feed. Regardless of exposure day, fungal treatment had no effect on sporozoite prevalence in the salivary glands, nor did the presence of malaria appear to affect subsequent fungal virulence. Overall, the results did not indicate a significant interaction between mosquito, fungus and parasite, and suggest that interaction with malaria infection is unlikely to have either
positive or negative consequences for the potential of the fungal biopesticide approach to reduce transmission.

**Methods**

**Mosquito rearing.** *Anopheles stephensi* mosquitoes were raised under standard insectary conditions at 27°C and 75% relative humidity with a 12-hour light/12-hour dark cycle. Mosquito eggs were hatched in plastic tubs containing 1.5 l of distilled water. Four days later, larvae were placed into new tubs containing 400 individuals per tub and provided with 10 mg of powdered Tetrafin fish flakes (TetraFin, Melle, Germany) daily. Pupae were placed into cages for emergence, and adults were given a 10% glucose solution supplemented with 0.05% para-aminobenzoic acid (PABA) to enhance *P. yoelii* oocyst infection rate (Peters & Ramkaran 1980).

**Conidial production and formulation.** Oil suspensions of *B. bassiana* (isolate I93-825) conidia were prepared according to established protocols (Blanford et al. 2005, 2011). Conidia harvested from potato dextrose agar (Oxoid, UK) were suspended in sterile 0.05% Tween 80 (Sigma) at a concentration of 10⁶ conidia/ml. Liquid cultures containing 1 ml of suspension and 75 ml of sterile liquid culture medium (4% d-glucose, 2% yeast extract (Oxoid, UK) in tap water) were incubated on a shaker at 24°C and 160 rpm for three days then diluted with 75 ml distilled water. The mixture was used to inoculate sterile solid medium (1 kg barley flakes (Bobs Red Mill, Milwaukie, OR, USA) and 600 ml tap water), sealed in mushroom spawn bags (Unicorn, Garland, TX, USA) and incubated at 24°C for ten days. The bag contents were then dried in paper bags to a moisture level of <20%. Conidia were harvested using a Mycoharvester (Acis Manufacturing, Devon, UK), dried over silica gel to a moisture level of 5% and sealed in foil sachets for storage at 5°C. Prior to the experiment, conidial viability was assessed by suspending conidia in Isopar M oil and plating the suspension on Sabouraud dextrose agar (Oxoid, UK). Three replicate plates were incubated at 25°C for 20 hours, and 300 spores per plate were visually assessed under a compound microscope to ensure that more than 85% of conidia had successfully germinated. A new suspension was prepared by adding dry conidia to an oil mixture (80% Isopar M:20% Ondina) at a concentration of 10⁷ conidia/ml, which was verified using a hemocytometer. The suspension was then applied to clay tiles as described below.
**Substrate preparation.** The conidial suspensions were applied to clay tiles in a manner designed to simulate spray treatments on clay/mud walls of traditional African huts (World Health Organization 2006; Blanford et al. 2011, 2012a). Tiles were created by pouring a slurry of white earthenware clay (Clay King, Spartanburg, SC, USA) and distilled water into 150 mm petri plates. All tiles were air-dried for at least one week until they had hardened completely. The dry tiles were then affixed to the back wall of a fume hood, and a handheld airbrush sprayer was used to uniformly apply 20 ml of conidial suspension to a 0.5 sq m area for a final application rate of $8 \times 10^8$ conidia/sq m. At higher application rates, this fungal isolate kills mosquitoes within three to five days (Blanford et al. 2011), so this relatively low application rate was selected to allow mosquitoes to live long enough post-infection so that potential interactions with malaria could be observed. Control tiles were sprayed with a blank oil formulation, and all tiles were air-dried overnight.

*Plasmodium yoelii* assays. The rodent malaria model *P. yoelii* was used to assess the impact of fungal co- and superinfection on malaria oocyst prevalence and intensity. Three- to five-day-old female mosquitoes were starved overnight then allowed to feed on female six to eight-week-old C57 mice (Charles River, Malvern, PA, USA) for up to 30 min. Mosquitoes from the malaria infection treatments were fed on anesthetized mice which had been injected with $10^5$ *P. yoelii* parasites (*yoelii* strain, clone 17XNL, WHO Registry of Standard Malaria Parasites, University of Edinburgh, UK) four days prior, while mosquitoes from the control treatments were fed on uninfected mice. All blood feeds took place at 26°C to maximize feeding, and mosquitoes that were not fully engorged were removed from the experiment. The mosquitoes were then moved to a 24°C incubator to maximize *P. yoelii* growth and survival (Paaijmans et al. 2012).

Following a one-hour acclimation period, the mosquitoes were randomly allocated to a number of treatment groups. Half the mosquitoes were exposed to fungus-treated or control tiles on the same day as the blood meal (day 0 exposures). Groups of approximately 50 individuals were aspirated into standard WHO cones on tiles and left for a 30-min exposure period. Afterward, the mosquitoes were aspirated into nylon-covered cups and placed in a 24°C incubator. Mortality was monitored daily. The other half of the mosquitoes were aspirated directly into nylon-covered cups and placed into the 24°C incubator. In this group, delayed fungal exposure was simulated by exposing the mosquitoes to the tiles three days after the blood feed, coinciding with when they would have been expected to seek their next blood meal (day 3 exposure). Thus, there were four treatment groups for each of the two exposure time points: dual
infection (fungus and malaria), fungal infection alone, malaria infection alone and no infection. There were five replicate cups of approximately 50 mosquitoes per cup (~250 mosquitoes total) for each treatment and time point. All cups were held at 24°C and provided with a cotton ball saturated with glucose-PABA solution for nutrition. Mosquito mortality was monitored daily until all mosquitoes in the fungal treatments were dead (28 days in the day 0 exposures, 25 days in the day 3 exposures).

Seven days after the blood feed, 20 mosquitoes from each treatment group were dissected and examined for oocysts. Oocyst prevalence (the proportion of infected mosquitoes in each treatment) and oocyst intensity (the number of oocysts in each infected individual) were recorded, and each midgut was placed in 10 µl of 70% EtOH and stored at -80°C.

Quantitative PCR was used to estimate the number of sporozoites per oocyst, a measure of parasite replication rate. The Microelute Tissue DNA Kit (Omega Bio-tek) was used to extract and purify the DNA according to the manufacturer’s instructions with one exception: during the tissue lysis step, a stainless steel ball was added to each tube. The samples were then homogenized for 30 sec at 30 Hz on a TissueLyser (Qiagen) prior to being incubated at 55°C and processed according to the kit protocol. The purified DNA was eluted in 20 µL of buffer and stored at -20°C.

Real-time quantitative PCRs were performed using the *Plasmodium* primers and probe described by Bell *et al.* (2009). Reaction mixtures were prepared by adding 2 µL of purified DNA template to a reaction mix of 1.5 µL each of 5 µM dilutions of forward and reverse primers, 1 µL of 5 µM probe, 12.5 µL of PerfeCTa® qPCR FastMix® (UNG, Low ROX™ by Quanta BioSciences) and 6.5 µL of RNAse-free water. The reactions were run on a Prism 7500 Sequence Detection System (TaqMan) with an initial 20-sec activation step at 95°C followed by 40 cycles of denaturation at 95°C for 3 sec and annealing/extension at 60°C for 30 sec. Serial dilutions of *P. yoelii* DNA standard spanning five orders of magnitude (7.22 x 10^4 to 7.22 sporozoites) were used to generate a standard curve for absolute quantification of the samples. Three replicates of each standard were included in each reaction run.

*Plasmodium falciparum* assays. The human malaria parasite *P. falciparum* was used to assess interactions between fungal infection and sporozoites. *In vitro* gametocyte production followed established procedures (Kennedy *et al.* 2012). NF54 strain *P. falciparum* cultures were maintained *in vitro* in O+ erythrocytes in a culture medium of RPMI 1640 (25 mM HEPES, 2 mM L-glutamine), 50 µM hypoxanthine and 10% A+ serum in an atmosphere of 5% CO₂, 5% O₂,
and 90% N₂. At 5% hematocrit and 0.8-1% parasitemia (mixed stages), gametocyte cultures were initiated. Media was changed daily, and the cultures were maintained for up to 17 days. On the day of the feed, the gametocyte cultures were spun down, and the pelleted infected erythrocytes were diluted to 2% gametocytemia and 40% hematocrit with fresh A+ human serum and O+ erythrocytes.

Three- to five-day-old female mosquitoes were aspirated into replicate cups and allowed to feed on warmed membrane feeders containing either the infectious blood mixture or uninfected blood for up to 30 min. Unfed mosquitoes were removed from each cup as described above. Seven days after the blood meal, seven mosquitoes from each malaria-infected cup were dissected as described above to ensure that there were no significant differences in initial oocyst prevalence (mean ± SE 0.44 ± 0.05, Fisher’s exact test \( p = 0.116 \)) or intensity (1.52 ± 0.12; Kruskal-Wallis \( \chi^2 = 1.78, \text{df} = 3, p = 0.620 \)) among the malaria treatment groups.

As described in the \( P. \) yoelii experiment, the mosquitoes were exposed to either fungus-treated or control tiles at two time points following the blood feed, generating four treatment groups (dual infection, fungal infection alone, malaria infection alone and no infection) per time point. However, this experiment simulated late fungal exposure by exposing the mosquitoes to the tiles either eight or 11 days after the blood feed. The day 11 treatments each included three replicate cups of approximately 30 mosquitoes per cup, and the day 8 treatments each included three replicate cups of approximately 50 mosquitoes per cup to ensure that an adequate number survived to the sporozoite stage. All cups were held at 27°C and were supplied with a cotton ball soaked with glucose-PABA solution, and daily mortality was monitored for 14 days following the blood meal. Over the next two days (days 15-16 after the blood meal), the remaining mosquitoes in the malaria infection treatments (malaria alone and dual infection) were dissected, and their salivary glands were inspected under a microscope for the presence of sporozoites. In the malaria control treatments (fungus alone and no infection), daily mortality was monitored until all the fungus-exposed mosquitoes had died (22 days after fungal exposure in the day 8 exposures, 20 days in the day 11 exposures).

**Statistical analysis.** Median survival times for each treatment group were calculated using the Kaplan-Meier procedure. The effects of fungal exposure and malaria infection on mosquito survival were analyzed using a full factorial Cox proportional hazards model with backward stepwise elimination of non-significant interactions \( (p > 0.05) \). The impacts of fungal exposure and malaria infection on the various malaria infection parameters were evaluated using
generalized linear models (GLM) with the model error distributions and link functions adjusted to fit the data. For the *P. yoelii* experiments, oocyst prevalence was analyzed using a binomial GLM with a logit link function. Oocyst intensity and the mean number of sporozoites per oocyst were heavily right-skewed, so treatments were compared using a negative binomial GLM with a log link function. In the *P. falciparum* experiments, the data for sporozoite prevalence (the proportion of dissected mosquitoes with sporozoites in their salivary glands) were overdispersed, so treatment effects were analyzed using a quasibinomial GLM with a logit link function. In each case, the analyses began with a full factorial model including malaria infection and fungal exposure as variables. In the *P. falciparum* experiments, replicate cup was also included in the initial model but was not significant. Non-significant variables (p >0.05) were then removed from each model using backward stepwise elimination. All analyses were performed in R (R Development Core Team 2013).

**Results**

*Plasmodium yoelii* experiment. Mosquito mortality rate varied with treatment group (Figure 4.1) but was not significantly affected by *P. yoelii* malaria infection status (hazard ratio (HR) = 0.95, z = -0.77, p = 0.44). Fungal exposure significantly increased mortality rate (HR = 17.06, z = 21.11, p <0.001); mosquitoes exposed to fungus had median survival times of eight to ten days relative to >25 days in the controls. There was also a significant interaction between fungal exposure and exposure day (HR = 0.64, z = -3.42, p = 0.001), such that, even after accounting for the delay in fungal infection, the mosquitoes exposed to fungus on day 3 had median survival times about one day longer than those exposed on the same day as the blood meal.

None of the *P. yoelii* malaria infection parameters was significantly affected by fungal exposure or exposure timing (Table 4.1, Figures 2 and 3). Infection prevalence was slightly higher in the day 0 exposure groups (90% for both fungus and control mosquitoes) than in the day 3 groups (75% for fungus and 70% for controls), but the difference was marginally insignificant (z = -1.94, p = 0.053). Although the number of sporozoites per oocyst was lower in mosquitoes exposed to fungus (z = 2.15, p = 0.032), this result was driven by a single outlier. When this data point was not included in the analysis, neither fungal exposure ($\chi^2 = 1.22,$
p = 0.269) nor exposure timing ($\chi^2 = 1.06, p = 0.303$) significantly affected sporozoite density per oocyst.

**Plasmodium falciparum experiment.** There was no evidence that mosquito survival to the infectious stage (14 days after the blood feed) was affected by *P. falciparum* infection (HR = 1.02, z = 0.14, p = 0.888). There was a significant interaction between fungal exposure and exposure day (HR = 5.57, z = 2.90, p = 0.004), but this was likely due to the timing of the dissections. Mosquito survival is generally quite high in the first few days of fungal infection because the fungus requires time to develop and invade the hemocoel (Hajek & St. Leger 1994). Once the infection is established, however, mosquito survival plummets rapidly. Mosquitoes in the day 11 malaria treatments were dissected just three days after fungal exposure when very few mosquitoes had died (≤14% in all treatments, Figure 4.4). In contrast, mosquitoes in the day 8 treatments were dissected six days after fungal exposure, at which point mosquito mortality was much higher in the fungal treatment groups (>40%) than the controls (<10%). When only the first three days following exposure were evaluated in all the treatments, neither exposure day (HR = 0.98, z = -0.06, p = 0.95) nor fungal exposure (HR = 1.14, z = 0.34, p = 0.71) significantly influenced mortality. There was also no evidence that exposure day significantly affected mortality rate in the malaria control treatments (HR = 1.21, z = 1.79, p = 0.074), which were monitored for up to 22 days after fungal exposure (Figure 4.4 inset).

Although fewer mosquitoes in the day 8 fungal exposure treatments survived to the end of the EIP, sporozoite prevalence (the proportion of mosquitoes with sporozoites in their salivary glands) was consistent across all treatment groups (Table 4.2). There was no evidence that sporozoite prevalence in the surviving mosquitoes was affected by either fungal exposure ($F = 0.25, df = 1, p = 0.614$) or exposure day ($F = 0.37, df = 1, p = 0.544$).

