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Evaluating insecticide-resistance in the malaria vector *Anopheles gambiae* and its implications for malaria transmission

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Abstract

Insecticide resistance, in the mosquito vector, threatens the efficacy of current methods to control malaria. Yet evidence of control failure due to insecticide resistance is sparse, despite over 50 years since resistance was identified in the mosquito. In this thesis, laboratory experiments with mosquitoes, as well as mathematical modelling, are used to improve our understanding of how insecticide resistance might impact malaria transmission.

Firstly, demographic and environmental effects on the phenotypic expression of resistance are investigated. Decreasing expression of resistance with age and malaria infection, suggest resistance may not be as large a problem as once believed. Further factors that affect the phenotypic expression of resistance, such as infection by the microsporidian *Vavria culicis* and quantity of larval food, suggest that the phenotypic expression of resistance may even be manipulated to reduce its impact on disease transmission.

Secondly, costs of resistance are explored as they may reduce the ability of a mosquito to transmit malaria. It is demonstrated that, under environmental stress from parasites, costs to longevity can be increased. Mosquito longevity is a key parameter in malaria transmission so any reduction in longevity, due to costs of resistance, will reduce the ability of the mosquito to transmit malaria. Finally, the thesis examines if the behavioural avoidance of insecticides can be changed through environmental manipulation.

In summary, the phenotypic expression of resistance and the costs of resistance are two factors that will determine the threat insecticide-resistance poses to malaria control. It is demonstrated, in the laboratory, that these two factors can vary due to environmental and demographic factors, but to fully understand the threat of resistance these ideas have to be investigated in the field.

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Declaration of originality

I declare that all the work presented in this thesis is my own original research with the following acknowledgements for each chapter:

Chapter 2 - *Plasmodium berghei* infected mice were provided by the Heussler research group, University of Bern. Rebecca Stanway anesthetized the mice and performed the mosquito blood feeding.

Chapter 3 - The data was jointly collected with Katarzyna Kulma. Data from this chapter have bee published in *PLoS One* in paper with Katarzyna Kulma and Jacob C Koella.

Chapter 4 - The data was jointly collected with Thomas Karacs. Results are published in *Evolutionary Applications* as part of a paper including mathematical by Jacob C Koella

Chapter 6 - *Plasmodium berghei* parasites were prepared by Andrew Blagborough in Professor Robert E. Sinden's lab, Imperial College London

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Professor Jacob Koella

Copyright Declaration

Acronyms & Abbreviations

ACTs Artemisinin-based combination therapies

AIDS acquired immunodeficiency syndrome

CDC The Centers for Disease Control and Prevention

DDT dichlorodiphenyltrichloroethane

EIP Extrinsic Incubation Period

GFP Green fluorescent protein

GLM generalized linear model

GMAP Global Malaria Action Plan

HIV human immunodeficiency virus

ITNs Insecticide treated bed nets

IRAC Insecticide Resistance Action Committee

IRS Indoor residual spraying

MoA Insecticide Mode of Action

RBM Roll Back Malaria Partnership

SIT Sterile insect technique

WHO World Health Organisation

WHOPES World Heath Organisation Pesticide Evaluation Scheme

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Chapter 1

General Introduction

1.1 Background

1.1.1 Malaria Burden

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium*, transmitted to humans by infected mosquitoes. The majority of malaria cases occur in Sub-Saharan Africa but malaria is also a major disease in Asia, Latin America and the Middle East. Historically malaria was also found in North America (Faust, 1951) and much of Europe, including England (Dobson, 1989). Currently malaria infection accounts for 20% of childhood mortality and is particularly harmful to pregnant woman, their unborn children, and immunocompromised HIV/AIDS patients (World Health Organisation (WHO), 2010). There are in excess of 200 million cases of malaria per year. Symptoms are often flu-like, however, if they are not treated malaria infection can lead to death. The number of deaths caused by malaria is debated; The World Malaria Report 2012 (WHO, 2012a) estimates that 655,000 people were killed from malaria in 2010, whereas a study in Lancet journal estimates almost double the mortality in 2010 with 1.24 million deaths (Murray et al., 2012). Despite the differences in the estimates, the two studies do agree that deaths caused by malaria peaked in 2004 and have been in decline since, with 2010 estimates around 30% lower than 2004. This reduction in malaria deaths is believed to be due to an up-scaling of control measures in Sub-Saharan Africa.

1.1.2 Malaria transmission

Plasmodium falciparum is the most common, and most virulent, of the five species of the genus *Plasmodium* that contribute to malaria burden in humans, which include*; P.vivax, P.malariae, P.ovale* and *P.knowlesi*. *P.knowlesi*, a zoonotic disease transmitted to humans from macaques, was once believed to contribute to only a

handful of cases. However, recent advances in identification methods have shown it may play a significant role in the malaria burden in South-East Asia (Cox-Singh et al., 2008). *Plasmodium* is transmitted between human hosts by a mosquito vector (Figure 1.1). Female mosquitoes are infected by *Plasmodium* when they take a blood meal, which they require for egg production. The parasites go through a developmental period inside the mosquito, known as the sporogonic development, in which they start as gametocytes in the midgut and end as sporozoites in the mosquitoes' salivary glands (Figure 1.1). Sporozoites are then injected into a new host, along with saliva that acts as an anticoagulant during mosquito feeding. Thus, transmission of malaria parasites cannot take place before sporogonic development is complete (Bellan, 2010). This extrinsic incubation period (EIP) typically takes around 10-14 days, but varies with *Plasmodium* species and environmental conditions (Lefèvre et al., 2013).

1.1.3 Transmission parameters

A wide range of parameters connected with the parasite, mosquito vector and human host contribute to the intensity of transmission. The Ross-Macdonald equation (Macdonald, 1957; Equation 1.1) describes some the parameters involved:

$$R_0 = \frac{ma^2bce^{-\mu T}}{r\mu} \qquad \text{(Equation 1.1)}$$

R_0 is the basic reproductive number - defined as the number of secondary cases of infection generated by a single infective individual in a population of susceptible hosts. When R_0 is less than 1, malaria will decline, and eventually be lost from a population, and with an R_0 greater than 1, malaria will spread. Current estimates for the R_0 of malaria, using data from 121 African based studies, vary from 1 to over 3000 (Smith et al., 2007) The parameters are; m, the number of adult female mosquitoes per human host, a, the mosquito's biting rate on humans, μ, the daily mosquito mortality rate, T, the duration of the parasite's sporogonic development, b, the proportion of infective bites that leads to an infection in humans, c, the proportion of mosquito bites on infected humans that results in an infected mosquito and r is the recovery rate of humans from infection. The model suggests not all parameters affect

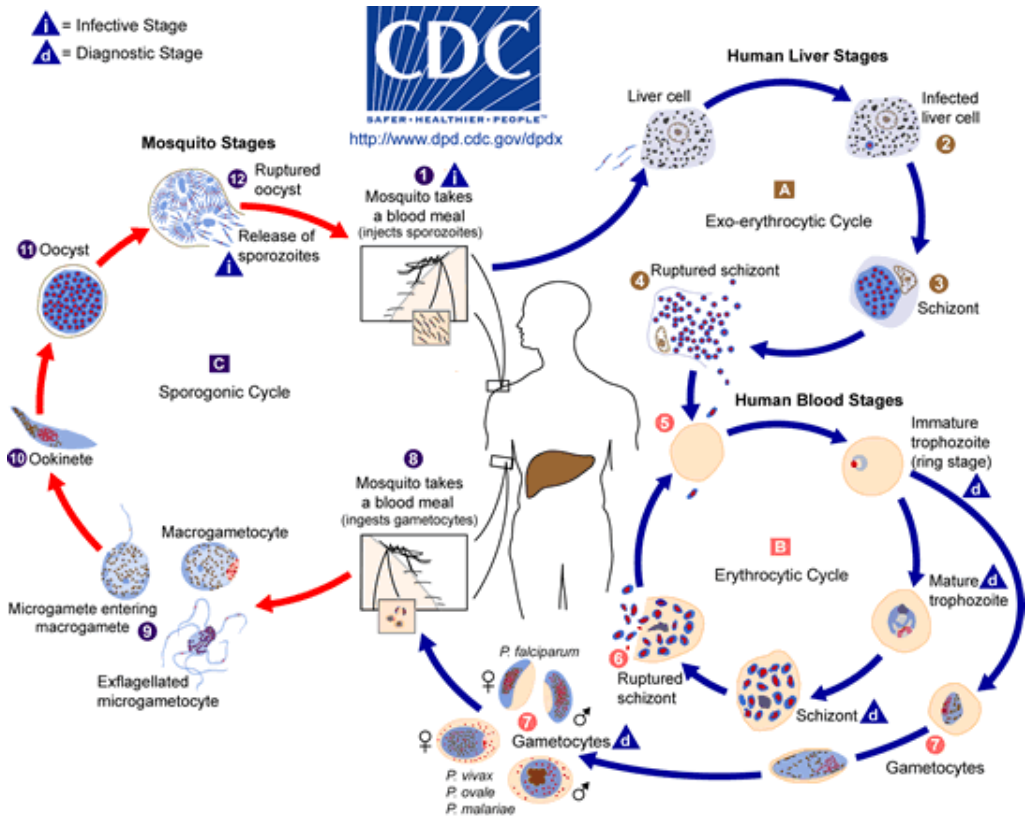


Figure 1.1: Life cycle of the malaria parasite in the human host and the mosquito vector, (Figure reproduced from DPDx: CDC's web site for laboratory identification of parasites). The sporogonic cycle in the mosquito (C), takes 10-14 days. Malaria cannot be transmitted, to humans from the vector, before sporozoites rupture from the oocyst (12) and find their way to the mosquito salivary glands. In humans, the parasite first infects and multiplies liver cells (2-4). Note, *P. vivax* and *P. ovale* can persist in the liver, in a dormant stage (hypnozoites), and reinvade the blood stream weeks or years later. The parasites then invade red blood cells and go through further multiplication (B). It is the blood stage parasites that are responsible for the clinical manifestations of the disease. Some of the blood stage parasites differentiate into gametocytes (7) and are injested by the mosquito during a blood meal – thus completing the malaria life cycle.

transmission equally, for example; if mosquito density, m, is reduced by 50% so is R_0, because the relationship is linear. But if daily mortality, μ, is doubled, R_0 decreases by around 80%, due to μ being involved in both the linear and exponential terms (Bailey & Duppenthaler 1980). The number of mosquitoes that reach infectivity (mosquitoes that harbour sporozoites in their salivary glands) is key to further transmission, thus the daily mosquito mortality has a greater influence on the number of mosquitoes that survive the incubation period of the malaria parasite than the total mosquito number.

Sensitivity analysis of the Ross-MacDonald equation indicates that daily adult mortality and mosquito biting rate as having the largest effect on malaria transmission (Koella, 1991). For this reason, vector control targeting adult mosquitoes is believed to be a particularly powerful tool in fighting malaria. Vector control and use of anti-malarial drugs are the two major tools used to fight malaria (WHO, 2010).