**Discussion**

This study found no significant evidence that *B. bassiana* exposure affected malarial parasite development. With *P. yoelii*, there was no evidence that fungal co- or superinfection affected oocyst prevalence, oocyst intensity or the number of sporozoites per oocyst. Similarly, with *P. falciparum*, there was no evidence of an effect of fungal superinfection on sporozoite
prevalence in surviving mosquitoes. Furthermore, there was no evidence in either experiment that malaria infection affected overall fungal virulence as measured by mosquito survival time.

Due to experimental constraints, many of the analyses associated with malaria infection parameters were based on small sample sizes. However, assuming \( \alpha = 0.05 \) and \( \beta = 0.2 \), all of the analyses would have been expected to detect proportional differences of \( \geq 0.8 \) except for the day 3 oocyst intensity assay (see Additional file 1). Although many of the analyses would have been insensitive to smaller impacts, low-level variation in malaria infection intensity or prevalence would likely be of limited importance in the context of transmission control. Mosquitoes with very few oocysts still become infectious, and the majority of mosquitoes that encountered fungus early in the malarial extrinsic incubation period (EIP) would not be expected to survive long enough to infect new hosts, particularly if higher fungal application rates were used (Blanford et al. 2011). For mosquitoes exposed late in the EIP, small reductions in sporozoite prevalence would likely have little impact on disease burden, particularly in high transmission areas where hosts may receive hundreds of infectious bites per year (Beier, Killeen & Githure 1999; Hay et al. 2005; Smith et al. 2005).

The results of this study generally support those of Blanford et al. (2005), who performed an experiment similar to the day 0 \( P. yoelii \) experiment using a different species of malaria (\( P. chabaudi \)) but the same mosquito species and fungal strain. At the oocyst stage, neither study found evidence of an impact of fungal co-infection on oocyst prevalence or density. At the sporozoite stage, Blanford et al. (2005) found that fungal co-infection significantly decreased the proportion of the initial (day 0) population which was both alive and infectious 14 days later. The sporozoite experiments in the current study differed from those in Blanford et al. (2005) in two ways: sporozoite prevalence was evaluated in mosquitoes exposed to fungus late in the EIP (day 8 or day 11) and the lethal and non-lethal effects of fungal exposure were analyzed separately. These analyses showed that, while fungal infection did significantly reduce mosquito survival in the day 8 exposures, there was no evidence of additional variation in sporozoite prevalence in the surviving population (i.e., those mosquitoes which were alive at the end of the EIP) for either exposure day. Given that there was also no evidence that malaria interacted with fungus to affect mosquito survival, these results suggest that the reductions in infectious mosquitoes in the late-exposure experiments were primarily mediated by mortality due to fungal infection rather than by interactions between \( P. falciparum \) and fungus.

Blanford et al. (2005) did note an upward trend in daily mortality rate in mosquitoes co-infected with fungus and malaria around the sporozoite release stage which was absent in the
treatment groups infected with fungus alone. The current study also found an apparent increase in daily mortality rate in the *P. yoelii* experiment starting at approximately day 12 in the ‘day 0 co-infection’ treatment relative to the ‘day 0 fungus alone’ treatment (see Additional file 2). However, the pattern was reversed in the equivalent super-infection treatments, with the ‘day 3 fungus alone’ treatment showing an increase in late stage daily mortality whereas the ‘day 3 superinfection’ treatment did not. Unfortunately, the very small sample sizes at the late stage of fungal infection make it difficult to interpret these patterns with great confidence, since differences of just one or two survivors would have led to large differences in proportional mortality.

The current study also agrees with a study by Fang *et al.* (2011) using a different mosquito-fungus combination (*Anopheles gambiae* and *M. anisopliae*). That group reported no effect of late superinfection on ultimate prevalence or density of *P. falciparum* sporozoites in the salivary glands. These consistent results across different species and application methods suggest that the lack of interaction between fungal and malarial infections may be a general phenomenon among common fungal entomopathogens, though these dynamics can be altered via genetic modification (2011).

Although malaria infection did not affect overall fungal virulence, mosquitoes exposed to fungus on the same day as the blood meal died marginally more quickly than those exposed three days later. Blood feeding greatly alters female physiology (Clements 1992), and previous work has shown that there could be trade-offs between immune response and reproduction in mosquitoes (Rono *et al.* 2010). Similar trade-offs could explain why mosquitoes might be more susceptible to fungal infection when they are actively digesting a blood meal. However, there is also evidence that blood feeding can temporarily increase resistance to fungal infection in *An. gambiae* and *Aedes aegypti* relative to individuals fed exclusively on glucose (Paula *et al.* 2011b; Mnyone *et al.* 2011), though no such increase was observed in studies on *An. stephensi* (Blanford *et al.* 2011).

There are a number of additional factors that could influence mosquito-malaria-fungus interactions. For example, hydric and nutritional stress can increase mosquito mortality associated with malaria infection (Ferguson & Read 2002b; Lambrechts *et al.* 2006; Aboagye-Antwi *et al.* 2010; Pollitt *et al.* 2013), and restricted diets are associated with a decrease in the mosquito melanization response (Koella & Sørensen 2002). The mosquitoes in this experiment were well-fed with easy access to sugar sources, potentially obscuring any resource competition or energetic tradeoffs involved in mounting an immune response that might occur under less favorable
nutritional conditions. There is also natural variation between and among mosquito species to malaria infection (Riehle et al. 2006, 2007), and the potential for complex effects of environmental variables, such as temperature, on mosquito immune function (Murdock et al. 2012a; b; Murdock, Moller-Jacobs & Thomas 2013). It is possible, therefore, that more diverse mosquito-parasite-pathogen interactions could be revealed under different contexts.

**Conclusion**

Overall there was little evidence for impacts of the fungal pathogen, *B. bassiana*, on infections of either rodent or human malaria within the mosquito host or for any reciprocal effects of malaria infection on fungal virulence. These results suggest that, compared to factors such as biopesticide coverage, dose and substrate (Blanford et al. 2011, 2012a), malaria-fungus interactions will have a relatively small impact on the potential of this fungus to reduce malaria transmission.

**Notes**

This work has been published at Malaria Journal with Matthew B. Thomas as coauthor (Heinig & Thomas 2015). The authors would like to acknowledge Janet Teeple for insectary and experimental support, Mark Kennedy, Courtney Murdock and Lillian Moller-Jacobs for assistance with the *P. falciparum* experiment, Derek Sim for animal support, Matt Jones for assistance with the molecular work, Nina Jenkins for providing fungal material, members of the Thomas and Read laboratory groups for helpful discussions and two anonymous reviewers for constructive feedback on the manuscript. The research leading to these results received funding from the European Union Seventh Framework Programme FP7/2007-2013 under grant agreement #306105 and from the Pennsylvania State University College of Agriculture Sciences. Additional materials may be found in Appendix C.
Figure 4.1. Cumulative proportional survival of adult mosquitoes in the *Plasmodium yoelii* experiment. Anopheles stephensi mosquitoes were fed either on *P. yoelii*-infected or control mice (indicated by line type), then exposed to tiles sprayed with *B. bassiana* conidial suspensions or blank oil (indicated by marker) either the same day as the blood meal (d0) or three days later (d3, indicated by line color). Each point represents the mean of five replicates (± standard error).
Figure 4.2. *Plasmodium yoelii* oocyst intensity (number of oocysts per midgut). Boxes represent the first and third quartiles, and whiskers encompass the values within 1.5 interquartile ranges of the lower and upper quartiles.
Figure 4.3. Number of *Plasmodium yoelii* sporozoites per oocyst ($10^3$) by treatment. Boxes represent the first and third quartiles, and whiskers encompass the values within 1.5 interquartile ranges of the lower and upper quartiles.
Figure 4.4. Cumulative proportional survival of adult mosquitoes in the *Plasmodium falciparum* experiment. *Anopheles stephensi* mosquitoes were fed on artificial membranes with either malaria-infected or uninfected human blood, then exposed to tiles sprayed with *B. bassiana* conidial suspensions ("fungus") or blank oil (indicated by marker) either eight (d8) or 11 days later (d11, indicated by line color). Each point represents the mean of three replicates (± standard error). (Inset) Proportional survival (y-axis) of malaria control treatments (fungus only and no infection groups) through day 20 following fungal exposure (x-axis).
Table 4.1. Summary of *P. yoelii* malaria infection parameters. Oocyst prevalence represents the proportion of An. stephensi mosquitoes with ≥1 oocyst in a sample of n = 20 mosquitoes per treatment. Of the infected mosquitoes (N), we report the mean (± standard error) and median (Med.) estimates for oocyst intensity and number of sporozoites per oocyst. The sample number decreases slightly in the sporozoite per oocyst because the PCR failed for some of the midgut samples.
<table>
<thead>
<tr>
<th>Exposure day</th>
<th>Fungal treatment</th>
<th>Proportion infectious (N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 8</td>
<td>Fungus</td>
<td>0.28 (39)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.32 (84)</td>
</tr>
<tr>
<td>Day 11</td>
<td>Fungus</td>
<td>0.33 (43)</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.38 (34)</td>
</tr>
</tbody>
</table>

Table 4.2. Summary of *P. falciparum* sporozoite prevalence. The proportion infectious *An. stephensi* mosquitoes is equal to the number of mosquitoes with successful sporozoite invasion of the salivary glands divided by the total number of mosquitoes dissected (N).
Chapter 5

Evaluating rate summation for predicting the influence of daily temperature variation

Abstract

A broad spectrum of ectotherm life history traits are strongly influenced by temperature, but most laboratory research on thermal performance is performed at constant temperatures representing long-term mean estimates of environmental conditions. Impacts of diurnal temperature variation are then predicted based on rate summation; rates at incremental temperature-time steps are summed to generate a composite rate. However, recent work suggests that rate summation may not predict the effects of realistic thermal variation when temperatures approach or exceed trait-specific thermal maxima.

This study evaluated in vitro radial growth rate of the fungus *Beauveria bassiana* at a number of constant temperatures to generate a thermal reaction norm curve. We used rate summation to predict the effects of a moderate and a high degree of realistic diurnal temperature variation, then tested the predictions empirically. Rate summation accurately predicted the impacts of moderate thermal variation but underestimated the impacts of high variation when temperatures exceeded the estimated critical thermal maximum. We explored whether this might result from carryover effects associated with exposure to extreme temperatures by performing a series of acute exposure assays in which fungal colonies were briefly exposed to extreme temperatures (34-42°C) then evaluated at the thermal optimum (26°C). We found that, under certain conditions, exposure to extreme temperatures delayed the resumption of growth when the fungus was returned to more favorable conditions. Once growth resumed, exposure time rather than temperature determined growth rates in the 40-42°C treatments but that growth rate was equivalent to the controls in the treatments closer to the critical thermal maximum (34-36°C).

This study demonstrates that, under certain conditions, rate summation does not accurately predict the impacts of realistic diurnal temperature variation and that these impacts are
determined in part by complex interactions between temperature and exposure time. Predictive models which rely on rate summation must therefore be interpreted with care, particularly when evaluating impacts near the extremes of an organism’s operative range.

**Introduction**

Temperature significantly impacts activity and development rates in ectotherms from a variety of disparate classes including insects, lizards, fish and fungi (Deutsch et al. 2008; Dell, Pawar & Savage 2011). Thus temperature is a crucial parameter in models estimating ectotherm performance in real-world scenarios. Examples include calculation of forensic post-mortem intervals based on necrophagous insect development rates (Catts & Goff 1992; Nabity, Higley & Heng-Moss 2006), development of food safety guidelines based on bacterial and fungal growth rates (Zwietering et al. 1991; Baert et al. 2007; Gougouli & Koutsoumanis 2010), estimation of insect-vectored pathogen extrinsic incubation periods to predict human disease dynamics (Paaijmans et al. 2010; Blanford et al. 2013; Chen et al. 2013) and projection of global climate change impacts (Deutsch et al. 2008; Paaijmans et al. 2013).

For practical reasons, the majority of studies assessing ectotherm performance are conducted at constant temperatures (e.g. the majority of studies cited by Deutsch et al. 2008). The resulting relationships may be described by asymmetric thermal performance curves defined by an operative range outside which performance is expected to be zero (Deutsch et al. 2008) (Figure 5.1). Because these relationships are nonlinear, thermal variation can have a positive, negative or neutral effect on performance, due to a mathematical phenomenon known as Jensen’s Inequality (Worner 1992). To account for these effects, rates under varying thermal conditions are often predicted by summing the incremental rates associated with some time interval (minutes, hours, etc.) to generate an overall rate estimate for a longer period of time (e.g. one day), a technique known as rate summation (Worner 1992). However, rate summation assumes that organisms have no thermal memory, i.e. temperatures experienced at one time point have no effect on performance at a later time point. This may not be a valid assumption, especially when temperatures approach or exceed the boundaries of the thermal operative range (Klass, Blanford & Thomas 2007a; Arrighi et al. 2013; Paaijmans et al. 2013). Even at moderate mean temperatures, exposure to temperatures outside the operative range can impact performance in ways that are not predicted by rate summation. For example, crossing the critical thermal
maximum may delay when growth or development resumes once an organism returns to favorable temperatures, perhaps due to heat-shock protein production and/or degradation (Parsell & Lindquist 1993; McMillan et al. 2005). Given that the vast majority of organisms experience at least some degree of temperature variation in the field, these types of carryover effects could significantly affect the reliability of ectotherm performance models under realistic thermal conditions.

To begin exploring these effects, we measured the impact of different thermal regimes using the entomopathogenic fungus, *Beauveria bassiana* (Bals.) Vuill. (isolate I93-825), as a model organism. We first evaluated *in vitro* growth rates at a number of constant temperatures and fit a common thermal performance model to the resulting data. We then used rate summation to predict rates under fluctuating thermal conditions and tested our predictions empirically for two different thermal regimes representing moderate and high degrees of realistic diurnal temperature variation. Having established that rate summation failed to predict the impact of the high variation treatment at the warm end of the operative range, we performed a series of assays evaluating carryover effects associated with movement between the thermal optimum (26°C) and temperatures exceeding CT_{max}. Our results demonstrated that, under certain conditions, realistic thermal variation affected pathogen performance in ways that were not predicted by rate summation and that these effects may have been mediated by complex interactions between temperature and exposure time.

**Methods**

**Design of radial growth rate experiments.** For these experiments, we used conidia from *Beauveria bassiana* isolate I93-825 which had been stored at -80°C on microporous beads (Pro-Lab Diagnostics, Austin, TX). Conidia were generated by placing 1-2 beads on either Sabouraud dextrose agar (SDA, Oxoid, UK) or potato dextrose agar (Oxoid, UK) slopes and incubating them at 25°C for approximately one week. We suspended the conidia in sterile 0.05% Tween (Sigma) and used a hemocytometer to adjust the concentration to 10^7 conidia ml^{-1}. We created germination plates by applying a drop of the conidial suspension to SDA in 60×15 mm petri plates of SDA (VWR, Radnor, PA) and spreading the solution with a microspatula. Three replicate plates were incubated for 20 hours at 26°C. We used a microscope to inspect approximately 300 conidia per plate for evidence of successful germination. Conidia were considered germinated if they had
hyphae at least the length of the conidium, and germination rate was calculated as a percentage of the total number of spores assessed. All suspensions used in the experiments had germination rates of >85%.

For each experimental treatment, we prepared 100×15 mm petri plates (VWR, Radnor, PA) containing approximately 18 ml SDA and drew two cardinal axes through the center of each plate base to use as measuring guides. We used a sterile 3-mm loop to inoculate the center of each plate with conidial suspension. The plates were then sealed with parafilm and placed into incubators which maintained the appropriate thermal regimen. Plates were always placed into incubators in the evening so that, for the fluctuating temperature treatments, initial temperatures were permissive for growth (i.e. within the operative range). We measured the diameter of each fungal colony on each axis starting from the first day on which growth was evident and once per day for seven days thereafter. If no growth occurred within eight days of inoculation, growth rate was considered zero. The daily radius for each plate was calculated by dividing the mean of the two diameter measurements by two. In a number of temperature treatments, particularly those near the thermal optimum, radial growth rate was initially rapid but slowed toward the end of the measuring period. We therefore selected the three days over which growth rate was highest and used that growth rate in our models for all temperature treatments. For each experiment, a reference set of plates was held at 26°C to act as a between-experiment control. All experiments were run using a 12-hour light/12-hour dark cycle at saturated humidity, and temperature was monitored using an OM-62 data logger (Omega Engineering Inc., Stanford, CN).

**Radial growth rate at constant and fluctuating temperatures.** We determined the effect of different constant temperatures on radial growth rate by measuring growth rate as described above at ten temperatures between 10-32°C. There were 4-7 replicate plates per temperature, and each temperature treatment was repeated at least twice over three separate experimental dates.

We also performed a separate set of growth assays under fluctuating thermal conditions. Incubators were programmed to simulate realistic thermal variation according to the model described by Parton and Logan (1981), and radial growth rates were measured as described above. Two different diurnal temperature ranges (DTR, the difference between the daily maximum and minimum temperature) were assessed: 8°C and 16°C. All 8°C assays were evaluated once (n=6-7 plates per thermal regime), and the 16°C assays were performed twice (n=4-7 replicate plates per thermal regime per experiment).

We used generalized linear models with a normal distribution and identity link to test for effects of temperature on radial growth rate. Because the experimental blocks were unbalanced
and the growth rate in the constant 26°C reference plates varied between experiments (Wald $\lambda^2=244.762$, df=6, p<0.001), each experiment was analyzed separately. Each model was evaluated for potential overdispersion based on deviance scores (deviance/df <5 for all models), and model fit was assessed visually.

**Thermal reaction norm models and predictions.** To make predictions about the impact of diurnal temperature variation on fungal growth rates, we began by fitting a number of common thermal performance models to our empirical constant temperature growth rates. Although thermal performance curves tend to be left-skewed, symmetrical models are commonly used to describe thermal performance and provide a baseline for comparison (Angilletta 2006). We fit two symmetric models: the Gaussian model, which describes a normal distribution, and the modified Gaussian model, which adds an additional exponential variable that allows for greater flexibility in curve breadth (Angilletta 2006). We also fit two skewed models developed specifically to describe thermal performance: the Logan and Briere models. The Logan model (Logan et al. 1976) is based on matched asymptotic expansions of theoretical enzyme kinetics models and empirical observations of rate decline above the thermal optimum. The Briere model (Briere et al. 1999) is derived exclusively from empirical observations but includes a minimal number of parameters, all of which are ecologically relevant. To minimize any effects of pseudoreplication, models were fit to the mean growth rates for each temperature treatment × experiment, and model parameters were estimated using the nonlinear regression procedure in SPSS 20.0 (IBM Corporation, Armonk, NY, USA).

We then calculated the corrected Akaike information criterion (AIC) for each model according to the following equation:

$$AIC = -2 \log \left( \frac{R}{n} \right) \left( 1 - n \right) + 2k + \frac{2k(k+1)}{n-k-1} \quad \text{eqn 5.1}$$

where $R$ is the residual sum of squares of the model, $n$ is the sample size (23) and $k$ is the number of parameters in the model including the error term. We used the AIC to compare the models because, while more traditional measures like $R^2$ describe how well a model describes the data, the AIC also accounts for model complexity in order to prevent overfitting (Burnham & Anderson 2002). We calculated the difference between each model’s AIC and the minimum AIC value for all the models ($\Delta_i$). Models with $\Delta_i$ of less than two were considered to have strong empirical support (Burnham & Anderson 2002).
We used GraphPad Prism (Windows v. 6.05) to estimate prediction bands around the best fit curve then used rate summation (Liu, Zhang & Zhu 1995; Paaijmans, Read & Thomas 2009) to predict daily growth rates under fluctuating thermal conditions as follows:

\[ \text{growth rate} = \sum_{i=0}^{24} g[T(i)] \, di \]

Eqn 5.2

where \( T(i) \) is mean temperature as a function of time of day as described by Parton and Logan (1981) and \( g(T) \) is the thermal reaction norm curve. We evaluated growth rate in time intervals of 15 minutes because it was the largest time interval which produced a relatively smooth curve (i.e. it accounted for the full thermal spectrum associated with various DTR treatments), but other time intervals produced similar patterns (Figure D.1). For time intervals in which mean temperatures exceeded \( CT_{\text{max}} \), we assumed incremental growth was equal to zero. Prediction envelopes for the fluctuating temperature treatments were estimated based on the prediction bands for the thermal reaction norm curve.

**Germination rate and temperature.** To assess whether differences in germination rate might be partially responsible for the observed impact of temperature on radial growth rate, we prepared germination plates as described above and placed them directly into incubators maintaining a number of constant temperatures (18, 26, 32, 34 and 36°C). Every 24 hours, we destructively sampled three plates from each temperature treatment and assessed approximately 300 conidia per plate for evidence of germination as described in the first section. Treatments were monitored until mean germination was >90%.

**High temperature assays.** Based on the assays described above, we determined that the thermal optimum for radial growth at constant temperatures was approximately 24-26°C, and the critical thermal maximum was approximately 32.5°C, though germination occurred at 34°C (see Results). We also established that, in the fluctuating temperature assays, growth occurred following transient exposure to temperatures up to 38°C (as in the 34°C treatment with an 8°C DTR) but was potentially blocked by repeated exposure to slightly higher temperatures (e.g. in the 32°C treatment with a 16°C DTR). We therefore performed a series of experiments to evaluate how growth rate was influenced by thermal history (i.e. how temperature at one time point might influence growth rate at a later time point).

We first explored how initial growth conditions affected our estimates of the critical thermal maximum. We prepared plates as described above and held them at 26°C or at temperatures above the estimated critical thermal maximum (34, 35 or 36°C). A parallel set of plates was
allowed to grow at 26°C for 1.5 days until the colony was established and had a diameter >3 mm (the size of the inoculation loop). We then recorded the initial radius of each plate and moved them to incubators maintaining constant 34, 35 or 36°C (“transfer” treatment). We continued to measure the plate radii daily for seven days. There were six replicate plates for each temperature treatment, and all measurements were standardized by subtracting the initial plate radius just prior to transfer (i.e. the standardized initial measurement was always 0 mm).

Next, we evaluated whether there were time- or temperature-dependent carryover effects associated with acute exposure to temperatures above the estimated critical thermal maximum that might explain the growth patterns observed in the fluctuating temperature treatments. We performed a series of experiments in which replicate plates were allowed to grow at a constant temperature of 26°C for 1.5 days, which was the point at which we could begin making reliable radial measurements (i.e. colony diameter was greater than the original 3-mm inoculation zone). The initial colony diameters were measured as described above, then the plates were moved to temperatures above the estimated critical thermal maximum (≥34°C) for a temporary exposure period (15 minutes to 12 hours) then returned to 26°C for the duration of the experiment. In the first assay, plates were exposed to either 34 or 36°C for periods of 4, 8, or 12 hours, and radii were measured once daily for eight days. Since growth resumed within 24 hours of the beginning of the exposure period in all of these treatment groups, we performed a second assay in which plates were held at 34 or 38°C for periods of 4, 8, or 12 hours and measured twice daily for three days. We also measured these plates immediately following the exposure period to determine whether growth continued during exposures. To evaluate the impact of temperatures near the maximum of the diurnal temperature ranges evaluated in the fluctuating temperature assays, we performed a final assay in which plates were held at 40 or 42°C for periods of 0.25, 0.5, 0.75, 1, 2, or 3 hours then measured twice per day for the first five days and once each day thereafter. Plates held at 26°C served as controls, and there were n=5-7 replicate plates per treatment.

All radial measurements were scaled to the initial radius as described above. Radial growth rates for assays 1 and 3 were estimated according to the procedure described in the first experiments and were compared using a generalized linear model with a gamma distribution and log link function. We began with a full factorial model including temperature and exposure time as factors and used backward stepwise elimination to remove nonsignificant (p<0.05) variables from the model. Where applicable, we compared growth rates within temperature treatments using the Bonferroni correction for multiple comparisons. Analyses were performed in SPSS 20.0
(IBM Corporation, Armonk, NY, USA). No growth rates were calculated for the second experiment due to the truncated observation period.

Results

**Growth rate at constant temperatures.** At constant temperatures, radial growth rate was left-skewed with the thermal optimum occurring at 24-26°C (Figure 5.2). The data were best described by the Briere model (Table 5.1). The fitted equation describing the thermal reaction norms for radial growth rate as a function of temperature ($T$) was:

$$f(T) = T(1.44 \times 10^{-3})(T + 1.58)\sqrt{(32.50 - T)}$$

Eqn 5.3

The model estimated the critical thermal maximum ($CT_{\text{max}}$) as 32.5°C. This was consistent with pilot data and the results from the acute thermal exposure experiments, which both indicated that no growth occurred at temperatures ≥34°C. The model also included an estimate of the thermal minimum (1.6°C). While this estimate was sufficient for our purposes, it should be interpreted with care since growth rate was not assessed at temperatures <10°C.

**Growth rate under conditions of moderate thermal variation.** Plates exposed to a moderate degree of thermal variation (diurnal temperature range [DTR]=8°C) exhibited changes in radial growth rate similar to those predicted by rate summation (Figure 5.3). At cooler temperatures (i.e. on the linear part of the curve), growth rates were similar to those observed at constant temperatures. At 26°C (i.e. on the concave portion of the curve), growth rate decreased relative to the constant temperature rate (mean difference ± SE: 0.359 ± 0.038, p<0.001). Thermal variation also “flattened” the top of the thermal performance curve such that there was no significant difference between growth rate at 22° and 26°C (mean difference ± SE: 0.073 ± 0.038, p=1.0). Although other experiments showed that the fungus does not grow at constant 34°C (see acute temperature exposure results), there was significant growth at this level of thermal variation (mean growth rate ± SE: 0.51 ± 0.03 mm/day). This rescue effect suggested that, while fungal growth likely stopped at inhospitable temperatures (>34°C), growth quickly resumed during the cooler periods of each day.

**Growth rate under conditions of high thermal variation.** Rate summation predicted the impact of high thermal variation (DTR=16°C) at temperatures up to 26°C but overestimated growth rates at warmer mean temperatures. As in the DTR=8°C experiment, rates were similar to
those observed at constant temperatures at the cool end of the curve and were depressed at the thermal optimum. At warmer temperatures, growth rates decreased rapidly. Interestingly, at 32°C, growth occurred in one experiment but not in the other. This may have been due to the fact that, although the mean temperatures for the two treatments were essentially the same (mean ± SE: 31.98 ± 0.17°C and 32.05 ± 0.17°C in the no growth and growth experiments, respectively), the daily maximum temperature was about 0.5°C higher in the no growth experiment (40.99 ± 0.08°C vs. 40.64 ± 0.34°C in the no growth and growth experiments, respectively). Spore germination rate and reference (constant 26°C) plate growth were also slightly higher in the growth experiment (93.1 ± 0.8 vs. 86.6 ± 3.5). The fact that such small differences between the treatments caused significant differences in growth rate suggested that the CT$_{max}$ for this magnitude of diurnal temperature variation was very close to 32°C.

**Impact of temperature on germination rate.** After 24 hours, mean germination was >90% in the 18, 26 and 32°C treatments (94, 99 and 98%, respectively, Figure 5.4). The 34°C treatment slowed but did not stop germination. Although a mean of only 37% of conidia germinated within 24 hours, 92% had germinated within 48 hours. In contrast, the 36°C treatment significantly reduced germination rates; mean germination remained <10% throughout the observation period (96 hours). While it is possible that additional germination might have occurred had we monitored the plates over a longer period, there was no significant difference in percent germination between the 72- and 96-hour time points (p=0.346).

**Impact of high temperatures on growth rate.** Among the plates which were initially allowed to grow at 26°C then permanently transferred to high temperatures, significant growth occurred in the 34°C transfer treatment (mean rate ± SE: 0.36 ± 0.07 mm/day) and, to a lesser degree, in the 35°C transfer treatment (overall radial increase ≤0.25 mm over 24 days, Figure 5.5). No growth occurred in the 36°C transfer treatment (monitored for 24 days) or in the plates held at constant 34, 35 or 36°C during the observation period (one month for the 36°C treatment and two months for the 34 and 35°C treatments).

Among the plates which were exposed to high temperatures then returned to the thermal optimum (26°C), acute exposure to high temperatures temporarily slowed or stopped growth in all of the treatments. Growth resumed within two days of exposure in all of the treatment groups. However, the time required for growth to resume depended on a combination of exposure temperature and exposure time. In the 34 and 36°C treatments, the colony radii one day after exposure decreased as both temperature and exposure period increased (Figure 5.6). Growth continued (albeit at a slower rate) during the 34°C four- and eight-hour treatments and the four-
hour 38°C treatment (Figure 5.7). In the eight-hour 38°C treatment, growth stopped (i.e. standardized mean radius was ≤0.1) but resumed within one day. In the 40 and 42°C assays, growth resumed within 0.5 days of the beginning of the shorter exposure treatments (≤1 hour at 40°C, ≤0.75 hours at 42°C), but longer exposures were associated with longer delays (Figure 5.8). In the 3-hour exposures, no growth was observed for 24 hours in the 40°C treatment, and in the 42°C treatment, growth did not resume until 1.5 days later.