1.1.4 Anti-Malarial Drugs

The first anti-malarial drug, Quinine, was isolated from the bark of a South American cinchona tree by Pierre Pelletier and Joseph Caventou in 1820 (Smith, 1979), 60 years before Alphonse Laveran identified the cause of the disease after the discovery *Plasmodium* parasites in blood samples (Bruce-Chwatt, 1981). Quinine is still used today although it was largely surpassed in the 1940s by the discovery of the synthetic anti-malarial drug Chloroquine (Achan, 2011). Chloroquine had several advantages over Quinine as it was easier to produce, easier to administer, it was cheaper and had fewer side effects. Chloroquine was produced in large quantities and used extensively from the 1940s onwards, however, resistance developed in South East Asia and South America in the late 1950s (Payne, 1987). Resistance then spread to Africa and was prevalent in the majority of malarious regions by 1987 (Payne, 1987). The threat of drug-resistance, to malaria control, lead to the search for new anti-malarial drugs and new ways to administer the drugs. Large-scale screenings of traditional Chinese medicines lead to the discovery of Artemisinin and its derivatives (Miller & Su, 2011). Artemisinin is now the recommended first-line treatment and is used in combination with other anti-malarial drugs with a different chemical class (Artemisinin Combination Therapy (ACTs)). The shift from traditional monotherapy to using a combination of drugs has the specific purpose of delaying drug resistance (Sinclair et al., 2011; White et al., 1999). ACTs have been successful at treating patients but also reducing transmission of malaria (Okell et al., 2008) and are a key tool in the current elimination effort. However, the efficacy of anti-malarial drugs is once again under threat as resistance to Artemisinin has now been recorded (Dondrop et al., 2009)

1.1.5 Vector Control

In the period between 1897-1899, research by Sir Ronald Ross and Battista Grassi identified anopheline mosquitoes as the vectors for the disease and established the malaria parasite life cycle (Ross, 1897; Cox, 2010). The discovery heralded the beginning of vector control as a form of malaria control, with early vector control methods focusing on larval source management (Kitron & Spielman, 1989; Fillinger & Lindsay 2011). Swamp drainage, oiling and larviciding were employed successfully for malaria control during the construction of the Panama canal (Darling, 1910), and were also used in early malaria control campaigns in Italy (Majori, 2012) and in the United States by the Tennessee Valley Authority (Derryberry & Gartrell, 1952). One of the most successful campaigns used the larvicide Paris Green, to eliminate *Anopheles gambiae* from Brazil after it was introduced from Africa and was causing large malaria epidemics (Killeen et al, 2002). Screening of houses, to reduce human contact with adult mosquitos, was also an early method to reduce malaria. However, after the discovery of the insecticide effects of dichlorodiphenyltrichloroethane (DDT), by Paul Müller (Raju, 1999), the control of adult mosquitos become the main focus of vector control. DDT was used to finally clear malaria from North America (Williams, 1963) and was used heavily, in parallel with chloroquine, in the Global Malaria Eradication Programme of the 1950s and 60s, which managed significant reductions in global malaria (Nájera et al. 2011).

Insecticide treated nets (ITNs) are now the main tool for vector control, however, indoor residual spraying (IRS) of insecticides remains commonly used. Both have been successful at reducing malaria transmission (Labbo et al., 2012; Loha & Lindtjørn, 2012; Pluess et al., 2010; Protopopoff et al., 2007). ITNs and IRS target specific mosquito behaviours. Two of the main malaria vectors, *Anopheles gambiae* and *Anopheles funestus,* preferentially take blood meals from humans (anthropophilic) and predominantly bite at night (Jones el al., 1966; Takken & Knols, 1999). ITNs therefore protect those sleeping underneath when the risk of being bitten is highest, yet, not all bed-net owners are under the nets when mosquito biting starts. Both species of mosquito also bite indoors (endophagic) and rest indoors once they have taken a blood meal (endophillic). This makes them susceptible to IRS. ITNs and

IRS are however limited to vector species that bite indoors and ITNs are further limited to mosquitoes that bite at night when people are underneath the nets. *Anopheles arabiensis* is also an important vector for malaria, however it often bites outdoors and therefore is less likely to be killed by the insecticides. The reduced probability of *An. arabiensis* being killed by IRS or ITNs, compared to *An. gambiae,* may even explain recent species shifts from *An. gambiae* to *An. arabiensis* seen in eastern Africa (Kitau et al. 2012). For *Culex* and *Aedes* mosquitoes, also vectors for disease, but not malaria, IRS is rarely used because they bite outdoors and during the day (Pates et al., 2005). Alternative vector control strategies, such as larval source management, are therefore needed in theses situations. As well as causing mosquito mortality, insecticides have excito-repellency properties, deterring mosquitoes from treated houses, thus protecting individuals from mosquito bites (Achee et al., 2012).

The Roll Back Malaria Partnership (RBM) developed a Global Malaria Action Plan (GMAP), which aimed to reduce malaria cases and malaria deaths in 2010 to 50% of the levels observed in 2000, and to reduce deaths to almost zero by 2015 (RBM, 2008). Although malaria deaths have fallen, the goals for 2010 were not achieved (WHO, 2010). Part of the action plan was to achieve universal coverage of vector control strategies (ITNs and IRS). People protected by IRS increased from 13 million to 75 million between 2005 and 2009 (covering 10% of people at risk). In a period between 2008 and the end of 2010, 289 million ITNs were supplied to infectious areas; this is enough to cover 76% of the people at risk. Despite this only 42% of houses have at least one ITN and only 35% of children sleep under one (WHO, 2010). Costs of nets and accessibility to nets are large barriers limiting ITN coverage, with rural areas having particularly low ownership levels (Sexton, 2011). Discomfort when using the nets appears to be the main reason for people not using the bed-nets, however, technical difficulties in putting up the net and perception that the nets are not needed are among several other reasons (Pulford et al, 2011).

1.1.6 Insecticides and resistance

The WHO recommend twelve different insecticides for IRS, these can be grouped into four chemical classes; organophosphates, carbamates, organochlorines (DDT)

and pyrethroids. Pyrethroids are the only class of insecticide used for ITNs (WHO, 2006). Furthermore these chemical classes can be described by their mode of action (MoA); the primary site of action organophosphates and carbamates are Acetylcholinesterase (AChE), where Pyrethroids and DDT are Sodium channel modulators. Resistance has been recorded for each of these classes of insecticide (Ranson et al., 2011; Ranson et al., 2009) and resistance to an insecticide in a class often confers resistance to others in the same class. Insecticide resistance is an inherited trait that allows a mosquito to survive an exposure to insecticide. Thus, in areas of high insecticide coverage there is strong pressure for insecticide resistance to evolve. Resistance has been recorded in all major vector species, and has been recorded in two thirds of countries with malaria problems (WHO, 2012b). Furthermore, wild populations of mosquitoes have now been documented to be resistant to all known classes of insecticide (Edi et al., 2012). Large-scale use of insecticides in agricultural practices are believed to be the main driving force behind the development of resistance (Chouaibou et al., 2008) however there are examples of IRS and ITNs leading to insecticide resistance (Czeher et al., 2008; Lines, 1988; Penilla et al., 2007; Vulule et al., 1994).

Four types of insecticide-resistance mechanisms have been identified; metabolic resistance, target site resistance, penetration resistance and behavioural resistance (IRAC, 2010; Rivero et al., 2010). Metabolic resistance increases the metabolism or detoxification of the insecticide through increases in enzyme production; variations of this type of resistance have been identified to all the major insecticides (Figure 1.2). Enzyme production is increased through an increase in gene copy number and/or an increase gene expression (Hemingway & Karunaratne, 1998). Target site resistance occurs when the neurological targets of insecticide are altered, due to point mutations, and become less sensitive (Ranson et al., 2000a; Hemingway & Ranson, 2000). Penetration resistance is when the rate of insecticide diffusion through the mosquito cuticle is slowed by cuticular sclerotization or an increase in the lipid and protein content of the cuticle (Mo et al., 2009; Nkya et al., 2013). Mosquitoes may also alter their behaviour so that they bite outdoors or earlier in the night to avoid ITNs, in what is know as behavioural resistance (Pates & Curtis, 2005; Moiroux et al., 2012). Separating what is true behavioural resistance, which would involve a change in

genetic background, or what is a result of the deterrence effects of the insecticides may however be difficult to determine (Russell et al., 2011).

The scale up in the use of the insecticides, as part of the RBM campaign, will only increase the evolutionary pressure for resistance to evolve. Large-scale use of insecticides in agriculture during the 1950s and 60s lead to an explosion in insecticide resistance in crop pests, questioning the sustainability of their use (Mallet, 1989). Current vector control is now in a similar situation and is compounded by the fact that new chemical insecticides are limited and have to go through rigorous assessment before they are passed for public health use (WHO, 2006; IRAC, 2010). Alternative methods of vector control should be employed in order to control the resistance problem (Thomas et al, 2012). Future vector control methods are being developed, however, existing methods such as larval source management and methods used in agriculture for resistance management may also help against the resistance problem (Tusting et al., 2013).

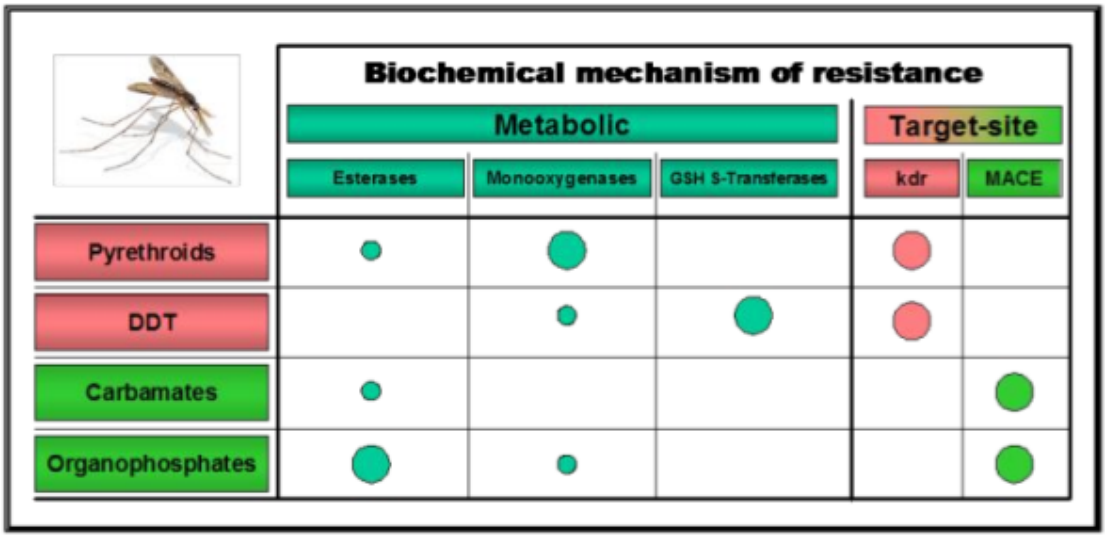


Figure 1.2 Major biochemical mechanisms conferring resistance to important classes of insecticides in adult mosquitoes (figure reproduced from (IRAC), 2010). Multiple resistance mechanisms have been identified to each class of insecticide used for vector control. Circle size reflects the relative impact of the mechanism on resistance.

1.1.7 Resistance Management

The Insecticide Resistance Action Committee (IRAC) have published an extensive document on how to slow or prevent the evolution of insecticide resistance (IRAC, 2010). Many of the techniques were first derived for the use in pest control in agriculture. Resistant management techniques use multiple insecticides, with different modes of action (MoA), in either mixtures, on rotation or in mosaics.

The theory behind the use of mixtures is that resistance to one MoA is rare but mosquitoes resistant to multiple MoA are even rarer, thereby delaying the emergence of resistance in a population. Results from mathematical modeling and from agriculture, indicate mixtures of insecticides in IRS can delay the onset of resistance (Curtis, 1985; Mani & Wood, 1984) with mixtures now being investigated for the use in bed-nets (Corbel et al., 2010; Curtis et al., 1998; Tungu et al., 2010). The same theory applies to mosaics and rotation methods however these methods also assume there is a fitness cost to resistance. In the absence of insecticide, and thus selection pressure, any costs of resistance to mosquito fitness would result in resistance being lost from the population (IRAC, 2010). Costs of resistance are therefore important to the success and cost of resistance management strategies (Brown et al., 2013). In Cambodia and Mexico resistance genes disappeared from mosquito populations when insecticide was removed (WHO, 2012b; Penilla et al., 2007), however after the use of insecticide DDT was ceased in Sri Lanka, DDT-resistance persisted, suggesting there was no costs to resistance in this case (Herath et al., 1988). Further recommendations are to use different insecticides for ITNs and IRS in the same huts to reduce the selection pressure for resistance (WHO, 2012b; Djenontin et al., 2009). The WHO also recommends that an area should use different insecticide in agriculture and vector control. Resistance management also relies on effective entomological surveillance, so that methods can change once resistance is detected (Casimiro et al., 2007; Kelly-Hope et al., 2008).