Once growth resumed, there was no evidence of an impact of exposure temperature or time on maximum radial growth rate in the 34, 36, or 38°C treatments (p<0.05). In the 40 and 42°C treatments, there was a statistically significant impact of the three-hour exposure time on maximum radial growth rate relative to the controls (Table 5.2), but the decrease was not biologically relevant.

**Discussion**

This study demonstrates that, under certain thermal conditions, rate summation can provide a good approximation of the effects of realistic daily temperature fluctuations. At a moderate degree of diurnal temperature variation (e.g. a diurnal temperature range (DTR) of 8°C), rate summation accurately predicted radial growth rates at mean temperatures 10-34°C. This was despite the fact that, in the high mean temperature assays (32 and 34°C), the fungal colonies spent extended periods at temperatures above the estimated critical thermal maximum of 32.5°C (Figure 5.9). For example, plates in the 34°C mean treatment experienced maximum temperatures of approximately 38°C for periods of three hours each day (Figure 5.9), and in the high-temperature transfer experiments, four-hour exposure to 38°C slowed radial growth for ≤0.5 days. Rate summation also accurately predicted radial growth rate under higher levels of thermal variation (DTR=16°C) at mean temperatures 10-26°C but overestimated rates at higher mean temperatures. This was in part due to the fact that the higher mean temperature treatments included longer periods at inhospitable temperatures. For example, plates in the 32°C mean treatment spent approximately three hours per day at temperatures 40-41°C, and in the high-temperature transfer experiments, three-hour exposure to 40°C stopped radial growth for a full day. However, the maximum temperatures (and time period at those temperatures) in the 30°C mean treatment were similar to those in the 34°C mean DTR=8°C treatment, which was
accurately predicted by rate summation. These results suggest that the conditions under which rate summation will or will not generate accurate predictions are not immediately apparent from the thermal reaction norm curve and may be mediated by complex interactions among both the mean temperature and the diurnal temperature range (DTR).

Within the operative range, our results were consistent with Jensen’s inequality, which predicts that thermal variation will decrease performance in concave portions of the thermal reaction norm curve, increase performance in convex portions and have no effect in linear portions (Ruel & Ayres 1999). These qualitative relationships have been demonstrated in multiple studies in a wide variety of organisms (e.g. Paaijmans et al. 2010, 2013; Gougouli & Koutsoumanis 2010; Arrighi et al. 2013). In nonlinear portions of the curve, we found that rate summation accuracy decreased as the magnitude of diurnal temperature variation increased. These results were consistent with previous studies in insects (Taylor & Shields 1990) and amphibians (Niehaus et al. 2012; Arrighi et al. 2013).

We initially hypothesized that, when treatment temperatures exceeded the critical thermal maximum (CT$_{\text{max}}$), carryover effects would decrease the accuracy of rate summation predictions. We also predicted that the “realized” CT$_{\text{max}}$ (rCT$_{\text{max}}$) observed under fluctuating conditions would be less than the CT$_{\text{max}}$ estimated from the thermal reaction norms (Paaijmans et al. 2013). These hypotheses were correct in the high diurnal temperature range (DTR=16°C) treatments; rate summation overestimated growth rates at 30 and 32°C treatments (peak temperatures ~38 and 40°C, respectively). However, rate summation accurately predicted growth rates at 34°C in the moderate DTR (8°C) treatment. This was despite the fact that the peak temperature in this treatment (~38°C) was similar to that of the 30°C/DTR 16°C treatment and that plates in these treatments spent more time at temperatures outside the operative range.

This may have been due in part to the influence of initial thermal conditions on later growth rates. In our transfer experiments, we found that plates initially held at 26°C continued to grow at 34-35°C, while control plates at those temperatures showed no evidence of growth even after extended observation periods, despite high conidial germination rates at 34°C. Within a single organism, different traits are often differentially affected by temperature (e.g. Paaijmans et al. 2010; Niehaus et al. 2012; Murdock et al. 2012; Arrighi et al. 2013), so physiological or metabolic processes associated with early fungal development may be less tolerant of extreme temperatures than those that occur in more established colonies (Skowronska & Gottlieb 1970). In the fluctuating temperature experiments, all plates were initially placed in the incubators during the evening when temperatures were cooler than the treatment mean, so our model may have
underestimated $CT_{\text{max}}$ for plates in which growth began before being exposed to temperatures $\geq 34^\circ\text{C}$. However, when we repeated the curve selection and fitting procedures including the transfer experiment growth rates for 34 and 35°C, the curve predictions did not change in a meaningful way (data not shown).

The acute exposure assays demonstrated that exposure to extreme temperatures could temporarily halt growth, and rate summation does not account for these delays. Warmer temperatures and longer exposure times led to longer delays in resumption of growth once plates were returned to cooler temperatures. However, in the majority of treatments, these acute exposures had no significant impact on long-term growth rates. While exposure to temperatures $\geq 40^\circ\text{C}$ for 3 hours did negatively impact long-term growth rates, the delays associated with these treatments (1-1.5 days) would be much more important in most modeling contexts. While delays may be incorporated into thermal performance models to improve fit (e.g. Klass, Blanford & Thomas 2007), they may not be predictable based on thermal reaction norms.

These experiments demonstrate that both mean temperature and magnitude of variation determine growth rates under realistic thermal conditions and that dynamics close to $CT_{\text{max}}$ are complex and may not be predictable based on thermal reaction norms alone. These dynamics could be particularly important in the context of climate change, where accurate predictions of both $CT_{\text{max}}$ and “thermal safety margins” are vital to predicting ecological impacts of increasing temperatures (Paaijmans et al. 2013). Estimates of $CT_{\text{max}}$ should be interpreted with care, and when accuracy is important, researchers should consider evaluating ectotherms under temperature conditions which are representative of those in the field.

**Notes**

The author would like to thank Nina Jenkins for assistance with the fungal cultures. Additional material may be found in Appendix D.
Figure 5.1. Example thermal performance curve. Performance occurs at temperatures within an operative range, which is defined by a critical thermal minimum ($CT_{\text{min}}$) and maximum ($CT_{\text{max}}$). Maximum performance occurs at the thermal optimum ($T_{\text{opt}}$).
Figure 5.2. Candidate fitted thermal performance curves. Points represent mean radial growth rates, and error bars represent one standard error.
Table 5.1. Thermal performance models and fit results. In the model equations, $T$ represents temperature and additional letters ($a$, $b$, $c$, $d$, $f$) represent fitted model parameters. $RSS$ is the residual sum of squares, and $k$ is the number of parameters in the model (including the error term) used to calculate the corrected Akaike information criterion (AIC). $\Delta_i$ represents the difference between the proposed model AIC and the minimum AIC value in the group of models (-31.3). Models with $\Delta_i$ values of $<2$ are considered strongly supported by the data (Burnham & Anderson 2002).

<table>
<thead>
<tr>
<th>Model</th>
<th>Equation</th>
<th>$R^2$</th>
<th>RSS</th>
<th>$k$</th>
<th>AIC</th>
<th>$\Delta_i$</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gaussian</td>
<td>$ae^{-0.5 \times (</td>
<td>T-b</td>
<td>/c)^2}$</td>
<td>0.91</td>
<td>0.84</td>
<td>4</td>
<td>-22.8</td>
</tr>
<tr>
<td>Modified</td>
<td>$ae^{-0.5 \times (</td>
<td>T-b</td>
<td>/c)^d}$</td>
<td>0.92</td>
<td>0.78</td>
<td>5</td>
<td>-20.2</td>
</tr>
<tr>
<td>Gaussian</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Logan</td>
<td>$a \left( \frac{1}{1 + f e^{-bt}} \right) - e^{-\frac{(c-T)}{d}}$</td>
<td>0.98</td>
<td>0.23</td>
<td>6</td>
<td>-28.7</td>
<td>2.6</td>
<td>Logan et al. 1976</td>
</tr>
<tr>
<td>Briere</td>
<td>$aT(T - b)\sqrt{c - T}$</td>
<td>0.96</td>
<td>0.36</td>
<td>4</td>
<td>-31.3</td>
<td>0.0</td>
<td>Briere et al. 1999</td>
</tr>
</tbody>
</table>
Figure 5.3. Observed and predicted radial growth rates under constant and fluctuating conditions. Points indicate mean (± one standard error) radial growth rates at constant (diurnal temperature range [DTR] = 0°C) or fluctuating (DTR = 8°C and 16°C) temperatures. Lines indicate best fit curves (at DTR=0°C) and rate summation predictions, and bands represent 95% prediction bands.
Figure 5.4. Impact of temperature on conidial germination rate. Bars represent the mean (± one standard error) of n=3 replicate plates per temperature treatment per time point. Temperature treatments were no longer monitored once germination reached 90%.
Figure 5.5. Impact of temporary exposure to 26°C on radial growth rate at high temperatures. Plates were held either at constant temperatures or at 26°C for 1.5 days then transferred to new temperatures (“transfer”). Radial measurements were standardized by subtracting the initial radius at the beginning of the temperature treatment (i.e. just prior to transfer to the warmer temperatures), and lines indicate the mean radial measurements (± one standard error) of n=6 replicate plates per treatment. No growth occurred in the 34, 35 and 36°C constant treatments and the 36°C transfer treatment (results not shown).
Figure 5.6. Impact of acute exposure to 34 or 36°C on radial growth. Color indicates temperature and line type indicates length of exposure. Mean standardized radial measurements (± one standard error) for n=5-6 replicate plates per treatment are shown.
Figure 5.7. Impact of acute exposure to 34 or 38°C on radial growth. Color indicates temperature and line type indicates length of exposure. Mean standardized radial measurements (± one standard error) for n=5 replicate plates per treatment are shown. Plates measured before and just following treatment (indicated by gaps in lines) then monitored for three more days.
Figure 5.8. Impact of acute exposure to 40 or 42°C on radial growth. Plates were exposed to either 40°C (above) or 42°C (below) for 0.25-3 hours (indicated by line type). Mean standardized radial measurements (± one standard error) for n=5-7 plates per treatment are shown.
<table>
<thead>
<tr>
<th>Treatment (temperature, time)</th>
<th>N</th>
<th>Mean ± SE</th>
<th>p&lt;0.05</th>
</tr>
</thead>
<tbody>
<tr>
<td>40°C 3 h</td>
<td>5</td>
<td>2.22 ± 0.05</td>
<td>c</td>
</tr>
<tr>
<td>2 h</td>
<td>6</td>
<td>2.27 ± 0.06</td>
<td>bc</td>
</tr>
<tr>
<td>1 h</td>
<td>5</td>
<td>2.40 ± 0.08</td>
<td>abc</td>
</tr>
<tr>
<td>0.75 h</td>
<td>6</td>
<td>2.44 ± 0.04</td>
<td>ab</td>
</tr>
<tr>
<td>0.5 h</td>
<td>6</td>
<td>2.51 ± 0.03</td>
<td>a</td>
</tr>
<tr>
<td>0.25 h</td>
<td>6</td>
<td>2.42 ± 0.07</td>
<td>abc</td>
</tr>
<tr>
<td>0 h (control)</td>
<td></td>
<td></td>
<td>ab</td>
</tr>
<tr>
<td>42°C 3 h</td>
<td>6</td>
<td>2.03 ± 0.06</td>
<td>c</td>
</tr>
<tr>
<td>2 h</td>
<td>6</td>
<td>2.24 ± 0.08</td>
<td>bc</td>
</tr>
<tr>
<td>1 h</td>
<td>6</td>
<td>2.36 ± 0.07</td>
<td>ab</td>
</tr>
<tr>
<td>0.75 h</td>
<td>6</td>
<td>2.39 ± 0.07</td>
<td>ab</td>
</tr>
<tr>
<td>0.5 h</td>
<td>5</td>
<td>2.39 ± 0.04</td>
<td>ab</td>
</tr>
<tr>
<td>0.25 h</td>
<td>7</td>
<td>2.52 ± 0.06</td>
<td>a</td>
</tr>
<tr>
<td>0 h (control)</td>
<td></td>
<td></td>
<td>ab</td>
</tr>
<tr>
<td>26°C control</td>
<td>4</td>
<td>2.51 ± 0.02</td>
<td></td>
</tr>
</tbody>
</table>

Table 5.2. Radial growth rate following acute exposure to 40 or 42°C. Each temperature group was analyzed separately with the 26°C treatment serving as the control for each analysis. Different letters indicate significant differences (p<0.05) between the estimated marginal means for each exposure time.
Figure 5.9. Experimental thermal regimes in which temperatures exceeded the estimated CT$_{\text{max}}$. Different mean temperatures are represented by different colors, and different diurnal temperature ranges (DTR) are represented by line type. Regimes were based on the thermal curves described by Parton and Logan (1981).
Chapter 6

Discussion

Fungal biopesticides are a promising technology for malaria mosquito control. In an environment where insecticide resistance is evolving more quickly than industry can develop new products, fungal entomopathogens offer a possible alternative to synthetic chemicals, not only killing mosquitoes which are no longer susceptible to traditional insecticides (Farenhorst et al. 2009; Blanford et al. 2011) but also minimizing selection for new resistant phenotypes via their slow rate of kill (Thomas & Read 2007; Read, Lynch & Thomas 2009; Koella et al. 2009; Lynch et al. 2012). However, as living pathogens of small-bodied ectotherms, fungi are strongly affected by environmental temperature (Blanford & Thomas 2001; Thomas & Blanford 2003; Kikankie et al. 2010), and it was not known how fungal virulence related to malarial extrinsic incubation period.

To answer this question, we evaluated virulence of the candidate fungal biopesticide *Beauveria bassiana* in *Anopheles stephensi* mosquitoes at temperatures between 10-34°C, which covers the range relevant to malaria transmission (Chapter 2). We found that, although fungal virulence varied significantly with temperature, this biopesticide consistently killed >90% of exposed mosquitoes before they would have been expected to begin transmitting malaria (i.e. before the predicted end of the malaria extrinsic incubation period). These results were consistent across a number of experiments involving different insect species (*Anopheles gambiae*) and levels of diurnal temperature variation (±0°C, ±6°C).

Having determined that fungus should successfully block malaria transmission in mosquitoes which became infected with both the biopesticide and the malaria parasite at the same time, we turned to an SEI model to determine how delayed fungal exposures might affect disease transmission outcomes. We found that, even when the daily probability that mosquitoes would encounter the fungus was low, the model predicted that fungus would significantly reduce densities of infectious mosquitoes.

Next, we combined the model predictions with mean monthly temperature data from across Africa to evaluate where and when fungus would have the greatest impact on infectious mosquito
densities. When the daily probability of fungal infection was low, temperature was an important
determinant of fungal efficacy. At higher daily probabilities of infection, reductions in infectious
mosquito densities were uniformly >90%. However, in areas with high entomological inoculation
rates, predicted EIR remained >20 for the majority of temperatures even at our benchmark “high”
daily probability of fungal infection (c=0.16). This could be addressed by increasing the daily
probability of infection via alternative delivery technologies, by making use of fungi genetically
altered to increase virulence (St. Leger et al. 1996; Fang et al. 2005; Wang & St. Leger 2007; Qin
et al. 2010) or by taking a multifaceted integrated vector management approach utilizing a more
diverse tool set to decrease disease transmission (Thomas et al. 2012).

One of the most attractive features of fungal entomopathogens is their potential to
significantly decrease selection for resistance traits. Fast-acting chemicals insecticides strongly
select for resistant phenotypes by killing susceptible individuals before they are able to reproduce.
In contrast, fungal biopesticides act much more slowly, allowing susceptible mosquitoes to
realize at least part of their potential reproductive output (Mouatcho et al. 2011; Blanford et al.
2011; Darbro et al. 2012). Simplified modeling suggests that even modest delays in mosquito
mortality can significantly decrease the relative fitness of resistant mosquitoes and extend the
potential useful life of a given vector control tool (e.g. Read, Lynch & Thomas 2009).
Mosquitoes’ potential to evolve resistance to late-life-acting pesticides in the context of malaria
control has been discussed at length (Thomas & Read 2007; Read, Lynch & Thomas 2009;
Koella et al. 2009; Lynch et al. 2012), but so far, there have been no reports of fungal resistance
in mosquitoes.

Lacking an understanding of what particular resistance mechanisms might evolve in mosquito
populations, it is difficult to predict whether evolution would favor resistant phenotypes, and, if
they were favored, how rapidly they would proliferate throughout the population. Fungal
resistance in other insects is highly variable. In aphids, resistance to Pandora neoaphidis fungus
appears to be mediated by a number of symbiotic bacterial species (Ferrari et al. 2001;
Scarborough, Ferrari & Godfray 2005; Łukasik et al. 2013a; b). In greater wax moths,
resistance may involve the “refocusing” of certain stress management and immunity
genes from the fat body to the integument (Dubovskiy et al. 2013). In fruit flies,
mechanisms of resistance are not clear, but there is variation in resistance in flies from
different locations, suggesting possible local adaptation (Tinsley, Blanford & Jiggins
2006). Finally, gregarious locusts may reduce their susceptibility to fungal infection by
upregulating antibacterial activity in the hemolymph, but they do so in a density-dependent manner (Wilson et al. 2002). In mosquitoes, resistance to chemical insecticides has often involved improvements in enzyme-mediated detoxification and sequestration of harmful compounds or by nucleotide changes which reduce or eliminate target site sensitivity (Hemingway et al. 2004), and many of these adaptations significantly reduce mosquito fitness in the absence of insecticides (Agnew et al. 2004; Labbé et al. 2007). Research suggests that these genetic mechanisms do not confer any resistance to fungal biopesticides (Farenhorst et al. 2009, 2010; Blanford et al. 2011), but behavioral resistance (Gatton et al. 2013) or “resilience” (Govella, Chaki & Killeen 2013) could have significant impacts fungal effective coverage rates.

Assuming a genetic basis of fungal resistance in mosquitoes, modeling by Lynch et al. (2012) suggested that fungal biopesticides would indeed reduce selection for resistance and extend product life, with a tradeoff occurring between malaria control and resistance selection. However, Govella et al. (2013) argue that plasticity rather than genetic variation may be responsible for behavioral resistance phenotypes. Regardless of the underlying source of behavioral variation, our research suggests that temperature could play a key role in these dynamics. This has been shown in other systems; Stacey et al. (2003) showed that temperature interacted with pea aphid strain to determine resistance to a fungal entomopathogen, and in crickets, there is a temperature-dependent tradeoff between resistance to certain bacterial pathogens and reproductive output (Adamo & Lovett 2011). Our work in Chapter 2 suggested how differential variation in mosquito, malarial and fungal performance could cause non-intuitive downstream impacts on predicted fungal efficacy. Particularly at low effective coverage levels, temperature could strongly impact the resistance-control tradeoff. For example, we demonstrated that mosquitoes exposed to fungus die more quickly at 26-28°C than at warmer or cooler temperatures. At the same time, gonotrophic cycle length decreases with temperature up to approximately 35°C (Mordecai et al. 2013), and larvae reared at higher temperatures tend to be smaller and develop more quickly (Bayoh & Lindsay 2003). Interactions between temperature, fungal virulence and mosquito life history traits could either reduce or enhance selection for fungus-resistant mosquito phenotypes depending on thermal environment.

In addition, our model did not account for effects of temperature on immature mosquito dynamics. Fungal biopesticides’ relatively slow rate of kill allows mosquitoes to realize some reduced proportion of their lifetime reproductive potential (Mouatcho et al. 2011; Blanford et al. 2011; Darbro et al. 2012). This reduced fecundity could alter density-dependent effects associated with larval population sizes (Timmerman & Brigel 1993; Gimnig et al. 2002) and could
potentially do so in a temperature-dependent manner. Increased competition at the larval stage has the potential to affect a variety of adult characteristics including pupal emergence rate, age at emergence, adult body size and fecundity (Reisen 1975; Lyimo, Takken & Koella 1992; Gimnig et al. 2002; Grech, Maung & Read 2007). The modeling framework utilized in this study does not account for larval development, but more complex stage-structured models may yield valuable insights into long-term mosquito population dynamics in the presence of fungal biopesticide interventions (e.g. Beck-Johnson et al. 2013). These could in turn be used to identify thermal conditions under which density-dependent processes might have unintended effects on mosquito population dynamics and/or disease transmission.

Co-infecting pathogens can interact with each other and with their hosts in ways that are not predictable from single-infection assays. For consistency, the experiments in Chapter 2 were all performed in mosquitoes that were not infected with malaria. However, it is possible that fungal co- or superinfection at certain key points in the malaria life cycle could impact parasite infection progress either directly, by competing for resources, or indirectly, by positively or negatively affecting immune responses. Malaria parasites, especially at high densities, could in turn affect fungal entomopathogen infection progress. In Chapter 3, we evaluated malaria infection parameters in A. stephensi infected with B. bassiana either on the same day as the blood feed (co-infection) or at a later date (superinfection). We found no effect of fungal co- or superinfection (three days later) on Plasmodium yoelii oocyst prevalence, oocyst density or sporozoite prevalence. We also found no significant impact of late fungal superinfection (eight or eleven days after the blood feed) on Plasmodium falciparum sporozoite prevalence. There was also no impact of malaria infection on fungal virulence in any of the experiments.

We are satisfied that, if malaria infections did have a negative impact on fungal virulence, we should have observed it in these experiments. However, these assays were performed under optimal conditions for the mosquitoes, so any potential immune trade-offs would not have been as pronounced as they might have been under more stressful conditions (e.g. lower nutrition and/or higher temperatures). We cannot rule out potential impacts of fungal infection on malarial development under less favorable conditions, but we would expect that they would only improve fungal virulence.

In Chapter 4, we explored how realistic diurnal temperature variation affected the in vitro growth rate of B. bassiana. We found that rate summation based on growth rates at constant temperatures tended to overestimate growth rates when there was a large degree of diurnal temperature variation which caused temperatures to exceed the critical thermal maximum. Both
mean temperature and the magnitude of thermal variation affected growth rate in ways that were not immediately obvious from the thermal reaction norm curve. We performed a number of acute thermal exposure assays that demonstrated that, while exposure to thermal extremes paused growth, it did not affect long-term growth rate after growth resumed.

These experiments identified a problem with the way we predict performance under realistic thermal conditions which has potentially far-reaching effects in a variety of fields (e.g. forensics (Catts & Goff 1992; Nabi, Higley & Heng-Moss 2006), food safety (Zwietering et al. 1991; Baert et al. 2007; Gougouli & Koutsoumanis 2010), disease forecasting (Paaijmans et al. 2010; Blanford et al. 2013; Chen et al. 2013) and climate change research (Deutsch et al. 2008; Paaijmans et al. 2013)). But while it is a known issue (Taylor & Shields 1990; Klass, Blanford & Thomas 2007a; Niehaus et al. 2012; Arrighi et al. 2013), there is as yet no solution. In part, this is because even different traits within the same individual may have different relationships with temperature (Paaijmans et al. 2010, 2013; Niehaus et al. 2012; Murdock et al. 2012a; Arrighi et al. 2013), so it is possible that there are no universal rules governing the behavior of traits at temperatures above the critical thermal maximum. It would be interesting to explore whether experimentally-determined delays like the ones in this experiment could be used to improve predictions at high temperatures.

Taken together, the results of this dissertation suggest that fungal biopesticides are a viable tool for malaria control, that fungal efficacy should not be impeded by a concomitant malaria infection and that care should be taken in using rate summation to predict quantitative impacts of realistic diurnal temperature variation on organismal performance.
Appendix A

Supplement to Chapter 2

Additional methods

**Mosquito rearing.** *Anopheles stephensi* and *Anopheles gambiae* mosquitoes were reared at 26-27°C and 75-80% relative humidity with a 12-h light/12-h dark cycle. Eggs were placed in plastic tubs containing approximately 1.5 L of distilled water. Four days after hatching, the larvae were separated into groups of 400 larvae per tub and provided with 10 mg powdered TetraFin fish food daily. After pupation, pupae were placed into cages for emergence. Adults were allowed to feed on a 10% glucose solution supplemented with 0.05% para-aminobenzoic acid (PABA) *ad libitum*, and experiments were performed exclusively on 3-5 day-old females.

**Preparation of clay tile substrates.** White earthenware clay (Clay King, Spartanburg, SC, USA) was mixed with distilled water and allowed to soak overnight. The next day, the clay-water mixture was homogenized using an electric paint stirrer. The mixture was poured into 150 mm-diameter petri plates and allowed to harden for approximately one week until the interior of the clay was completely dry.

**Production and formulation of fungal conidia.** Microporous beads coated in fungus were retrieved from long-term storage at -80°C and incubated at 25°C on potato dextrose agar (Oxoid, UK) slopes to produce conidia. The conidia were then suspended in sterile 0.05% Tween 80 (Sigma) at a concentration of $10^6$ conidia ml$^{-1}$. One ml of suspension was added to 75 ml of sterile liquid medium (4% d-glucose, 2% yeast extract (Oxoid, UK) in tap water), and the culture was incubated on a shaker at 24°C and 160 rpm for three days. The culture was then combined with 75 ml of sterile water and used to inoculate mushroom spawn bags (Unicorn, Garland, Texas, USA) containing a sterile mixture of 1 kg barley flakes (Bobs Red Mill, Milwaukie, Oregon, USA) and 600 ml tap water. The bags were sealed and incubated at 24°C for 10 days. The contents were then dried in paper bags for 4 days to reduce their moisture content below 20%. A
Mycoharvester (Acis Manufacturing, Devon, UK) was used to harvest conidia from the barley, and the harvested conidia were dried over silica gel to a moisture level of 5%. The conidia were then sealed in foil sachets and stored at 5°C.

Dry conidia were suspended in an oil mixture of 80% Ondina:20% Isopar M at concentrations of either $10^7$ or $10^9$ conidia ml$^{-1}$. Viability was assessed by plating three samples of the $10^7$ conidia ml$^{-1}$ suspension on Sabouraud dextrose agar (Oxoid, UK). The plates were incubated at 25°C for 20 h. Following incubation, a microscope was used to assess germination visually. Those conidia which had germination tubes at least the length of the conidium were classified as germinated. Approximately 300 conidia were assessed on each plate. All suspensions used in the exposures had mean percent germination rates of ≥85%.

**Rate summation.** We used rate summation (Liu, Zhang & Zhu 1995; Paaijmans, Read & Thomas 2009) to predict the impact of the experimental diurnal temperature range (12°C) on malarial extrinsic incubation period (EIP, parameter $t_E$). Corrected EIP ($t_{E*}$) was estimated as follows:

$$t_{E*} = \left( \sum_{i=0}^{24} r(T(i))\, di \right)^{-1}$$  \hspace{1cm} \text{eqn A.1}$$

where $T(i)$ is mean temperature as a function of time of day (evaluated in hourly increments) as described by Parton and Logan (1981) (see below) and $r(T)$ is parasite development rate as a function of mean temperature, $T$, as estimated by Mordecai et al. (2013):

$$r(T) = T(1.11 \times 10^{-4})(T - 14.7)(34.4 - T)^{1/2}$$  \hspace{1cm} \text{eqn A.2}$$

Where daily fluctuation included peak temperatures above the critical maximum temperature for parasite development (i.e. where growth is zero, ~34.4°C), we constrained parasite growth to the standard curve. This was a conservative assumption that likely underestimated the impact of high temperature fluctuations since it is expected that even transient exposures above the critical maximum temperature could have additional negative effects on growth even when temperatures return to permissive levels (Paaijmans et al. 2013). However, there are currently no data available to enable us to characterize these effects explicitly.

**Parton and Logan temperature model.** Parton and Logan (1981) developed a model which described realistic fluctuating daily temperature patterns using a sine function during the day and an exponential decline to a thermal minimum at night. For these experiments, we assumed a 12-
hour day/12-hour night cycle. Temperature was calculated as a function of time, \( i \) (expressed as hours past midnight; 6:00 pm = 18.0), according to the following equation:

\[
T(i) = \begin{cases} 
T_{\text{min}} + (T_{\text{max}} - T_{\text{min}}) \sin \left( \frac{\pi(i - 6)}{15} \right), & i < 12 \\
T_{\text{min}} + T_s e^{-i} + \frac{(T_s - T_{\text{min}})e^{-1}}{1 - e^{-3}}, & i \geq 12
\end{cases}
\]

where \( T_{\text{max}} \) and \( T_{\text{min}} \) are the daily maximum and minimum temperatures, respectively, and \( T_s \) is the temperature at sunset. \( T_{\text{max}} \) and \( T_{\text{min}} \) were calculated as follows:

\[
T_{\text{max}} = T_{\text{median}} + \frac{D}{2}
\]

\[
T_{\text{min}} = T_{\text{median}} - \frac{D}{2}
\]

where \( D \) is the diurnal temperature range (DTR) and \( T_{\text{median}} \) is the median daily temperature, estimated based on the mean daily temperature, \( T_{\text{mean}} \), according to the following equation:

\[
T_{\text{median}} = T_{\text{mean}} + 0.057582D
\]

The temperature at sunset, \( T_s \), was calculated as follows:

\[
T_s = T_{\text{min}} + (T_{\text{max}} - T_{\text{min}}) \sin \left( \frac{12\pi}{15} \right)
\]

See Figure A.1 for example temperature readouts based on the Parton and Logan (1981) model.