Nets using a chemical synergist have proven to be successful against resistant mosquitoes (N'Guessan et al., 2010; Koudou et al., 2011; Okia et al., 2013; Van Bortel et al., 2009) however the effectiveness of mixtures and rotations for resistance

management of vector species in IRS is unproven and could be very costly (Thomas et al., 2012). Cross-resistance between insecticides and multiple-resistance strains of mosquitoes have already been identified and threaten the effectiveness of resistance management strategies (Nauen, 2007). Combined with the evolution of resistance to artemisinin anti-malaria drugs (Dondorp et al., 2009) there is an increasing need for new methods to control malaria (Greenwood et al., 2008)

1.1.8 Future methods of malaria control

The search for a malaria vaccine has been extensive, with over 40 projects reaching the clinical trial stage in the last 10 years alone (Schwartz et al., 2012). However, one of the most promising vaccines, using whole *P.falcipurum* sporozoites (Seder et al., 2013), is still a long way from being used for global malaria control (Kaiser, 2013).

A method that has regained some popularity because of new methods of genetic manipulation is the Sterile Insect Technique (SIT) (Alphey et al., 2010). SIT was first used in agriculture and involves the release of male insects that have been sterilised by radiation. Wild females will not produce offspring when mated with the sterile males and so pest, or vector, populations will decrease. A similar effect can also be achieved with genetically modified males carrying a dominant lethal transgene. Lab-reared sterile males can be less competitive than to wild males therefore SIT requires large numbers of mosquitoes being released into an area. Despite these challenges recent releases of *Aedes aegypti* mosquitoes have achieved population control in the field (Harris et al., 2012).

Another target for GM mosquitoes is to produce mosquitoes that are refractory to the malaria parasite and thus will stop transmission to humans (Marshall & Taylor, 2009). The drawback is genetic modification can result in a fitness cost to the mosquito. However, various systems are being explored to drive the desired genetic elements into mosquito populations despite there being a cost to the mosquito fitness (Lambrechts et al., 2008; Marshall & Taylor, 2009; Sinkins & Gould, 2006). Genetically modified symbiotic bacteria of mosquitoes can be used to suppress malaria development in the mosquito effectively making refractory mosquitoes (Wang et al., 2012). Another bacterium, *Wolbachia*, spreads quickly through

mosquito populations though cytoplasmic incompatibility. It has many advantages for disease control such as the suppression of dengue virus (Walker et al., 2011), suppression of malaria parasites (Bian et al., 2013) and can shorten the life-span of a mosquito (Hoffmann et al., 2011).

1.1.9 Late Acting Insecticides

The use of late acting insecticides, such as the microsporidian *Vavraia culicis* and entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*, has been suggested as evolution-proof way of controlling malaria (Koella et al., 2009a; Koella et al., 2009b; Lynch et al., 2012; Read et al., 2009). The theory behind the proposal combines principles of evolutionary biology with malaria epidemiology. As discussed, a mosquito must survive the extrinsic incubation period, of at least ten days, before it can transmit malaria. Therefore, in terms of malaria control, killing a newly emerged adult mosquito with a conventional insecticide would have the same effect on transmission as killing a 9-day-old mosquito with a late acting insecticide. However, killing a newly emerged mosquito imposes a much greater pressure to evolve resistance than killing an older mosquito. This theory that selection pressure reduces with age is well established (Hamilton, 1966; Charlesworth, 1993). Killing a young mosquito before it has a chance to reproduce means it will have a fitness of zero, whereas, a late acting insecticide would allow the mosquito to lay multiple clutches of eggs before it is killed and thus reduce the pressure for resistance to evolve.

It is clear that late-acting insecticides will exert less selection pressure than traditional insecticides, however, they may produce very little selection pressure at all. The daily mortality of adult mosquitoes is often reported to be around 10% (Charlwood et al., 2009; Costantini et al., 1996; Killeen et al., 2000; Midega et al., 2007; Okech et al., 2007) thus relatively few mosquitoes will live long enough to be killed by the late-acting insecticide (most mosquitoes will die of natural causes). Resistance to late-acting insecticides would therefore bring little benefit. Furthermore, if resistance against late-acting insecticides is associated with a fitness cost, which is common with chemical insecticides (Kliot & Ghanim, 2012), the cost to young mosquitoes may

outweigh any benefit they will receive in later life. Essentially blocking the evolution of resistance against late acting pesticides.

Moreover it is hypothesized that, when using a biological larvicide, even if resistance occurred it may not be detrimental to malaria control (Koella et al., 2009b). An immune response required to tolerate infection in the larval stage may trade-off with lifespan of adult mosquitoes, thus reducing the ability of the mosquito transmit malaria (Koella et al., 2009b). If the same theory is applied to resistance to traditional insecticides, it may suggest there are trade-offs with resistance and mosquito life history traits that reduce their ability to transmit malaria.

1.2 Thesis Introduction

Insecticide-resistance is considered a serious threat to malaria control programs because it is predicted that it will lead to an inability to control vector species and therefore an inability to control malaria (Chanda et al., 2011). The threat is so serious that resistance to DDT contributed to the original malaria eradication campaign of the 1950s being abandoned (Kelly-Hope et al., 2008; Davidson & Zahar, 1973), although it is believed the funding issues, technical difficulties in implementing the control measures and environmental concerns over DDT were the main reasons for the abandonment of the campaign (Najera, 1989; Smith et al., 2013). Despite the threat of resistance little is know about the impact of insecticide-resistance on malaria control; the current literature is reviewed below. The topic of the thesis is then introduced in which specific aspects of mosquito biology are investigated to explain the impact of insecticide-resistance on malaria control.

1.2.1 Insecticide Resistance and Malaria Transmission

Resistant mosquitoes were identified soon after the insecticides were first used for vector control in the 1950s, yet the impact of resistance on the efficacy of control programs is still unclear. A systematic review of malaria resurgence events, since the 1930s, connected 14 out of 75 resurgence events with insecticide resistance in the

vector (Cohen et al., 2012). 91% of all resurgence events were due to weakening of control programs from issues such as funding problems or war; thus resistance was seen as a secondary cause of resurgence and largely only supported by anecdotal evidence.

The most widely accepted example of resistance leading to a rise in malaria was in an area of South Africa from 1996 to 1999 (WHO, 2012b; Hargreaves et al., 2000). Up until 1996 the area had been using DDT in its IRS program, it was then switched to the more environmentally friendly deltamethrin, however the incidence of malaria then increased dramatically. The study run in 1999 indicated that a major malaria vector *Anopheles funestus* had returned to the area, after eradication with DDT in the 1950s, and showed signs of pyrethroid resistance. It was concluded that pyrethroid resistance lead to reinvasion of *A.funestus* and the increase in malaria. Although this study may indicate malaria control failure due to resistance, there may be other explanations; perhaps the simplest was that deltamethrin was not as effective at control compared to DDT? After DDT was introduced again the malaria incidence plummeted (Maharaj et al., 2005).

Connecting resistance with an increase in malaria transmission is however an inherently difficult relationship to measure, as you need a very good entomological monitoring paralleled with thorough disease surveillance (WHO, 2012b). There are also many confounding factors, such as environmental conditions, that affect malaria incidence, so it regularly fluctuates year to year. More recently, an increase in malaria in Senegal was associated with an increase in insecticide resistance (Trape et al., 2011) but the short time period of the investigation cast doubts over the conclusions (Keating & Eisele, 2011). It was recommended the experiment be extended to confirm if the trend was down to insecticide resistance.

Other studies indicate that IRS, but in particular ITNs, still work when introduced into areas with high insecticide resistance. Protopopoff et al (2008) showed a decrease in malaria prevalence with IRS and ITNs despite increasing insecticide resistance. Similarly bednets can significantly reduce the incidence of malaria even in an area with extremely high (80%) resistance (Henry et al., 2005). Hougard shows no difference between the effectiveness of bed-nets in resistant and sensitive populations

(Hougard et al., 2003). In Malawi increasing pyrethroid resistance did not increase malaria transmission, however the investigators believed that the presence of resistance meant that the control efforts did not reduce malaria to the levels expected (Wondji et al., 2012). As these results were primarily seen in situations with ITNs and not IRS and alternative explanation could be that people were being bitten before they went underneath the net, thus reducing the expected efficacy of the nets.

Due to the difficulties in detecting a relationship between resistance and malaria transmission, entomological outcomes, such as mosquito numbers and biting rates, are believed to be a good proxy for disease transmission (WHO, 2012b). Hut trials have shown that there is significantly less mortality and more blood feeding of mosquitoes in areas where there is resistance (Asidi et al, 2012; Rowland et al., 2007). However, it is possible to have a reduction on sporozoite rates (the percentage of mosquitoes harbouring sporozoites in their salivary glands) even when there was no reduction in numbers of *An.gambiae* (Sharp et al., 2007), the likely explanation being that there is a shift towards a younger population structure.

1.2.2 When is resistance a threat?

From the field evidence it is unclear how insecticide-resistance affects malaria control. Although entomological parameters suggest resistance may lead to a failure to control mosquito numbers and biting, there is little evidence of failure to control malaria. The threat of resistance poses to malaria control is therefore more complicated than it first appears. Discussed in the following sections, and throughout the thesis, are two parameters that could influence how insecticide resistance affects malaria transmission and may explain the unexpected results seen in the field; (i) The vector capacity of resistant mosquitoes (the ability of the mosquito to transmit malaria) and (ii) Phenotypic expression of resistance. Non-lethal effects of control measures may also partially maintain their effectiveness; ITNs remain a barrier to mosquito biting and resistant mosquitoes can still be repelled by insecticides (Sharma et al., 2005), thus control interventions still confer some personal protection to the user.

1.2.3 Vector capacity and costs of resistance

The vector capacity of resistant and sensitive mosquitoes may differ for several reasons (Rivero et al., 2010). First, the resistance mechanisms may affect parasite development or susceptibility of the mosquito to infection. Insecticide resistance in the mosquito *Culex quinquefasciatus* has been shown to have negative effect on the parasitic worm *Wuchereria bancrofti*, which causes lymphatic filariasis in humans (McCarroll & Hemingway, 2002; McCarroll et al., 2000). Notably, a gene encoding for resistance to pyrethroids, was up-regulated in the mosquito mid-gut when infected with malaria parasites (Félix et al., 2010; Stevenson et al., 2011) suggesting a possible link between resistance and mosquito immunity. A recent study indicated that strain of *Anopheles gambiae* with kdr resistance do indeed have lower malaria parasite loads when compared to sensitive mosquitoes, however, the same study indicated that the resistant mosquitoes had higher initial infection rates (percentage of mosquitoes that are infected after a blood meal) (Alout et al., 2013). *Anopheles gambiae* with metabolic resistance also had increased infection rates compared to controls (Alout et al., 2013) however esterase resistance (metabolic) or acetylcholinesterase resistance (target site) did not appear to effect the infection rates or parasitic load in *Culex pipiens* mosquitoes (Vézilier et al., 2010).