**Malaria and fungus infection model.** We utilized the susceptible-exposed-infectious (SEI) model developed by Hancock *et al.* (2009) to predict how environmental temperature and fungal coverage would impact the ability of *B. bassiana* to reduce infectious mosquito population densities (Figure A.2). The model tracks mosquitoes in three classes: susceptible (\( S_1 \)), exposed (\( E_1 \) – mosquitoes infected with malaria but not infectious) and infectious (\( I_1 \) – mosquitoes able to transmit malaria). At the same time, it tracks if and when mosquitoes become infected with fungus in a parallel set of classes (\( S_2, E_2, I_2 \)). The numbers of mosquitoes in each class depend upon time overall (\( t \)), time since malaria infection (\( p \)), and time since fungal infection (\( u \)) and are represented by the following equations:

\[
S_1(t) = \int_0^t \varepsilon \theta S_1 [t - \tau] d\tau
\]
where \( \theta_{s_1}[t - \tau] = \exp[-(t - \tau)(\mu + abx + F)] \) eqn A.9

\[ S_2(t, u) = S_1(t - u) F \theta_{s_2}[u] \] eqn A.10

where \( \theta_{s_2}[u] = \exp\left[-\int_0^u \mu + M_F(\xi) + abx \, d\xi \right] \) eqn A.11

\[ E_1(t, p) = S_1(t - p) abx \theta_{E_1}[p], \quad 0 \leq p < t_E \] eqn A.12

where \( \theta_{E_1}[p] = \exp[-p(\mu + F)] \) eqn A.13

\[ E_2(t, p, u) = H[u, p] E_1(t - u, p - u) F \theta_{E_2}[u, u] \]
\[ + (1 - H[u, p]) S_2(t - p, u - p) abx \theta_{E_2}[p, u], \quad 0 \leq p < t_E \] eqn A.14

where \( \theta_{E_2}[t_1, t_0] = \exp \left[- \int_{t_0}^{t_1} \mu + M_F(\xi) \, d\xi \right] \) eqn A.15

and \( H[u, p] = \begin{cases} 1, & u \leq p \\ 0, & u > p \end{cases} \) eqn A.16

\[ I_1(t) = \int_0^t E_1(\tau, t_E) \theta_{I_1}[t - \tau] \, d\tau \] eqn A.17

where \( \theta_{I_1}[t - \tau] = \exp[-(t - \tau)(\mu + F)] \) eqn A.18

\[ I_2(t, u) = I_1(t - u) F \theta_{I_2}[u, u] + \int_{t-u}^t E_2(\tau, t_E, u - (t - \tau)) \theta_{I_2}[t - \tau, u] \, d\tau \] eqn A.19

where \( \theta_{I_2}[t_1, t_0] = \exp \left[- \int_{t_0}^{t_1} \mu + M_F(\xi) \, d\xi \right] \) eqn A.20

where \( \varepsilon \) is the adult mosquito recruitment rate, \( a \) is the human bite rate, \( b \) is malaria transmission efficiency, \( x \) is the proportion of humans with transmissible malaria, \( t_E \) is the length of the malarial EIP, \( \mu \) is the background mosquito mortality rate, \( F \) is the rate of fungal infection, and \( M_F \) is additional mosquito mortality due to fungal infection:

\[ M_F(u) = \beta \mu_F(\mu_F u)^{\theta - 1} \] eqn A.21

where \( \mu_F \) and \( \theta \) are temperature-dependent constants. See Table A.1 for additional information about the parameters.

The equilibrium densities of mosquitoes in each class are calculated based on the equations below. One asterisk (e.g. \( E_1^* \)) indicates the equilibrium density of mosquitoes in a given class at
time \( t \) and two asterisks (e.g. \( E_1^{**} \)) indicates the equilibrium total density of mosquitoes in that class.

\[
S_1^* = \frac{E}{F + abx + \mu}
\quad \text{eqn A.22}
\]

\[
E_1(p) = abxS_1^* \exp(-p(F + \mu))
\quad \text{eqn A.23}
\]

\[
E_1^{**} = \frac{abxS_1^* \left(1 - \exp(-t_E(F + \mu))\right)}{F + \mu}
\quad \text{eqn A.24}
\]

\[
I_1^* = \frac{abxS_1^* \exp(-t_E(F + \mu))}{F + \mu}
\quad \text{eqn A.25}
\]

\[
S_2^*(u) = S_1^* F \theta_{S_2}[u]
\quad \text{eqn A.26}
\]

\[
S_2^{**} = \int_0^\infty S_2^*(u) \, du
\quad \text{eqn A.27}
\]

\[
E_2^*(p, u) = H[u, p]E_1^*(p - u)F \theta_{E_2}[u, u] + (1 - H[u, p])S_2^*(u - p)abx \theta_{E_2}[p, u]
\quad \text{eqn A.28}
\]

\[
E_2^{**} = \int_0^\infty \int_0^{T_E} E_2^*(p, u) \, dp \, du
\quad \text{eqn A.29}
\]

\[
I_2^*(u) = I_1^* F \theta_{I_2}[u, u] + \int_0^u E_2^*(t_E, \tau) \theta_{I_2}[u - \tau, u] \, d\tau
\quad \text{eqn A.30}
\]

\[
I_2^{**} = \int_0^\infty I_2^*(u) \, du
\quad \text{eqn A.31}
\]

Additional model details may be found in Hancock et al. (2009). Proportion reduction in infectious mosquito density was calculated as follows:

\[
\text{Proportional reduction} = \frac{I_0^{**} - (I_1^{**} + I_2^{**})}{I_0^{**}}
\quad \text{eqn A.32}
\]

where \( I_0^{**} \) was the predicted total equilibrium infectious mosquito density in the absence of fungus, and \( I_1^{**} + I_2^{**} \) was the density in the presence of fungus at a given temperature and AR.

**Sensitivity analysis.** Since there was a degree of uncertainty in both the estimates used to parameterize the curves fit by Mordecai et al. (2013) and in the curve fits themselves, we performed a local sensitivity analysis to assess the impact of variation in each parameter on the predicted proportion reduction in infectious mosquito density. Each parameter estimate was increased or decreased by 5% or 10% at each temperature, and the model was reevaluated using
the revised estimates. We found that, although the equilibrium total density of infectious mosquitoes was affected by changes in the parameter estimates, the proportion reduction in infectious mosquito density was relatively robust to this level of variation, particularly at the higher daily probability of fungal infection ($c = 0.16$) (Figure A.8). We were primarily concerned with decreases in proportion reduction in infectious mosquito density (i.e. reductions in predicted fungal efficacy), and at the high AR, this level of variation reduced the predicted proportion reduction in infectious mosquito density by less than 0.03 at $c = 0.016$ and less than 0.04 at $c = 0.04$ across all temperatures. Results at the low AR were similar (Figure A.9).

Different parameters affected the model in different ways. The proportion reduction in infectious mosquito density was most sensitive to changes in EIP, with decreases in EIP leading to decreases in proportion reduction. This was due to the fact that decreasing the EIP always increased the predicted equilibrium total infectious mosquito density, but it had more of an impact on density when fungus was present than when it was absent (i.e. $c = 0$, Figure A.11). Variation in $\mu_F$ produced similar patterns. In contrast, decreases in background mosquito mortality rate ($\mu$) increased the proportion reduction in infectious mosquito density, despite the fact that decreases in mortality increased infectious mosquito densities. This was because background mortality rate had a much larger impact on infectious mosquito density in the absence of fungus than in the presence of fungus.

Since the predictions were most sensitive to variation in the EIP, we reevaluated the model at the high AR with EIP increases and decreases of 40%, a range which encompassed all of the original data used to fit EIP curve by Mordecai et al. (2013) and which, at 30°C, resulted in an EIP estimate of just 5.6 days. At the higher daily probability of fungal infection ($c = 0.16$), the model predicted that $B. bassiana$ should still reduce infectious mosquito densities by $>75\%$ (Figure A.12). We also looked at how other parameters in the model would interact with EIP to decrease the proportion reduction in infectious mosquito density. We reduced EIP by 10%, repeated the sensitivity analysis, and found that increases or decreases of 10% in the other parameters had minimal impact on the predicted reduction, decreasing predictions by $\leq 0.016$ at $c=0.04$ and $\leq 0.014$ at $c=0.16$. 
Figure A.1. Example temperature readouts from fluctuating temperature treatments. Incubators were programmed to mimic realistic diurnal temperature variation as described by Parton and Logan (1981). Points represent one day of temperature readings taken every 15 min in incubators programmed with mean temperatures between 20-32°C (represented by point color) and a diurnal temperature range of 12°C.
Figure A.2. Schematic of SEI model. Malaria infection dynamics are tracked in susceptible (S), exposed (E; infected with malaria but not able to transmit disease), and infectious (I, able to transmit disease) classes. $S_1$, $E_1$, and $I_1$ represent mosquitoes which are not infected with fungus, and $S_2$, $E_2$, and $I_2$ represent mosquitoes which are infected with fungus. Mosquitoes enter the susceptible class at constant rate, $\varepsilon$, and become infected with malaria at rate $abx$, where $a$ is the human bite rate, $b$ is malaria transmission efficiency, and $x$ is the proportion of humans with transmissible malaria. Mosquitoes move to the infectious class following a fixed delay ($t_E$, the length of the malarial EIP). Background mortality occurs at rate $\mu$. Mosquitoes become exposed to fungus at rate $F$ and experience additional mortality ($M_F$) which occurs as a function of time since fungal exposure. Figure adapted from Hancock et al. (2009).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Description</th>
<th>Value</th>
<th>Species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\varepsilon$</td>
<td>adult recruitment rate to $S_1$ class</td>
<td>0.2 day$^{-1}$</td>
<td></td>
<td>Hancock et al. 2009</td>
</tr>
<tr>
<td>$c$</td>
<td>daily probability of fungal infection</td>
<td>$1 - \exp(-F)$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$F$</td>
<td>rate of fungal infection</td>
<td>varies (day$^{-1}$)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\beta, \mu_F$</td>
<td>shape and rate parameters describing relationship between time since fungal infection and risk of mosquito mortality</td>
<td>see Table S2</td>
<td>An. stephensi</td>
<td></td>
</tr>
<tr>
<td>$x$</td>
<td>proportion of humans with transmissible malaria</td>
<td>0.5</td>
<td></td>
<td>Hancock et al. 2009</td>
</tr>
<tr>
<td>$b^2$</td>
<td>vector competence</td>
<td>$-0.54T^2 + 25.2T - 206$</td>
<td>An. quadrimaculatus and P. vivax</td>
<td>Mordecai et al. 2013</td>
</tr>
<tr>
<td>$\mu$</td>
<td>background mosquito mortality rate</td>
<td>$-\ln(-0.000828T^2 + 0.0367T + 0.522)$</td>
<td>An. gambiae</td>
<td>Mordecai et al. 2013</td>
</tr>
<tr>
<td>$a$</td>
<td>mosquito biting rate</td>
<td>$0.000203T(T - 11.7)(42.3 - T)^{0.5}$</td>
<td>An. pseudopunctipennis</td>
<td>Mordecai et al. 2013</td>
</tr>
<tr>
<td>$t_E$</td>
<td>$P. falciparum$ EIP</td>
<td>$1/[0.000111T(T - 14.7)(34.4 - T)^{0.5}]$</td>
<td>An. gambiae, An. culicifacies, An. stephensi, An. quadrimaculatus, An. atroparvus</td>
<td>Mordecai et al. 2013</td>
</tr>
</tbody>
</table>

Table A.1: Model parameters and values. Table adapted from Hancock et al. (2009) and Mordecai et al. (2013).
<table>
<thead>
<tr>
<th>AR</th>
<th>Temperature (°C)</th>
<th>( \mu_F \pm SE )</th>
<th>( \beta \pm SE )</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>10</td>
<td>0.071 ± 0.000</td>
<td>9.19 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.115 ± 0.001</td>
<td>9.86 ± 0.81</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.159 ± 0.001</td>
<td>11.67 ± 0.94</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.175 ± 0.002</td>
<td>7.09 ± 0.55</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.200 ± 0.001</td>
<td>8.78 ± 0.74</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.235 ± 0.002</td>
<td>6.90 ± 0.62</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>0.253 ± 0.002</td>
<td>8.93 ± 0.91</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.254 ± 0.002</td>
<td>8.41 ± 0.92</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.225 ± 0.002</td>
<td>6.00 ± 0.45</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>0.194 ± 0.002</td>
<td>5.92 ± 0.55</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>0.125 ± 0.001</td>
<td>7.47 ± 0.29</td>
</tr>
<tr>
<td>Low</td>
<td>10</td>
<td>0.056 ± 0.000</td>
<td>7.93 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>0.083 ± 0.000</td>
<td>7.16 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>18</td>
<td>0.113 ± 0.001</td>
<td>7.01 ± 0.34</td>
</tr>
<tr>
<td></td>
<td>20</td>
<td>0.131 ± 0.001</td>
<td>5.51 ± 0.43</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>0.124 ± 0.002</td>
<td>3.64 ± 0.32</td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>0.158 ± 0.002</td>
<td>4.40 ± 0.25</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>0.175 ± 0.002</td>
<td>4.82 ± 0.30</td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>0.182 ± 0.001</td>
<td>5.44 ± 0.20</td>
</tr>
<tr>
<td></td>
<td>30</td>
<td>0.162 ± 0.001</td>
<td>5.30 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>0.136 ± 0.001</td>
<td>5.78 ± 0.37</td>
</tr>
<tr>
<td></td>
<td>34</td>
<td>0.093 ± 0.001</td>
<td>4.32 ± 0.36</td>
</tr>
</tbody>
</table>

Table A.2. Summary of Weibull rate (\( \mu_F \)) and shape (\( \beta \)) parameter estimates for nonlinear least-squares fits of *An. stephensi* constant temperature survival curves for all application rate (AR) and mean temperature combinations. Since the model described by Hancock *et al.* (2009) includes a separate term for background mortality rate, we used Abbott’s correction (Abbott 1925) to adjust daily proportional survival (s) in the fungus-treated groups as follows:

\[
s = 1 - \frac{X - Y}{X}
\]

where \( Y \) was the proportion survival in a given treatment cup and \( X \) was the mean proportional survival in the corresponding controls. On days in which the mean control survival was lower than that of the treatment (e.g. at the beginning of the experiments in which survival was high in all cups), corrected proportional survival was assumed to equal one. We used the *nls* function in R (R Development Core Team 2013) to fit Weibull curves to the corrected survival data for each treatment:

\[
s(u) = \exp\left[ -\left( \mu_F u \right)^\beta \right]
\]

where \( s(u) \) was proportional survival as a function of time since fungal infection, \( u \). Goodness of fit was confirmed visually (Figure A.3, Figure A.4).
Figure A.3. Graphical evaluation of Weibull parameter fits of high application rate survival curves. Points represent corrected daily proportional survival from treatment replicates, and lines represent best fit curves for each temperature treatment with the associated $R^2$ noted in the legend.
Figure A.4. Graphical evaluation of Weibull parameter fits of low application rate survival curves. Points represent corrected daily proportional survival from treatment replicates, and lines represent best fit curves for each temperature treatment.
Table A.3. Summary of *An. stephensi* survival following fungal exposure. For each application rate and temperature combination, we report the total pooled number of mosquitoes which died (N events) and the number which survived (censored), median survival time (MST) ± standard error (SE) and MST 95% confidence intervals (CI). Results from both groups (1 and 2) are summarized; the 26°C treatments were included in both groups. In a number of the control treatments, more than 50% of mosquitoes survived to the end of the observation period (21 days in group 1, 23 days in group 2), so we did not estimate MST (indicated by ‘na’). Starred control treatments had replicate cups removed from the analysis due to fungal contamination (n=2 cups for 10 and 26°C group 2; n=3 cups for 14, 18 and 28°C; n=4 cups for all other treatments).

<table>
<thead>
<tr>
<th>Application rate</th>
<th>Temperature (group)</th>
<th>N events (censored)</th>
<th>MST ± SE</th>
<th>MST 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>High</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 (2)</td>
<td>119 (0)</td>
<td>14 ± 0.19</td>
<td>13.64-14.36</td>
</tr>
<tr>
<td></td>
<td>14 (2)</td>
<td>116 (0)</td>
<td>9 ± 0.11</td>
<td>8.78-9.22</td>
</tr>
<tr>
<td></td>
<td>18 (2)</td>
<td>112 (0)</td>
<td>7 ± 0.04</td>
<td>6.92-7.08</td>
</tr>
<tr>
<td></td>
<td>20 (1)</td>
<td>121 (0)</td>
<td>6 ± 0.10</td>
<td>5.80-6.20</td>
</tr>
<tr>
<td></td>
<td>22 (1)</td>
<td>115 (0)</td>
<td>5 ± 0.09</td>
<td>4.83-5.17</td>
</tr>
<tr>
<td></td>
<td>24 (1)</td>
<td>115 (0)</td>
<td>4 ± 0.11</td>
<td>3.79-4.21</td>
</tr>
<tr>
<td></td>
<td>26 (1)</td>
<td>113 (0)</td>
<td>5 ± 0.08</td>
<td>4.85-5.15</td>
</tr>
<tr>
<td></td>
<td>26 (2)</td>
<td>114 (0)</td>
<td>4 ± 0.04</td>
<td>3.93-4.07</td>
</tr>
<tr>
<td></td>
<td>28 (1)</td>
<td>115 (0)</td>
<td>4 ± 0.07</td>
<td>3.86-4.14</td>
</tr>
<tr>
<td></td>
<td>30 (1)</td>
<td>112 (0)</td>
<td>5 ± 0.08</td>
<td>4.83-5.17</td>
</tr>
<tr>
<td></td>
<td>32 (2)</td>
<td>118 (0)</td>
<td>5 ± 0.11</td>
<td>4.79-5.21</td>
</tr>
<tr>
<td></td>
<td>34 (1)</td>
<td>117 (0)</td>
<td>8 ± 0.13</td>
<td>7.74-8.26</td>
</tr>
<tr>
<td><strong>Low</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 (2)</td>
<td>110 (0)</td>
<td>16 ± 0.46</td>
<td>15.11-16.89</td>
</tr>
<tr>
<td></td>
<td>14 (2)</td>
<td>116 (0)</td>
<td>12 ± 0.18</td>
<td>11.65-12.35</td>
</tr>
<tr>
<td></td>
<td>18 (2)</td>
<td>113 (0)</td>
<td>9 ± 0.17</td>
<td>8.67-9.33</td>
</tr>
<tr>
<td></td>
<td>20 (1)</td>
<td>108 (0)</td>
<td>7 ± 0.20</td>
<td>6.61-7.39</td>
</tr>
<tr>
<td></td>
<td>22 (1)</td>
<td>128 (0)</td>
<td>7 ± 0.24</td>
<td>6.53-7.47</td>
</tr>
<tr>
<td></td>
<td>24 (1)</td>
<td>119 (0)</td>
<td>6 ± 0.13</td>
<td>5.74-6.26</td>
</tr>
<tr>
<td></td>
<td>26 (1)</td>
<td>111 (0)</td>
<td>6 ± 0.13</td>
<td>5.74-6.26</td>
</tr>
<tr>
<td></td>
<td>26 (2)</td>
<td>123 (0)</td>
<td>5 ± 0.15</td>
<td>4.71-5.29</td>
</tr>
<tr>
<td></td>
<td>28 (1)</td>
<td>121 (0)</td>
<td>6 ± 0.11</td>
<td>5.79-6.21</td>
</tr>
<tr>
<td></td>
<td>30 (1)</td>
<td>120 (0)</td>
<td>6 ± 0.17</td>
<td>5.68-6.32</td>
</tr>
<tr>
<td></td>
<td>32 (2)</td>
<td>118 (0)</td>
<td>7 ± 0.14</td>
<td>6.73-7.27</td>
</tr>
<tr>
<td></td>
<td>34 (1)</td>
<td>118 (0)</td>
<td>10 ± 0.30</td>
<td>9.42-10.58</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>10 (2)</td>
<td>14 (45)</td>
<td>&gt;23</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>14 (2)</td>
<td>3 (84)</td>
<td>&gt;23</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>18 (2)</td>
<td>8 (80)</td>
<td>&gt;23</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>20 (1)</td>
<td>8 (104)</td>
<td>&gt;21</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>22 (1)</td>
<td>15 (105)</td>
<td>&gt;21</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>24 (1)</td>
<td>29 (78)</td>
<td>&gt;21</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>26 (1)</td>
<td>23 (89)</td>
<td>&gt;21</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>26 (2)</td>
<td>19 (42)</td>
<td>&gt;23</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>28 (1)</td>
<td>23 (59)</td>
<td>&gt;21</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>30 (1)</td>
<td>93 (39)</td>
<td>18 ± 0.60</td>
<td>16.82-19.18</td>
</tr>
<tr>
<td></td>
<td>32 (2)</td>
<td>104 (18)</td>
<td>19 ± 0.44</td>
<td>18.14-19.86</td>
</tr>
<tr>
<td></td>
<td>34 (1)</td>
<td>103 (13)</td>
<td>18 ± 0.39</td>
<td>17.24-18.76</td>
</tr>
</tbody>
</table>
Figure A.5. Cumulative proportional survival of *An. gambiae* maintained at 20°C, 26°C or 32°C following exposure to clay substrates treated with fungal biopesticide at high (top) or low (middle) application rates. Control substrates (bottom) were treated with blank oil formulation. Mortality was monitored for 35 days; survival curves are truncated for consistency.
<table>
<thead>
<tr>
<th>AR</th>
<th>Temperature</th>
<th>N</th>
<th>MST ± SE</th>
<th>MST 95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>High</td>
<td>20</td>
<td>110</td>
<td>5 ± 0.07</td>
<td>4.86-5.14</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>118</td>
<td>4 ± 0.01</td>
<td>3.97-4.03</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>115</td>
<td>4 ± 0.07</td>
<td>3.87-4.13</td>
</tr>
<tr>
<td>Low</td>
<td>20</td>
<td>119</td>
<td>7 ± 0.24</td>
<td>6.53-7.47</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>117</td>
<td>5 ± 0.11</td>
<td>4.78-5.22</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>119</td>
<td>5 ± 0.11</td>
<td>4.78-5.22</td>
</tr>
<tr>
<td>Control</td>
<td>20</td>
<td>85</td>
<td>18 ± 0.57</td>
<td>16.9-19.1</td>
</tr>
<tr>
<td></td>
<td>26</td>
<td>124</td>
<td>12 ± 0.31</td>
<td>11.4-12.6</td>
</tr>
<tr>
<td></td>
<td>32</td>
<td>121</td>
<td>8 ± 0.32</td>
<td>7.37-8.63</td>
</tr>
</tbody>
</table>

Table A.4. Summary of *An. gambiae* survival following fungal exposure. For each application rate (AR) and temperature combination, we report the total pooled number of mosquitoes in each treatment, median survival time (MST) ± standard error (SE) and MST 95% confidence intervals (CI). We also report the mean time required for 90% of exposed mosquitoes to die (LT90 ± one standard error) with each cup serving as a replicate. There were n=4 cups of approximately 30 mosquitoes/cup for all treatments except one (20°C control), for which n=3. Mortality was monitored until all mosquitoes died (35 days).
Figure A.6. Cumulative proportional survival of blood fed adult An. stephensi maintained at a range of temperatures (indicated by line color) following exposure to tiles treated with fungus at high or low application rates or blank oil (‘control’). The non-blood fed 26°C treatment (‘no blood’) is included for reference. Lines represent mean daily survival (± one standard error) from three replicates of approximately 30 mosquitoes.
<table>
<thead>
<tr>
<th>Diet</th>
<th>Application rate</th>
<th>Temperature</th>
<th>N events (censored)</th>
<th>MST ± SE</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood fed</td>
<td>High</td>
<td>10</td>
<td>83 (0)</td>
<td>6 ± 0.37</td>
<td>5.27-6.73</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>14</td>
<td>84 (0)</td>
<td>7 ± 0.31</td>
<td>6.40-7.60</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>18</td>
<td>88 (0)</td>
<td>6 ± 0.23</td>
<td>5.56-6.44</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>22</td>
<td>83 (0)</td>
<td>5 ± 0.08</td>
<td>4.84-5.16</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>26</td>
<td>86 (0)</td>
<td>4 ± 0.13</td>
<td>3.74-4.26</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>30</td>
<td>87 (0)</td>
<td>5 ± 0.06</td>
<td>4.89-5.11</td>
</tr>
<tr>
<td></td>
<td>High</td>
<td>32</td>
<td>87 (0)</td>
<td>5 ± 0.18</td>
<td>4.65-5.35</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>10</td>
<td>85 (0)</td>
<td>7 ± 0.46</td>
<td>6.10-7.90</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>14</td>
<td>101 (0)</td>
<td>9 ± 0.53</td>
<td>7.97-10.03</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>18</td>
<td>87 (0)</td>
<td>8 ± 0.66</td>
<td>6.71-9.29</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>22</td>
<td>88 (0)</td>
<td>6 ± 0.19</td>
<td>5.64-6.36</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>26</td>
<td>83 (0)</td>
<td>5 ± 0.18</td>
<td>4.64-5.36</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>30</td>
<td>81 (0)</td>
<td>6 ± 0.11</td>
<td>5.78-6.22</td>
</tr>
<tr>
<td></td>
<td>Low</td>
<td>32</td>
<td>91 (0)</td>
<td>7 ± 0.15</td>
<td>6.70-7.30</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>10</td>
<td>78 (0)</td>
<td>6 ± 0.63</td>
<td>4.77-7.23</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>14</td>
<td>70 (12)</td>
<td>10 ± 0.85</td>
<td>8.34-11.66</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>18</td>
<td>46 (35)</td>
<td>20 ± 6.00</td>
<td>8.24-31.76</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>22</td>
<td>46 (46)</td>
<td>29 ± na</td>
<td>na</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>26</td>
<td>55 (36)</td>
<td>26 ± 1.58</td>
<td>22.90-29.10</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>30</td>
<td>79 (7)</td>
<td>16 ± 0.22</td>
<td>15.57-16.43</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>32</td>
<td>88 (1)</td>
<td>20 ± 1.56</td>
<td>16.93-23.07</td>
</tr>
<tr>
<td></td>
<td>No blood</td>
<td>High</td>
<td>26</td>
<td>83 (0)</td>
<td>5 ± 0.06</td>
</tr>
<tr>
<td></td>
<td>No blood</td>
<td>Low</td>
<td>26</td>
<td>88 (0)</td>
<td>6 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>No blood</td>
<td>Control</td>
<td>26</td>
<td>43 (44)</td>
<td>na</td>
</tr>
</tbody>
</table>

Table A.5. Summary of blood fed *An. stephensi* survival following fungal exposure with non-blood fed treatment ('no blood') included for reference. For each application rate and temperature combination, we report the total pooled number of mosquitoes which died prior to the end of the observation period (N events) and the number which survived (censored), median survival time (MST) ± one standard error (SE) and MST 95% confidence intervals (CI). MSTs could not be calculated for treatments in which more than 50% of mosquitoes survived to the end of the observation period (21 days in group 1, 23 days in group 2), so we did not estimate median survival time (indicated by ‘na’).
### Table A.6. Summary of the impact of diurnal temperature variation on *An. stephensi* survival following fungal exposure.

Mosquitoes were exposed to either high or low application rates (AR) of fungus then held at a mean temperature of 20, 26 or 32°C. Temperatures were either held constant (diurnal temperature range or DTR=0°C) or allowed to fluctuate (DTR=12°C). The mosquitoes were monitored for at least 15 days or until the last fungus-treated individual died (23 days in the 20°C treatments). For all mean temperature, diurnal temperature range and application rate combinations, we report the total pooled number of mosquitoes which died prior to the end of the observation period (N events) and the number which survived (censored), median survival time (MST) ± one standard error (SE) and MST 95% confidence intervals (CI). Within each mean temperature and application rate combination, we tested for significant differences between the two diurnal temperature range treatments using a Kaplan-Meier log-rank test; the resulting χ² and p-values are reported. There were n=4 cups of approximately 30 mosquitoes per cup for all treatment groups except one (mean temperature=20°C, DTR=0, low AR), for which n=3. In a number of the control treatments, more than 50% of mosquitoes survived to the end of the observation period (15 days in the 26 and 32°C treatments, 23 days in the 20°C treatments), so we did not estimate median survival time (indicated by ‘na’).