Secondly, traits are often negatively correlated, so the evolution of one life-history trait often comes at the cost of another (Stearns, 1992). Thus, as malaria transmission is dependent on mosquito life-history traits, in particular longevity, any costs associated with resistance may impact the ability of a mosquito transmit malaria. Indeed, the assumption that there is a cost to being resistant is key to the success resistance management techniques such as pesticide rotation and pesticide mosaics (WHO, 2012b). Insecticide resistance does affect life-history traits of many species (Carriere et al., 1994; Foster et al., 2003; Janmaat & Myers, 2005; Kliot & Ghanim, 2012), including mosquitoes (Agnew et al., 2004; Gazave et al., 2001; Martins et al., 2012). Studies with *Culex pipiens* have shown that the over-winter survival is reduced in resistant mosquitoes (Gazave et al., 2001), they are subject to higher predation (Berticat et al., 2004) and they have reduced adult survival (Agnew et al., 2004). For the malaria vector *Anopheles gambiae,* resistant mosquitoes have been shown to have

lower pupae survival (Djogbenou et al., 2010). Costs of resistance will likely depend on the resistance mechanism, the mosquito species and environmental conditions.

1.2.4 Environmental impacts on resistance costs

Genetic correlations, or genetic trade-offs, can also be influenced by the environment (Sgro & Hoffmann, 2004); with insecticide-resistance it is no different. Experiments with both *Culex pipiens* and *An.gambiae* mosquitoes have shown food stress decreases survival in resistant mosquitoes more than sensitive (Agnew et al., 2004; Djogbenou et al., 2010). The cost of resistance in *C. pipiens* to organophosphates is increased if the mosquitoes are infected by the microsporidian parasite *Vavraia culicis* (Agnew et al., 2004) or reared at high larval densities (Bourguet et al. 2004). The fitness cost of permethrin resistance of *C. pipiens* is enhanced if the mosquito is exposed to temephos, another insecticide (Hardstone et al., 2009). In the diamondback moth, the cost of resistance to spinosad is low at the optimal temperature and increases at unfavourably low and high temperatures (Li et al., 2007), and the cost of resistance to *Bacillus thuringiensis* increases in harsh and competitive environments (Raymond et al., 2005).

Trade-offs between immunity and insecticide resistance may lead to an increase in parasitism, could explain an increase in infection rates seen in resistant mosquitoes (Alout et al., 2013). It has also been observed that insecticide resistance can lead to an increase in parasite load of *Wolbachia*, which in turn leads to decreased survival (Berticat et al., 2002; Duron et al., 2005). Resistant mosquitoes can have lower energy reserves, which may detract from mosquito immunity and allow *Wolbachia* to reach larger parasite loads (Duron et al., 2006; Echaubard et al., 2010; Vidau et al., 2011). Resistant mosquitoes are also more susceptible to the biopesticides *Metarhizium anisopliae* and *Beauveria bassiana* (Howard et al., 2010a). In these studies parasitism is increasing the cost of resistance. However, an infection by *Vavraia culicis* in *C.pipiens,* has been shown to reduce the relative fitness costs between resistant and sensitive mosquitoes (Agnew et al., 2004). In other words, when the mosquitoes are not infected there is a clear cost of resistance in longevity and numbers of mosquitoes

making it to adulthood, but when the parasite is introduced the difference between sensitive and resistant mosquitoes is reduced.

1.2.5 Phenotypic expression of resistance

Insecticide-resistance is often thought of in black and white terms i.e. genetically resistant mosquitoes will survive an insecticide exposure and sensitive mosquitoes will be killed. It is however becoming increasingly clear that this is not the case. Studying the factors behind the phenotypic expression of resistance is therefore vitally important if we are to understand the impact of resistance on disease transmission.

Insecticide resistance in the diamondback moth is determined by the interaction between genes and the environment (Raymond et al., 2005); and detoxification genes in honey bees, associated with resistance, can be up-regulated by particular dietary components (Mao et al., 2013). Symbiotic bacteria can also increase insecticide resistance in crop pests (Kikuchi et al., 2012). Natural and un-natural xenobiotics ingested by mosquito larvae can affect tolerance of adult mosquitoes to insecticides (Nkya et al., 2013; Poupardin et al., 2008). Temperature (Hodjati & Curtis, 1999; Polson et al., 2012), parasitism (Farenhorst et al., 2009), larval diet (Oliver & Brooke, 2013) and age (Curtis & Hodjati, 1999; Hunt et al., 2005; Jones et al., 2012; Rajatileka et al., 2011; Rowland & Hemingway, 1987) can increase sensitivity to insecticides in genetically resistant mosquitoes; whereas a blood-meal is believed to increase resistance (Hunt et al., 2005; Rajatileka et al., 2011). It is the phenotypic expression of resistance that will threaten disease control so these environmental and demographic factors may play an important role in determining the threat of resistance. In particular, a decline in resistance with mosquito age could have significant implications for malaria transmission as the incubation period of the parasite means only older mosquitoes can transmit malaria.

1.2.6 Resistance prevention

Investigations into how insecticide resistance affects malaria transmission will also allow us to determine new ways of preventing resistance. Resistance costs are at their

highest when resistance first emerges and can diminish with time due to gene replacement (Guillemaud et al., 1998; Labbe et al., 2005). A similar effect is achieved with modifier genes that reduce the cost of resistance in Australian blowfly (Mckenzie & Ofarrell, 1993). The vector capacity of resistant mosquitoes may therefore increase with the diminishing costs. It is therefore vitally important we that we still investigate ways to prevent resistance emerging.

1.3 Research aims

In this thesis laboratory experiments, with insecticide resistant and sensitive mosquitoes*, as well as mathematical modeling are used to improve our understanding of how insecticide resistance impacts malaria control. First, demographic and environmental effects on the phenotypic expression of resistance are investigated. In chapters 2 and 3 laboratory experiments are used to assess the impact of malaria infection and mosquito age on the expression of resistance. Both factors could have a significant impact on the number of mosquitoes surviving to infectivity and thus the ability to transmit malaria. The effect of larval food on the expression of resistance is also investigated in chapter 3 with infection by the microsporidian *Vavria culicus* on the phenotypic expression of resistance is investigated in chapter 4. Chapter 5 brings together ideas from the previous chapters by investigating the impact of phenotypic expression of resistance on malaria transmission through mathematical modelling. In chapter 6, the importance of resistance costs on the vector capacity of a mosquito is discussed; with particular attention on costs to longevity when under environmental stress from parasitic infection. Finally, chapter 7 examines if the behavioural avoidance of insecticides can be changed through environmental manipulation.

* See Appendix A for colony information and maintenance procedures

Chapter 2

The effect *Plasmodium berghei* infection on DDT resistance in the malaria vector *Anopheles gambiae*

2.1 Introduction

Insecticide-resistance, in the mosquito vector, threatens the control of malaria transmission (Maxmen, 2012; Moszynski, 2012; Baleta, 2009). Although there are still gaps in our knowledge, much is known about the mechanisms of insecticide-resistance including the genes and mutations involved (IRAC, 2010). However, to fully understand the impact of insecticide resistance on malaria transmission, factors affecting the phenotypic expression of resistance must be studied. Only mosquitoes harbouring malaria parasites in their salivary glands can transmit malaria to humans, so the resistance level of these mosquitoes is vitally important in the control of malaria.

Environmental stresses such as temperature (Hodjati & Curtis, 1999; Polson et al., 2012) and food source (Bourguet et al., 2004; Oliver & Brooke, 2013) can influence the expression of resistance. Recent work with biopesticides *Vavraia culicis* (Koella et al., 2012) *Metarhizium anisopliae* and *Beauveria bassiana* (Farenhorst et al., 2009) have indicated that parasitic infection of the mosquito can increase sensitivity to insecticide. It has been proposed that these biopesticides, in combination with conventional chemical insecticides, could even block the evolution of insecticide resistance (Koella et al., 2012). The mechanisms behind the increase in sensitivity with parasitism are unknown but they may be due to increased metabolic stress caused by the pathogens or, alternatively, the re-allocation of detoxification enzymes towards defence against the fungal pathogen and not insecticide (Koella et al., 2012, Farenhorst et al., 2009). It is therefore possible that natural parasites of mosquitoes may also impact the expression of resistance; the most important for malaria control being the malaria parasite itself.

Plasmodium parasites cause damage to mosquito cells and can trigger a potentially energetic costly immune response (Dimopoulos et al., 1998; Maier et al., 1987). Recently, it was demonstrated that infection of *Plasmodium yoelii* or *Plasmodium chabaudi* did not increase the sensitivity of permethrin-sensitive *Anopheles stephensi* mosquitoes to low doses of permethrin (Glunt et al., 2011). However, the effect of *Plasmodium* infection on the expression of resistance has yet to be investigated in insecticide-resistant mosquitoes. A larger effect of malaria infection on the sensitivity of resistant mosquitoes may be expected for two reasons; first, malaria parasites are more virulent in unnatural parasite-mosquito interactions (Ferguson & Read, 2002), and insecticide-resistance has been suggested to provide an unnatural environment for malaria parasites (Rivero et al., 2010). Secondly, resistant mosquitoes store fewer lipids, sugars and energetic reserves than sensitive ones (Rivero, 2011), and the reduced energy reserves could influence the success of a potentially energetically costly immune response (Richman et al., 1997; Schmid-Hempel, 2005; Sheldon & Verhulst, 1996). Indeed, *Plasmodium* infection has a greater effect on mortality in nutritionally compromised mosquitoes (Takken et al., 2013). Furthermore, immune responses differ due to the developmental stage of the parasite (Dimopoulos et al., 1998; Dimopoulos, 2003) and could also affect the expression of resistance. In the extreme case, if malaria infection were to make resistant mosquitoes completely sensitive to insecticide, then insecticide-resistance would not be a threat to malaria control. More realistically, malaria infection might partially restore sensitivity to insecticide but this could still have a significant effect on malaria transmission.

Before a mosquito can transmit malaria it has to survive the development of the malaria parasite, of around 10-14 days, thus, any changes in the expression of resistance with mosquito age could be important for malaria control. Indeed, several studies have now indicated that the expression of resistance declines with mosquito age, suggesting older malaria-transmitting mosquitoes are more susceptible to malaria control interventions (Curtis & Hodjati, 1999; Hunt et al., 2005; Jones et al., 2012; Rajatileka et al., 2011; Rowland & Hemingway, 1987). Thus mosquitoes that can transmit malaria, which are older and carry the malaria parasite, may have significantly lower resistance levels than the majority of the mosquito population.

Here, the impact of *Plasmodium berghei* infection on the phenotypic expression of resistance in mosquitoes with genetic resistance to the chemical insecticide dichlorodiphenyltrichloroethane (DDT) is investigated. Resistance is tested at two time points after blood feeding in order to capture parasites at the oocyst and sporozoite stages. It also allows the effect of age on resistance to be investigated.

2.2 Methods

2.2.1 Experimental design

The mosquitoes used for the experiment were a DDT-resistant colony of *An.gambiae* (ZANU), from Zanzibar with increased metabolism of the insecticide, catalysed by members of the glutathione S-transferase enzyme family (Ranson et al., 2000b). Insecticide exposures were carried out on mosquitoes 10 and 19 days after blood feeding in order to capture two life stages of the malaria parasite, oocysts (day 10) and sporozoites (day 19). On these days infected and uninfected mosquitoes were exposed to filter paper treated with; 2% DDT, 1% DDT or 0% DDT (paper containing oil as a control), for 1hr using the standard WHO test kits (WHO, 1998). Mosquito survival was recorded 24 hours after exposure and used in the analysis.

4% DDT is the standard dose used to discriminate resistant mosquitoes from sensitive mosquitoes (WHO, 1998). However due to dramatic decrease in resistance with age in the ZANU mosquitoes (Kulma et al., 2013; (chapter 3)), preliminary experiments indicated that 4% DDT caused close to 100% mortality in mosquitos of equivalent age to ones used in this experiment. For this reason 2% and 1% DDT concentrations were used for the resistance test despite them being lower than the standard discriminating dose. Typically survival of 3-4 day old ZANU mosquitoes is greater than 85% for an exposure of 1hr using 4% DDT (Appendix A).

2.2.2 Mosquito Rearing

Mosquitoes were reared at a temperature of 26 (+/-2) °C and 70 (+/-10) % relative humidity with a 12 h:12 h light/dark cycle. 1500 larvae were reared individually in 12-well plates and feed a set amount of Tetramin fish food; 0.04 mg on day 2, 0.08 mg (day 3), 0.16 mg (day 4), 0.32 mg (day 5), 0.6 mg on day 6 and following days until pupation.

The mosquitoes pupated over two days and were transferred to adult cages and allowed to emerge. The mosquitoes remained in the emergence cages for 48hrs to give them time to mate; then female mosquitoes were transferred equally to 8 cages for blood feeding. Adults were supplied with 10% glucose solution, which was removed 24hrs before blood feeding to starve the mosquitoes and increase the probability that they would take a blood meal. Mosquitoes emerged over two days thereafter 4-5 day old mosquitoes were blood fed on mice.

2.2.3 Blood feeding and infection with *Plasmodium berghei*

24hrs before blood feeding mosquitoes were transferred to a new chamber with the lower temperature of 19 (+/-2) °C; the optimum temperature for *Plasmodium berghei* development (Rastogi et al, 1987). Mosquitoes remained in this chamber until the completion of the experiment, including during DDT exposures.

Four *Plasmodium berghei* infected Balb/c mice and four uninfected control mice were obtained from the Heussler research group, University of Bern, Switzerland. The *P.berghei* line expressed Green Fluorescent Protein (GFP) and were easily identified under a fluorescent microscope. One mouse was assigned randomly to each cage and the mosquitoes were allowed to feed for 45 minutes.

24 hrs after feeding, mosquitoes that had taken a blood meal were divided into six holding cups per cage, one for each treatment group, and supplied daily with fresh sugar water until insecticide exposure

After survival was recorded, 24 hours post insecticide exposure, the mosquitoes were checked for *Plasmodium* infection. For all *Plasmodium* fed mosquitoes midguts were dissected and oocysts were counted under 100x magnification using a fluorescent microscope. For the mosquitoes exposed to DDT 19 days after the blood meal, additional dissections of the salivary glands were carried out to identify the presence of sporozoites.

2.2.4 Statistical Analysis

The analyses were carried out with the statistical package JMP 10.0.0 (SAS Institute, Cary, NC). Mosquito survival 24hrs after exposure was analysed with a binomial generalized linear model (GLM) with logit link. The mouse the mosquitoes fed on was included as a random factor in a mixed model, however it had little impact on the model so was left out of further analysis. Day of exposure and concentration of DDT were included in the models as nominal factors. Malaria was treated as a nominal factor but used in two separate ways. First, a model was used where the mosquitoes were categorised on whether they had fed on an uninfected or malaria infected mouse. A second model was run where the mosquitoes that feed on an infected mouse were split into two groups depending on infection status, thus resulting in three categories; mosquitoes that fed on an uninfected mouse, mosquitoes that fed on an infected mouse but were not infected themselves and mosquitoes that fed on infected mice and were infected. For the categorical variables that contained more than two levels, treatment contrasts (automatically given in JMP output) or *a posteriori* contrasts (Crawley, 2012) were used to determine the significance of each level. Contrasts were also used to explain any interactions observed with the multi-level categorical variables.

Initial analysis indicated complete or quasi-complete separation, this statistical phenomenon occurred due to 0% or 100% mortality in different treatment groups of the same analysis, in other words the outcome (dead or alive) is completely separated by the predictor variables. The term ‘separation’ was coined by Albert and Anderson (1984) and results in infinite parameter estimates, meaning maximum likelihood

estimates do not exist. The data was therefore analysed with firth logistic regression, which uses a penalized likelihood estimation method (Heinze & Schemper, 2002).

2.3 Results

2.3.1 Analysis based on mouse infection status

Mosquito blood feeding on uninfected mice was more successful, giving an average of 30 mosquitos per treatment group that were fed on uninfected mice and 23 mosquitoes per treatment group that fed on infected mice (the combined total from four cages). Mortality increased with DDT dose, with 1% DDT (χ^2 =29.8, $p<0.001$) and 2% DDT (χ^2 =196.7, $p<0.001$) causing significantly higher mortality than exposure to the 0% control (figure 2.1). Higher mortality was observed on day 19 compared to day 10 (χ^2 =13.32, d.f =1, $p<0.001$), however, malaria (as defined by infection status of the mouse that the mosquitos fed on) was not significant as a main effect (χ^2 = 0.51, d.f =1, p=0.473). A significant interaction between malaria and dose (χ^2 =8.84, p=0.012) is reflected by higher mortality in mosquitoes that fed on malaria infected mice using 1% DDT (χ^2 =7.2, p=0.007) but no significant difference using 0% DDT (χ^2 =2.25, p=0.133) (figure 2.1). Statistical separation prevented the same contrast being calculated for 2% DDT. There was no mortality in mosquitoes that fed on infected mice using 0% DDT paper, indicating the mortality seen in the 1% treatment group was not caused by the presence of the parasite alone. No other interactions were significant.

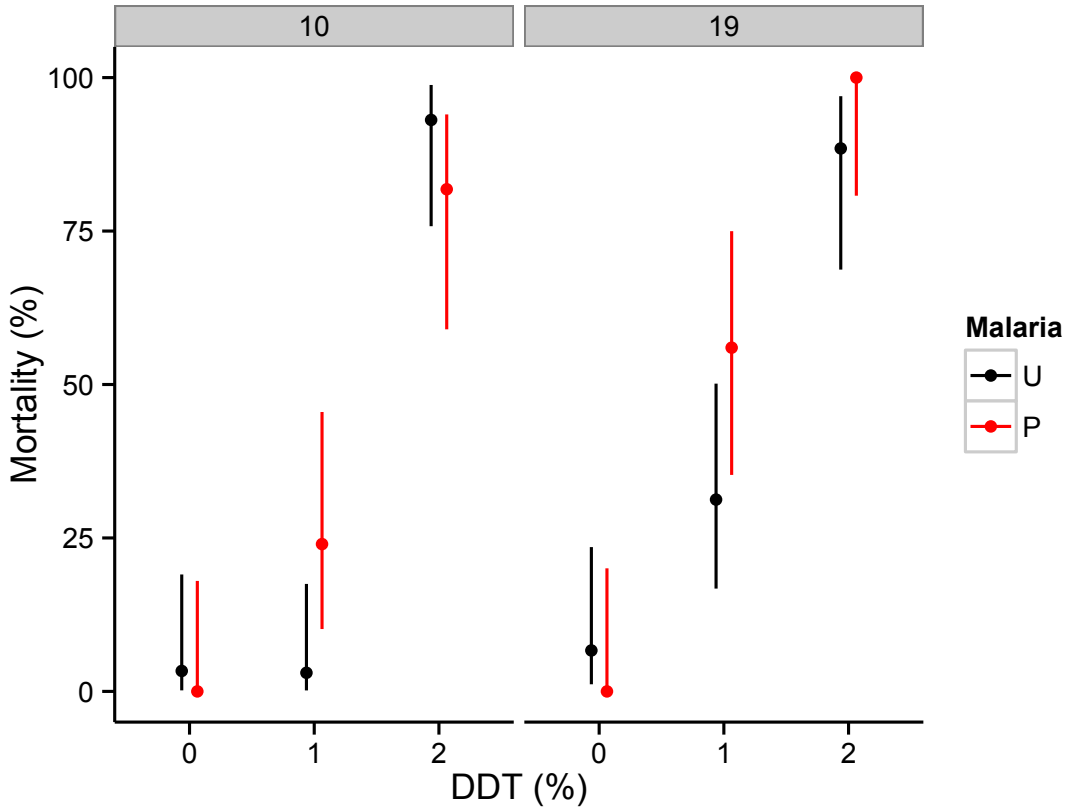


Figure 2.1: Percentage mortality of mosquitoes after exposure to 0%, 1% and 2% DDT. Exposures occurred on 2 days, 10 days (left) and 19 days (right) after blood feeding on mice infected with *Plasmodium* (P, red 95% CIs) and mosquitoes that fed on uninfected blood (U, black 95% CIs)

2.3.2 Analysis based on mosquito dissections

Of the mosquitoes exposed to insecticide ten days after blood feeding, on infected mice, 75.4% contained at least one oocyst. Oocysts numbers in the mid-gut ranged from 1-232, with a median of 50.5 per mosquito. 61.9% of mosquitoes exposed at day 19 were found with oocysts and 44.4% with sporozoites in the salivary glands.

0% DDT was left out for the second analysis as all malaria infected mosquitoes survived at this dose. As before, mortality increased with DDT dose (χ^2 =110.12, d.f =1, p<0.0001) and day of exposure (χ^2 =12.39, d.f = 1, p<0.0001) but this time malaria infection status was significant as a main effect (χ^2 =10.34, d.f = 2, p=0.006). Mortality in mosquitoes the fed on infected mice but were not infected themselves was significantly higher than mosquitoes that fed on an uninfected mice (χ^2 =10.3,

p=0.001), however, the mosquitoes that were infected were not significantly different the mosquitoes that fed on the uninfected blood (χ^2 =0.99, p=0.32). This result looks clear for 1% DDT (Figure 2.2) and although different trends were not seen using 2% DDT, no interactions were significant. The lack of an interaction between malaria and day of exposure suggests the parasite is having the same effect on both days, thus the oocysts and sporozoites are having the same effect. Results from this analysis have to be viewed cautiously as the number of mosquitoes that fed on infected mice but were not infected themselves was very low (ranging from 5-12 mosquitoes). This was due to the malaria infection rate of between 61.9% and 75.4%, leaving few mosquitoes uninfected. The low mosquito numbers is reflected in the large confidence intervals seen in figure 2.2.

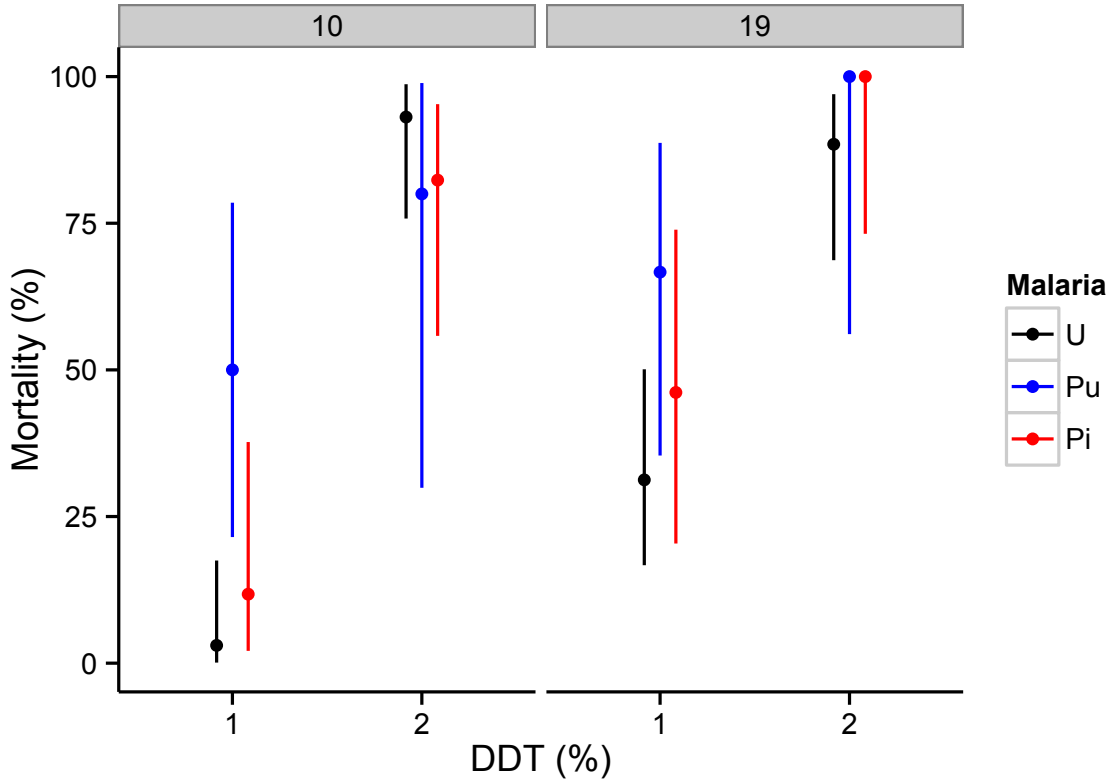


Figure 2.2: Percentage mortality after exposure to 1%, 2% DDT in mosquitoes that fed on uninfected mice (U, black 95% CIs), mosquitoes that fed on *Plasmodium* infected mice but were uninfected themselves (Pu, blue 95% CIs) and mosquitoes infected with *Plasmodium* (Pi, red 95% CIs). Exposures occurred on 2 days, 10 days (left) and 19 days (right) after blood feeding.