<table>
<thead>
<tr>
<th>Mean temperature</th>
<th>AR</th>
<th>DTR</th>
<th>N events (censored)</th>
<th>MST ± SE</th>
<th>MST 95% CI</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 High</td>
<td>0</td>
<td>0</td>
<td>115 (0)</td>
<td>6 ± 0.048</td>
<td>5.91-6.09</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>118 (0)</td>
<td>6 ± 0.088</td>
<td>5.83-6.17</td>
<td>15.743</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0</td>
<td>86 (0)</td>
<td>8 ± 0.289</td>
<td>7.43-8.57</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>110 (0)</td>
<td>8 ± 0.256</td>
<td>7.50-8.50</td>
<td>1.069</td>
<td>0.301</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>7 (113)</td>
<td>na</td>
<td>na</td>
<td>4.105</td>
<td>0.043</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>16 (103)</td>
<td>na</td>
<td>na</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>26 High</td>
<td>0</td>
<td>123 (0)</td>
<td>4 ± 0.071</td>
<td>3.86-4.14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>119 (0)</td>
<td>5 ± 0.083</td>
<td>4.84-5.16</td>
<td>85.140</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0</td>
<td>116 (0)</td>
<td>6 ± 0.142</td>
<td>5.72-6.28</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>114 (0)</td>
<td>7 ± 0.123</td>
<td>6.76-7.24</td>
<td>0.277</td>
<td>0.598</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>5 (110)</td>
<td>na</td>
<td>na</td>
<td>2.848</td>
<td>0.091</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>1 (118)</td>
<td>na</td>
<td>na</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>32 High</td>
<td>0</td>
<td>115 (0)</td>
<td>5 ± 0.098</td>
<td>4.81-5.19</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>112 (0)</td>
<td>6 ± 0.120</td>
<td>5.77-6.23</td>
<td>30.914</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Low</td>
<td>0</td>
<td>119 (0)</td>
<td>7 ± 0.165</td>
<td>6.68-7.32</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>119 (0)</td>
<td>8 ± 0.145</td>
<td>7.72-8.28</td>
<td>11.363</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>9 (110)</td>
<td>na</td>
<td>na</td>
<td>61.563</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>78 (35)</td>
<td>14 ± 0.293</td>
<td>na</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure A.7. Predicted impact of fungal biopesticide on infectious mosquito density at different mean temperatures. The lines represent the predicted equilibrium density of malaria infectious mosquitoes at different daily probabilities of fungal infection ($c$, indicated by line type) at the high and low fungal application rates (distinguished by line color). The ‘$c = 0$’ line represents the predicted equilibrium total density of infectious mosquitoes in the absence of fungus.
Figure A.8. Impact of variation in parameter estimates on proportion reduction in infectious mosquito density at the high fungal application rate. Each parameter (indicated by line color/type) was reduced or increased by 5% or 10% (change in parameter estimate). We evaluated the model at 2°C intervals from 18-34°C (n=9 temperatures) assuming the high application rate and daily probability of fungal infection (c) equal to 0.16 (above) or 0.04 (below) and calculated the mean (± one standard error) change in proportion reduction in infectious mosquito density relative to the reduction calculated using the original parameter estimate. Mosquito-related parameters (bite rate, a, transmission efficiency, b, and background mortality rate, µ) are black, fungal parameters (β and µF) are blue, and extrinsic incubation period (EIP) is red.
Figure A.9. Impact of variation in parameter estimates on proportion reduction in infectious mosquito density at the low fungal application rate. Each parameter (indicated by line color/type) was reduced or increased by 5% or 10% (change in parameter estimate). We evaluated the model at 2°C intervals from 18-34°C (n=9 temperatures) assuming the low application rate and daily probability of fungal infection (c) equal to 0.16 (above) or 0.04 (below) and calculated the mean (± one standard error) change in proportion reduction in infectious mosquito density relative to the reduction calculated using the original parameter estimate. Mosquito-related parameters (bite rate, α, transmission efficiency, b, and background mortality rate, μ) are black, fungal parameters (β and μF) are blue, and extrinsic incubation period (EIP) is red.
Figure A.10. Impact of parameter variation on equilibrium total infectious mosquito density. Mean change in total density (± one standard error) in the absence of fungus ($c = 0$) at the high application rate (above) and in the presence of fungus ($c = 0.16$) at the high application rate (below). Mosquito-related parameters (bite rate, $a$, transmission efficiency, $b$, and background mortality rate, $\mu$) are black, fungal parameters ($\beta$ and $\mu_F$) are blue, and extrinsic incubation period (EIP) is red.
Figure A.11. Impact of EIP (above) and background mortality ($\mu$, below) variation on equilibrium total infectious mosquito density in the absence ($c = 0$) and presence ($c = 0.16$, indicated in figures) of fungus at the high application rate. Decreases in both parameters increased the equilibrium total infectious mosquito density both in the presence and absence of fungus.
Figure A.12. Predicted proportion reduction in infectious mosquito density at different levels of variation in extrinsic incubation period (EIP). We reduced or increased EIP by 5%, 10%, or 40% and evaluated the model at 2°C intervals from 18-34°C (n=9 temperatures) assuming the high application rate and daily probability of fungal infection \((c)\) equal to either 0.16 or 0.04 (indicated on figure). The predicted reduction based on the original EIP estimates (‘null’) is included for reference.
Appendix B
Supplement to Chapter 3

Relationship between EIR and PR

Let $m$ equal the number of mosquitoes per human, $a$ equal the number of human bites per mosquito per day (human feeding rate) and $Z$ equal the proportion of mosquitoes which are infectious (sporozoite rate). The entomological inoculation rate, $E$, can be calculated as follows (Smith & McKenzie 2004):

$$E = maZ$$  \hspace{1cm} \text{eqn B.1}

If eaves tubes reduced the number of infectious mosquitoes by some proportion ($\rho$), the new entomological inoculation rate, $E^*$, would be:

$$E^* = maZ(1 - \rho)$$  \hspace{1cm} \text{eqn B.2}

Assuming a homogeneous, infinite host population, the proportion of hosts infected with malaria (parasite rate, $P$) can be estimated from $E$ as follows (Hay et al. 2005):

$$P = s \log E + k$$  \hspace{1cm} \text{eqn B.3}

Correcting for the presence of eaves tubes would generate the following:

$$P^* = s \log[E^*(1 - \rho)] + k$$  \hspace{1cm} \text{eqn B.4}

Therefore:

$$E^* = P + s \log(1 - \rho)$$  \hspace{1cm} \text{eqn B.5}
Figure B.1. Parasite rate (PR) in the absence of fungus (“no fungus”) or in the presence of fungus with $c=0.16$ estimated based on the relationship described by Smith et al. (2005):

$$P = 1 - (1 + 0.45 \times 0.42 \times E)^{1/4.2}$$

eqn B.6
Appendix C
Supplement to Chapter 4

Power analyses for malaria infection parameters

A series of power analyses were performed to estimate statistical ability to detect differences in the various malarial infection parameters given the experimental sample sizes. For simplicity, comparisons were separated by exposure day. The analytical framework used depended on the parameter being evaluated.

The detectable effect sizes associated with the binomial analyses (Plasmodium yoelii oocyst prevalence and Plasmodium falciparum sporozoite prevalence) were estimated using the pwr package in R (R Development Core Team 2013). Cohen’s effect size (h) was calculated assuming α = 0.05, β = 0.2, and sample sizes equivalent to those used in each experiment (Cohen 1988). Cohen’s effect size is defined as:

\[ h = 2 \times \arcsin(\sqrt{P_0}) - 2 \times \arcsin(\sqrt{P_0 - DP_0}) \]  

Eqn C.1

where \( P_0 \) is the prevalence in one population and \( D \) is the proportional change in prevalence in the second population. Thus the detectable proportional change in prevalence could be calculated as follows:

\[ D = 1 - \left( \sin \left( \frac{\arcsin(\sqrt{P_0}) - h}{2} \right) \right)^2 \]  

Eqn C.2

The analyses assumed that \( P_0 \) was equal to the prevalence in the control population. Results are summarized in Table C.1.

The count data for the \( P. yoelii \) oocyst intensity and number of sporozoites per oocyst were both overdispersed, and traditional power analyses tend to overestimate power associated with small samples of overdispersed data (Seavy et al. 2005). For this reason, a Bayesian framework was employed in these analyses. The rnegbin function in R (R Development Core Team 2013) was first used to simulate a negative binomial distribution with a sample size, mean, and variance equivalent to the control population at one of the exposure time points (day 0 or day 3). The same
procedure was then used to generate a second population equal in size to the corresponding fungal treatment group with its mean increased or decreased by a factor of 0.5-0.9. As in the experimental analysis, the two populations were compared using a negative binomial generalized linear model (GLM) with a log link. The procedure was repeated 5000 times, and power was estimated as the proportion of replicates in which the GLM detected a significant difference between the populations (p <0.5, Table C.2).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Exposure day</th>
<th>N₀</th>
<th>N₁</th>
<th>P₀</th>
<th>h</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. yoelii</em> oocyst prevalence</td>
<td>Day 0</td>
<td>20</td>
<td>20</td>
<td>0.90</td>
<td>0.89</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Day 3</td>
<td>20</td>
<td>20</td>
<td>0.70</td>
<td>0.89</td>
<td>0.62</td>
</tr>
<tr>
<td><em>P. falciparum</em> sporozoite</td>
<td>Day 8</td>
<td>84</td>
<td>39</td>
<td>0.32</td>
<td>0.54</td>
<td>0.67</td>
</tr>
<tr>
<td>prevalence</td>
<td>Day 11</td>
<td>34</td>
<td>43</td>
<td>0.38</td>
<td>0.64</td>
<td>0.70</td>
</tr>
</tbody>
</table>

Table C.1. Detectable effect size estimates for *Plasmodium yoelii* oocyst prevalence and *Plasmodium falciparum* sporozoite prevalence analyses. For each prevalence assay and exposure day, sample sizes for the control and treatment groups (‘N₀’, ‘N₁’), control prevalence (‘P₀’), detectable effect size (‘h’), and corresponding proportional difference in prevalence between the control and treatment populations (‘D’) were reported. All analyses assumed α = 0.05 and β = 0.2.
Table C.2. Power estimates for analyses of *Plasmodium yoelii* oocyst intensity and number of sporozoites per oocyst. Exposure day determined the baseline sample sizes, mean, and variance in each set of analyses. Power was estimated for a variety of different proportional increases or decreases in mean (‘D’) for the analyses of oocyst intensity and number of sporozoites per oocyst (SPO) assuming $\alpha = 0.05$.  

<table>
<thead>
<tr>
<th>Exposure day</th>
<th>Oocyst intensity power</th>
<th>SPO power</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D Decrease</td>
<td>Increase</td>
</tr>
<tr>
<td>Day 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.49</td>
<td>0.50</td>
</tr>
<tr>
<td>0.6</td>
<td>0.60</td>
<td>0.64</td>
</tr>
<tr>
<td>0.7</td>
<td>0.72</td>
<td>0.76</td>
</tr>
<tr>
<td>0.8</td>
<td>0.81</td>
<td>0.85</td>
</tr>
<tr>
<td>0.9</td>
<td>0.90</td>
<td>0.92</td>
</tr>
<tr>
<td>Day 3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5</td>
<td>0.29</td>
<td>0.18</td>
</tr>
<tr>
<td>0.6</td>
<td>0.42</td>
<td>0.24</td>
</tr>
<tr>
<td>0.7</td>
<td>0.56</td>
<td>0.28</td>
</tr>
<tr>
<td>0.8</td>
<td>0.71</td>
<td>0.34</td>
</tr>
<tr>
<td>0.9</td>
<td>0.88</td>
<td>0.40</td>
</tr>
</tbody>
</table>
Figure C.1. Summary of mean (± standard error) daily percent mortality rate (e.g. number dead on a particular day divided by the number alive at the start of that day). *Anopheles stephensi* mosquitoes were fed either on *P. yoelii*-infected or control mice (indicated by line type), then exposed to tiles sprayed with *B. bassiana* conidial suspensions or blank oil (indicated by marker) either the same day as the blood meal (d0) or three days later (d3, indicated by line color).
Figure D.1. Impact of time interval variation on rate summation estimates. A Briere curve (with prediction intervals) was fit to empirical radial growth rates observed at constant temperatures (represented in black/gray). Rate summation was then used to predict growth rate under fluctuating temperature conditions with a diurnal temperature range (DTR) of 8°C (blue) or 16°C (red). Points indicate empirical growth rates observed under each thermal regime (± 95% confidence intervals), lines represent optimal curves (for DTR=0°C) or rate summation predictions (for DTR>0°C), and bands indicated prediction bands. Each graph indicates a different time interval over which mean temperature and growth rate were evaluated (0.25-8 hours); a time step of 24 hours would correspond to the overall daily mean temperature (i.e. the DTR=0°C estimate).


Łukasik, P., Guo, H., van Asch, M., Ferrari, J. & Godfray, H.C.J. (2013b) Protection against a fungal pathogen conferred by the aphid facultative endosymbionts *Rickettsia* and *Spiroplasma* is expressed in multiple host genotypes and species and is not influenced by co-infection with another symbiont. *Journal of Evolutionary Biology*, 26, 2654–61.


VITA
Rebecca Heinig

Education
The Pennsylvania State University, State College, PA 2010-2015
   Ph. D., Entomology
Cornell University, Ithaca, NY 1998-2003
   B. A., Biology

Publications

Selected Honors and Awards
Vartkes Miroyan Memorial Award, PSU Department of Entomology 2013
Microbial Control Division Student Travel Award, Society for Invertebrate Pathology 2013
College of Agricultural Sciences Travel Award, PSU 2013
College of Agricultural Sciences Competitive Research Grant, PSU 2012
EEID Ecology Workshop Travel Award, NSF 2012
Sigma Xi Grant in Aid of Research 2011
Alex and Jessie Black Graduate Fellowship, PSU 2010

Research Experience
Graduate Assistant 2010-2015
   Thomas Lab, Penn State; State College, PA
Biological Technician 2006-2010
   Nagai Lab, Hawaii Agriculture Research Center; Honolulu, HI
Biological Technician 2004-2006
   Chang Lab, USDA ARS; Honolulu, HI
Lab Assistant 2001-2003
   Caillaud, Gray and Hoy Labs, Cornell University; Ithaca, NY

Service
Society for Invertebrate Pathology 2013
   46th Annual Meeting website designer 2013
Penn State Center for Infectious Disease Dynamics Grad Student Assn. 2013-2014
   President 2013-2014
   Vice President 2012-2013
   Founding Treasurer 2011-2012
Penn State Entomology Department 2013-2014
   Awards Committee member 2013-2014
   Candidacy Committee member 2012-2013