2.4 Discussion

To control malaria it is only necessary to kill mosquitoes that transmit the malaria parasites, therefore, if malaria-transmitting mosquitoes differ in their level of resistance to the rest of the population it could have significant implications for malaria control. Infectious mosquitos differ from the rest of the population in two main ways; (i) they harbour the malaria parasite in their salivary glands and (ii) as the mosquitoes have had to survive the developmental period of the *Plasmodium* parasite, which is typically 10-14 days (Lefèvre et al., 2013), and the daily mortality of mosquitoes is around 10% (Charlwood et al., 2009; Costantini et al., 1996; Killeen et al., 2000; Midega et al., 2007; Okech et al., 2007), malaria-transmitting mosquitoes are older than the majority of the mosquito population.

Here, the results indicate that both malaria infection and age can reduce the phenotypic expression of insecticide resistance, suggesting that malaria-transmitting mosquitoes may still be killed with insecticide despite possessing a resistant genotype. For example, an exposure to 1% DDT caused only 3% mortality in uninfected mosquitoes 10 days after blood feeding but 46% mortality in infected mosquitoes 19 days after a blood meal (Figure 2.2). Furthermore no mosquitoes that fed on infected mice, whether they were subsequently infected or not, survived an exposure to 2% DDT 19 days after the blood meal. This result would mean that the genetically resistant ZANU mosquitoes would be classed as sensitive by the time they can transmit the parasite (according WHO recommendations of 4% DDT as the discriminating dose (WHO, 1998)).

Mosquito age appears to be the main determining factor in the reduced phenotypic expression of resistance in malaria transmitting mosquitoes. The extent to which malaria affects the expression of resistance is less clear. The first analysis showed that mosquitoes which fed on malaria infected mice were more sensitive to DDT, although this was not observed using 2% DDT 10-days after the blood meal (Figure 2.1). Furthermore, once infection status was determined, the mortality of the infected

mosquitoes, although higher, was not significantly different from the mosquitoes that fed on the control mice. Mosquitoes that fed on an infected mouse, but were not infected themselves, had the highest mortality; although once again the results were unclear when using 2% DDT (Figure 2.2). The reasons why a different pattern were seen using 2% DDT are uncertain and the results were made more difficult to interpret due to statistical separation. Furthermore, the small sample size of mosquitoes that were fed infected blood, but were not infected themselves, means the results from this analysis have to be have to be viewed cautiously. If mosquitoes that are able to clear a malaria infection are subsequently more sensitive to insecticide it would suggest that there is trade-off between immunity to *Plasmodium* and resistance to insecticides. Further investigation is warranted, as the pattern would imply insecticides are selecting against mosquitoes that are refractory to the malaria parasites, which would prove worrying for malaria control.

Contrary to the results, it may have been expected that the mortality of the two uninfected malaria groups (mosquitoes that were fed uninfected blood and mosquitoes that were fed infected blood but were not infected themselves) would be more similar to each other compared to the infected group. One explanation could be that the mosquitos that were not infected because they mounted a stronger immune response to malaria infection, which potentially weakened the mosquitoes, leaving them more susceptible to the subsequent insecticide exposure. Detoxification enzymes expressed by the mosquito during *Plasmodium* infection have been linked to enzymes expressed for insecticide-resistance (Félix et al., 2010), thus diverting these enzymes in the defence against malaria may leave the resistant mosquitoes more vulnerable to insecticide exposure (Farenhorst et al., 2010). Alternatively, there may be some experimental bias. Dissecting mosquitoes becomes more difficult the longer they have been dead and expression of GFP protein also fades the longer the tissue has been dead; both of which could have reduced malaria detection and thus make it appear the dead mosquitoes had lower infection rates. If experimental bias occurred it would suggest that the results from the first analysis, based on the infection status of the mouse, would be more valid.

The results indicate that the threat resistance poses might not be as great as once believed. However, before determining what the implications are for malaria

transmission, investigations using mosquitoes with other resistance mechanisms and using natural vector-parasite combinations are needed. The resistance mechanism in ZANU mosquitoes is one of many different mechanisms that mosquitoes have evolved to survive an exposure to insecticide (Hemingway & Ranson, 2000). Resistance mechanisms can be divided into two main categories; increases in detoxification enzymes that break down insecticide (metabolic resistance) and alterations in the neurological targets of insecticide (target-site resistance) (Ranson et al., 2000a; Hemingway & Ranson, 2000). It is not know why parasitism increases the sensitivity of genetically resistant mosquitoes to insecticides, however, one theory is that detoxification enzymes, or energetic resources, are re-allocation towards defence against the parasite and not insecticide (Farenhorst et al., 2009). As the allocation of detoxification enzymes is irrelevant to target site resistance, it may therefore be expected that parasitism may affect these two main types of resistance in different ways. As target site resistance involves a structural change in the mosquitoes nervous system, it is unlikely to be directly affected by available resources. However, target-site resistance in *Culex* mosquitoes has been associated with reduced energetic reserves, presumably due to hyperactivity of the nervous system (Rivero et al., 2011). Target site resistance has also been associated with lower body mass and longer development time of *Lepidoptera* (Kliot & Ghanim, 2012) also suggesting an energetic cost. Parasitism may therefore have a greater impact on the general condition of mosquitoes carrying target site resistance compared to sensitive mosquitoes, which in turn may affect resistance. Studies investigating the effects of parasites on mosquitoes with target site resistance are however lacking, so further investigations into this area are needed.

Conversely the decline in resistance with age has been observed several times; in several different species of mosquito, in mosquitoes with metabolic resistance and in mosquitos with target site resistance (Chouaibou et al., 2012; Hunt et al., 2005; Jones et al., 2012; Kulma et al., 2013; Lines & Nassor, 1991; Rajatileka et al., 2011; Rowland & Hemingway, 1987). Although it has been hypothesised that the decrease in resistance with mosquito age is a result of declining expression of detoxification enzymes (Rajatileka et al., 2011), resistance can decline when there is no change in enzyme levels (Christian et al., 2012; Hunt et al., 2005). With particular relevance to this study, Rajatileka et al. (2011) demonstrate that the expression of the

detoxification enzyme in ZANU mosquitoes does not significantly change over a period of 3 – 14 days. Furthermore resistance can decline in mosquitos with target site resistance suggesting that there are other mechanisms behind changes in resistance other than changes in enzyme levels. Mosquitoes do senesce, with several traits declining with age (Styer el al., 2007), it is possible therefore that the increase in sensitivity with age is due to non-specific reduction in mosquito condition, or potentially a reduction in energetic reserves with age.

Here, an unnatural parasite and vector combination was used, it is important to determine if the same result is seen with natural combinations particularly with *Plasmodium* species that cause disease in humans. In unnatural combinations of parasite and vector species the virulence of the parasite is higher compared to natural combinations (Ferguson & Read, 2002). Thus, a natural malaria parasite, which will cause less metabolic stress, may not increase the sensitivity of a genetically resistant mosquito. Although further lab work could answer many of these questions, there are specific differences between mosquitoes infected in the lab and mosquitoes infected by malaria in the field. On one hand, mosquitoes infected in the lab often have a much higher parasitic load than field mosquitoes. The mean number of oocysts in field mosquitoes is around 1 - 4 (Pringle, 1966), whereas in this study the mean number of oocysts per infected mosquito was 50.5. Thus, as the virulence of the malaria parasite increases with parasitic load (Dawes et al., 2009), the effect of plasmodium infection on resistance may be enlarged in a lab scenario. On the other hand, mosquitoes in the lab are kept in a stable environment with access to an unlimited supply of sugar. Mosquitoes may therefore increase uptake of sugar to compensate for the damaging effects of the parasite (Rivero & Ferguson, 2003). The effect of malaria infection on resistance may therefore be greater in more stressful field conditions and where food may not be instantly accessible to replenish resources used up by parasitism.

What would the impact of increasing sensitivity with mosquito age and malaria infection be for mosquito control and malaria transmission? Old and malaria-infected mosquitoes only make a small proportion of the mosquito population so if these mosquitoes are killed by insecticide it is unlikely to have a significant effect on overall mosquito numbers. Even in high transmission zones, where the chances of a mosquito taking a blood meal from an infected human are high, it is unlikely that the

impact on mosquito populations will be drastic. However, of most concern is the number of mosquitoes reaching infectivity rather than overall mosquito numbers; this will depend on the rate resistance declines with age (Chapter 5) and the extent to which oocytes increase sensitivity to insecticides.

In conclusion, *Plasmodium* infection modestly increased sensitivity of resistant mosquitoes to insecticide in comparison to increasing mosquito age. However, both factors in combination could mean few genetically resistant mosquitoes are still phenotypically resistant by the time they can transmit malaria. The next step is to determine the phenotypic resistance of malaria transmitting mosquitoes in the field.

Chapter 3

Effects of age and larval nutrition on phenotypic expression of insecticide-resistance in *Anopheles* mosquitoes

3.1 Introduction

Mosquito control with insecticides has been a vital tool in the fight against malaria since Paul Müller discovered the effect insecticide properties of DDT in the late 1930s (Raju, 1999). Bed-nets treated with insecticides are now the mainstay of malaria control with indoor residual spraying (IRS) of insecticide also contributing significantly to the malaria control effort (WHO, 2010). However, concerns over the future efficacy of these techniques have arisen with the evolution of insecticide resistance. Resistance to each chemical class of insecticide used for malaria control has now been recorded, and resistance can be found in many malarious regions across the globe (Edi et al., 2012; Maxmen, 2012). Research into resistance is therefore vital to understanding and counteracting the problem. Many of the molecular mechanisms leading to insecticide-resistance have already been identified (Hemingway et al., 2004) and resistance test protocols have been developed allowing easy identification and monitoring of resistant mosquitoes in the field (CDC 2012, WHO 2008). It is, however, the phenotypic expression of resistance that presents that threat to malaria control, so it is vital that any environmental or demographic effects on the expression of resistance are understood.

The effect of mosquito age on the expression of resistance has received significant interest with many studies showing a decline in resistance with mosquito age (Chouaibou et al., 2012; Hunt et al., 2005; Jones et al., 2012; Lines & Nassor, 1991; Rajatileka et al., 2011; Rowland & Hemingway, 1987). This is particularly important for malaria control as it takes at least 10 days for the malaria parasite to develop

inside a mosquito. Thus, by the time a mosquito can transmit the malaria parasite to a new human host, it has significantly lower resistance levels and may be killed by insecticide interventions despite its resistant genotype. Environmental factors such as temperature can also influence the expression of resistance (Hodjati & Curtis, 1999). It has even been suggested that biopesticides be used to manage resistance, as it has been shown that parasitism can increase the sensitivity of genetically resistant mosquitos to insecticide (Farenhorst et al., 2010; Koella et al., 2012). The mechanism behind this increase in sensitivity is unknown, but may be linked to the metabolic stress the parasite imposes on a mosquito and the resulting reduction in energetic reserves (Rivero et al., 2007). Resistant mosquitoes store fewer lipids, sugars and energetic reserves than sensitive mosquitoes (Hardstone et al. 2010; Rivero et al., 2011), which suggests resistance is dependent on these energetic reserves and, thus, resource availability would limit the expression of resistance. This appears to be the case as mosquitoes that receive a blood meal are more resistant than mosquitoes that do not (Hunt et al., 2005; Rajatileka et al., 2011). However changes in resistance levels after blood feeding may be related to changes in enzyme expression, so further investigation is warranted.

To test the hypothesis that available recourses limit the expression of resistance, DDT-resistant and sensitive *Anopheles gambiae* mosquitoes were reared on three different feeding regimes, at the larval stage, and their resistance levels were measured as adults. Larval diet was manipulated as it is correlated with adult condition and energy reserves in several insects (Boggs & Freeman, 2005; Dmitriew & Rowe, 2011; Rolff, 2004) including mosquitoes (Araújo, 2012; Takken, 2013; Suwanchaichinda,1998). It was expected that the negative effects of low diet would be stronger in the resistant mosquitoes than in sensitive mosquitoes because of the resource demands related to insecticide resistance. It was further hypothesised that manipulating larval food could also influence the expression of resistance throughout the mosquitos' lifespan. The reduction in resistance with mosquito age is often thought to occur due to a reduction in the expression of detoxification enzymes (Rajatileka et al., 2011), however, this is not always true (Christian et al., 2012; Hunt et al., 2005). The reduction in resistance may therefore be due to a reduction in mosquito condition and energetic reserves. Consequently it was predicted then that

the reduction in resistance with mosquito age would be most dramatic in mosquitoes that were fed a low diet as larvae and which resulted in lower mosquito condition at eclosion.

3.2 Materials and methods

3.2.1 Feeding regime

All the experimental procedures were carried out at a temperature of 26 (+/−1)°C and 70 (+/−5) % relative humidity with a 12 h: 12 h light/dark cycle. Two colonies of *Anopheles gambiae* mosquitoes were used: a DDT-resistant ZANU colony with increased metabolism of the insecticide, catalyzed by members of the glutathione S-transferees enzyme family (Ranson et al., 2000b) and the DDT-sensitive Kisumu colony from western Kenya (Vulule et al., 1994). Two-day old larvae were transferred to 12-well plates and reared individually in 3ml of de-ionized water. For each colony we reared 480 mosquitoes at each of three feeding regimes: 100% (high), 70% (medium) or 40% (low) of the standard amount of TetraMin® Baby fish food (Table 3.1). Given food quantities were administered to each well in 100uL of de-ionized water (which partially compensates for evaporative loss). Emerged females were moved to plastic cups and supplied with cotton balls moistened with saturated 10% sugar solution, males were discarded**.** Although only 150 female mosquitoes were required per feeding regime, 480 mosquitoes were reared in order to take account of; male mosquitoes, mosquitoes not surviving to become adults and mosquitos that would not survive to 15 days after emergence (the final insecticide exposure day).

Days after hatch	LOW	MEDIUM	HIGH
1	0.016	0.028	0.04
2	0.024	0.042	0.06
3	0.032	0.056	0.08
4	0.064	0.112	0.16
5	0.128	0.224	0.32
6 and later	0.240	0.420	0.60

Table 3.1: Daily amounts of food (in mg) for the three different diet levels

3.2.2 Insecticide exposures

The resistance of mosquitoes was measured with the standard World Health Organization test-kit according to WHO guidelines (WHO, 1998). 50 adult females from each feeding regime and colony were exposed to insecticide at each of three ages: 5, 10 or 15 days after emergence. They were individually exposed to DDT-treated filter paper (4%) for 100 minutes (resistant ZANU colony) or 40 minutes (sensitive Kisumu colony). Exposure times were based on earlier experiments, so that about half of the mosquitoes were expected to die within 24 hours of exposure when they were 5 days old. After exposure the mosquitoes were moved back into insecticide-free plastic cups and survival was recorded 24h later. After survival was recorded mosquito wings were removed, fixed onto glass slides, scanned and measured from the tip (excluding the fringe) to the distal end of the allula using ImageJ software (http://rsb.info.nih.gov/ij/). Wing length was recorded as an average of both wings, however if a wing was damaged and a measurement couldn't be taken a single wing was used.

3.2.3 Statistical analysis

All analyses were carried out separately for ZANU and Kisumu mosquitoes. Wing length measurements were normally distributed and comparisons of the mean wing length between mosquitoes of each food regime were carried out with Analysis of

Variance (ANOVA) and a Tukey HSD *post hoc* test. Note, only the wing lengths of the mosquitos that were used in the DDT exposures were compared, wing lengths of males and discarded females were not included.

24hr survival data from the DDT exposures was analysed with a binomial GLM with logit link, with a correction for over-dispersion if necessary. As age at exposure had a close to linear effect (Figure 3.2), it was considered a continuous factor. Feeding regime was considered an ordinal factor. Age of mosquito emergence was also included in the analysis, however, as age at emergence determined the date of exposure (so that age at exposure could be fixed), it was considered a nominal factor. The analysis included only the interaction between age at exposure and feeding regime, as including interactions with age at emergence would have led to a very unbalanced analysis.

The analyses were carried out with the statistical package JMP 8.0.2 (SAS Institute, Cary, NC).

3.3 Results

ZANU mosquitoes emerged over a period of 5 days (9 to 13 days after hatching) and Kisumu mosquitoes emerged over 3 days (9 to 11 days after hatching). Comparison of wing lengths (of mosquitos that were exposed to DDT) indicates a reduction on larval food reduced the mean wing length in both ZANU ($F = 68.5$, d.f. = 2,299, $p < 0.001$) and Kisumu colonies ($F = 17.5$, d.f. = 2,320, $p < 0.001$). Tukeys HSD test indicated that the means from each pairwise comparison were statistically significant; the closest to non-significance was the comparison of mean wing length between the low and medium food groups of Kisumu ($p = 0.0485$; Figure 3.1).

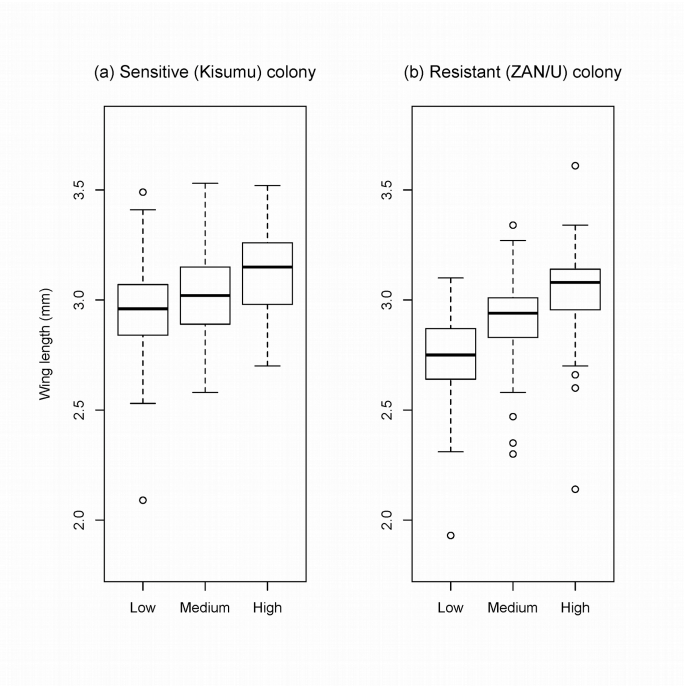


Figure 3.1: Boxplot of wing length by colony and diet. A positive correlation between diet and wing length is observed in both: a) Kisumu and b) ZANU colonies. Thick horizontal lines represent median, bottom and upper edges of the boxes first and third quartiles, whiskers demonstrate minimum and maximum values.

Factor	df	X^2	P
(a) resistant ZANU			
Age at emergence	4	5.49	0.241
Age at exposure	1	68.01	**<0.001**
Food regime	2	3.63	0.163
Food* Age at exposure	2	4.38	0.112
(b) sensitive Kisumu			
Age at emergence	2	4.95	0.084
Age at exposure	1	10.72	**0.001**
Food regime	2	10.50	**0.005**
Food* Age at exposure	2	8.65	**0.013**

Table 3.2. GLM analysis of 24 hours post-exposure survival in a) resistant and b) sensitive colonies of *Anopheles gambiae* mosquitoes

In both colonies, a significant negative slope in the analysis indicated that increasing age at exposure decreased the likelihood of survival after exposure to DDT (Figure 3.2, Figure 3.3, Table 3.2). The drop in resistance with age was particularly rapid in ZANU mosquitos, however, a non-significant interaction between larval food indicted the decline in resistance with age was not affected by larval food quantity. Moreover larval food level did not effect the survival after insecticide exposure in ZANU mosquitoes (Table 3.2).

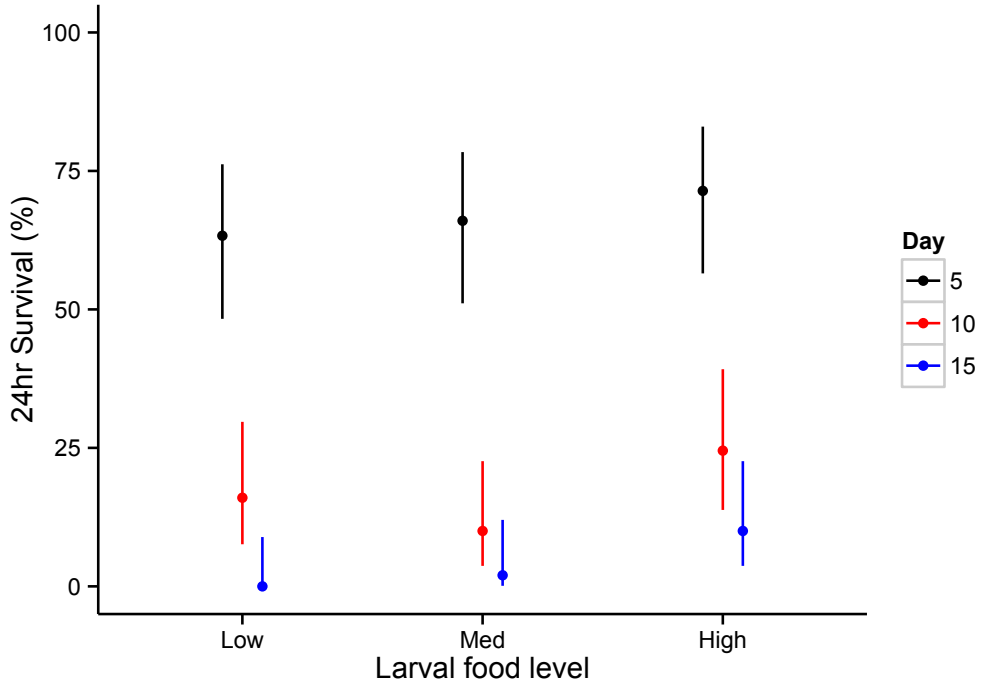


Figure 3.2 Survival of adult ZANU mosquitoes 24 hrs after a 100-minute exposure to DDT. Mosquitoes received one of three larval food regimes (indicated on the x-axis) and were exposed to DDT 5, 10 or 15 days after emergence (indicated by the 95% confidence intervals and accompanying legend)

For the sensitive Kisumu mosquitoes a reduction in larval food decreased survival (Figure 3.3, Table 3.2). *A posteriori* contrasts (Crawley, 2012), with the interaction between larval food and age at exposure removed, indicate that survival was significantly higher in the medium feed group compared to the low fed group (χ^2 =4.18, p=0.041), and higher significance between the low fed group and the high fed group (χ^2 =6.05, p=0.013). A significant interaction between a continuous variable (age) and categorical variable (larval food) means the slope of the continuous variable is different in one or more of the levels. The analysis indicated that there was no difference in the decrease in age with in mosquitoes that received low and medium larval food (χ^2 =0.64, p=0.424) however the decline in resistance with age was steeper in high food group (χ^2 =8.17, p=0.004). Finally age at emergence did not have a significant effect on survival (Table 3.2).

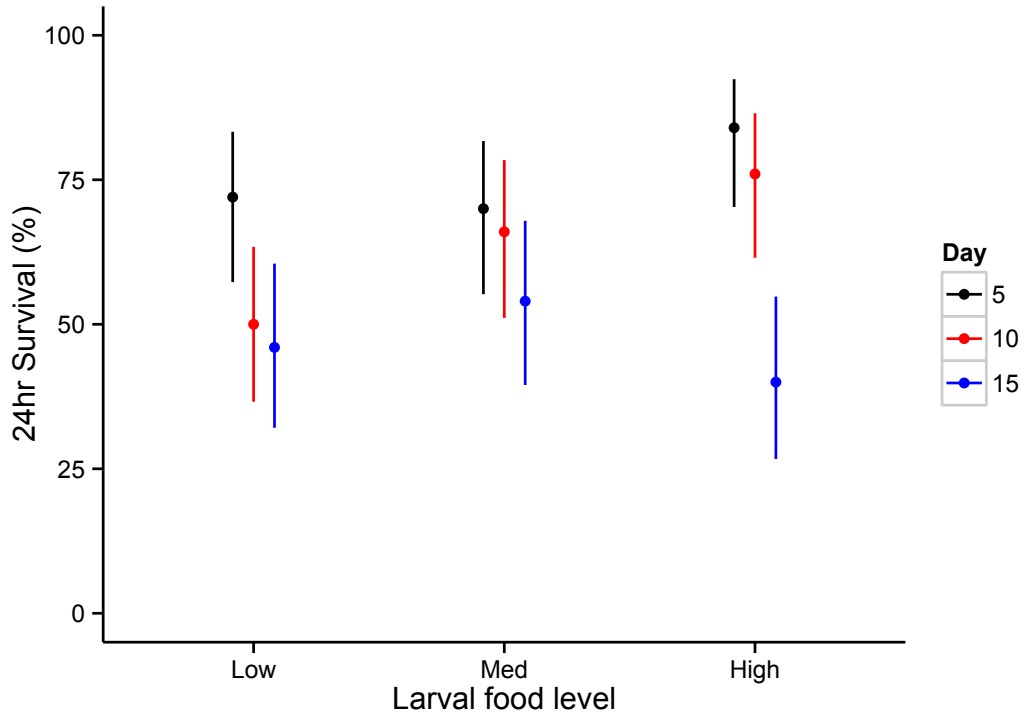


Figure 3.3 Survival of adult Kisumu mosquitoes 24 hrs after a 40-minute exposure to DDT. Mosquitoes received one of three larval food regimes (indicated on the x-axis) and were exposed to DDT 5, 10 or 15 days after emergence (indicated by the 95% confidence intervals and accompanying legend)

3.4 Discussion

Manipulating the quantity of larval food had mixed effects regarding the sensitivity of the two mosquito colonies to insecticide. In the DDT sensitive Kisumu colony, decreasing the food levels further decreased mosquito survival post insecticide exposure, and although a similar trend was seen in ZANU mosquitoes it was not significant. This suggests that the expression of detoxification genes in ZANU is less sensitive to environmental variation than the more general immunological mechanisms that presumably help Kisumu mosquitoes survive exposure to insecticides. Note, however, that any difference between ZANU and Kisumu must be interpreted with caution as their genetic backgrounds differ by more than just the site of resistance (David et al., 2005).

The result in ZANU mosquitoes was somewhat surprising. It was believed that restricting the available resources would restrict the resistance mechanism, as the resistance mechanism increases the production of metabolic enzymes and therefore presumably dependent on the resources available. Although resistance mechanisms have been associated with energetic costs in mosquitoes (Hardstone et al. 2010; Rivero et al., 2011), studies with *Drosophila* (McCart et al., 2005) indicate that this may not always be the case. The energetic cost of resistance in ZANU mosquitoes has not been measured, thus it could be minimal and the expression of resistance would not be effected. Establishing the energetic costs of ZANU mosquitoes may therefore help provide answers to the results observed here. Repeating the experiment with resistant strains that have shown to have energetic costs (Hardstone et al. 2010; Rivero et al., 2011) may result in greater effects of larval nutrition on the expression of resistance.

It is also plausible that the expression of resistance may not be influenced by energetic reserves at all, even if there is an energetic cost found. Resistance is vital to fitness of resistant mosquitoes such as ZANU, so allocation of resources to resistance may be maintained despite low availability. Indeed, a recent study by Brooke and Oliver (2013), showed that the overexpression of GST enzymes associated with resistance was equivalent in adult mosquitoes whether they were starved or fed a normal diet as

larvae. As the resistance of ZANU mosquitoes is mediated by the expression of GST, a similar situation would explain why larval diet did not influence the expression of resistance in this study. The resistant strain of *Anopheles arabiensis* used by Brooke & Oliver (2013) overexpressed other detoxification enzymes (Esterase and Cytochrome P450) that did have reduced expression in starved mosquitoes. Subsequently the resistant *Anopheles arabiensis* were more sensitive to DDT if starved as larvae, highlighting that larval diet could affect different resistance mechanism in contrasting ways. If expression of detoxification enzymes is maintained it may be detrimental to other mosquito traits that are determined by resource based trade-offs. Indeed, low diet appears to have a greater impact on the development time and size of the ZANU mosquitoes. Again care has to be taken in the interpretation of the result as the genetic backgrounds of the two colonies differ in more than just the site of resistance. Finally the experimental set-up may have effected the results; the underfed larvae may have compensated for their low energy reserves by feeding more in the adult stage where food was not limited (Aboagye-Antwi & Tripet, 2010). Further experiments manipulating adult food may therefore prove fruitful in determining if energetic reserves influence the expression of resistance.

In contrast to ZANU, larval diet did affect the tolerance of Kisumu mosquitoes to low doses of DDT. The defence against DDT in Kisumu mosquitoes is likely mediated by general immune response mechanisms. Reducing larval diet can reduce immunity in adult mosquitoes (Suwanchaichinda,1998), and damselflies (Rolff, 2004), so may explain why the tolerance of Kisumu mosquitoes to low doses of DDT is reduced when fed a low diet as larvae. The focus of this study was to assess the quantity of larval food on the expression of resistance, but the composition or quality of larval food can also affect adult traits. Fellous and Lazzaro (2010) showed that mosquitoes on a high protein larval diet had a stronger immune response than mosquitoes on other diets, even though the diets had no apparent effect on mosquito size or quality. Indeed, the expression of insecticide resistance in the cotton aphid is determined by the plant it is reared on (Godfrey, 2001). In nature larval habitats and the organic matter contained within them can vary dramatically, affecting; mosquito development time, adult condition and susceptibility to malaria infection (Okech, 2007b). Larval density and competition can also influence; adult body size, condition and

susceptibility to dengue virus (Alto 2008; Lyimo et al., 1992). Could these factors also affect the tolerance or resistance of mosquitoes to insecticides? Composition and quality of food can influence the expression of resistance in several insect species (Gordon, 1961). Toxins found in certain plant material can also influence the expression of enzymes associated with insecticide resistance (David et al., 2006), and even oxygen and ammonia levels in water can affect tolerance to insecticides (Fossog et al., 2012). Predicting if certain larval habitats will produce more or less resistant adult mosquitoes could be very valuable in the fight against insecticide resistance and the fight against malaria.

Mosquitoes reared on restricted diets have reduced longevity, presumable because of the lower energetic reserves at eclosion and reduced immune response. The second hypothesis therefore predicted that the reduction in resistance with age would be more dramatic in mosquitoes that were given the low diet. The results corroborated other studies that resistance does indeed decline with mosquito age (Chouaibou et al., 2012; Hunt et al., 2005; Rajatileka et al., 2011), however, the only evidence it was affected by larval food quantity was in Kisumu mosquitos where decline resistance with age was greatest in the best fed larva, again going against expectations. The interaction was highly influenced by one treatment in particular (the high diet mosquitoes exposed 15 days after emergence (Figure 3.2)) so could be down to experimental error. There may be other explanations, for instance, higher bacterial loads accumulate in water used to rear the larvae when more food is available, which in turn could have affected the resistance of high diet mosquitoes.

In conclusion, although the effect of larval diet did not affect the expression of resistance in ZANU mosquitoes, much is still unknown about the impact of larval and adult mosquito diets on the phenotypic resistance. Considering environmental variation is important in understanding the expression of insecticide resistance and therefore the threat it poses to mosquito borne diseases. The reduction in resistance with age could significantly reduce the probability of resistant mosquitoes to transmit malaria and although it is now well described in the laboratory, field studies are needed to assess its impact on malaria control.

Chapter 4

Restoring sensitivity of insecticide-resistant malaria vectors with a microsporidian

4.1 Introduction

Insecticides, used for indoor-residual spraying (IRS) or on bed-nets (ITNs), are vital tools in the fight against malaria (WHO, 2012a). The current malaria eradication campaign has halved the malaria burden in several African countries with the use of ITNs, IRS and anti-malarial drugs (WHO, 2012a). However, insecticide-resistance, in the mosquito vector, has been recorded in two thirds of countries with malaria problems (WHO, 2012a) and threatens the control of malaria transmission (Maxmen, 2012; Moszynski, 2012; Baleta, 2009). Insecticide-resistance, by its very definition, allows mosquitoes to survive an exposure to insecticide, thus, the ability to manipulate the expression of resistance would be a powerful tool in maintaining the effectiveness of control methods. One potential method to achieve this is through environmental manipulation as the phenotypic expression of resistance is determined by both genetic and environmental factors.

For example, toxins found in certain plant material can influence the expression of enzymes associated with insecticide resistance if eaten by mosquito larvae (David et al., 2006), oxygen and ammonia levels in water can affect tolerance of mosquitoes to insecticides (Fossog et al., 2012). The resistance of cotton aphids to insecticides depends on the characteristics of their host plant (Godfrey & Fuson, 2001). Most relevant to this study is that infecting adult *Anopheles* that are resistant to DDT and pyrethroids (with mechanisms involving metabolic and target-site mechanisms) with

pathogenic fungi *Beauveria bassiana* or *Metarhizium anisopliae* restores their sensitivity to the insecticides (Farenhorst et al., 2009).

Thus, stress caused by environmental conditions and in particular by parasitic infection can affect the expression of resistance. Here, the microsporidian biopesticide *Vavraia culicis* is used to show, at least in simple laboratory situations, that environmental manipulation can be used to increase the sensitivity of genetically resistant mosquitoes to insecticide. *V. culicis* has been proposed as an alternative vector control method as it reduces adult mosquito longevity (Koella et al., 2009a; Lorenz & Koella, 2011) and can reduce malaria infection in the mosquito (Bargielowski & Koella, 2008). *V. culicis* also reduces the energetic reserves of a mosquito (Rivero, 2007) which may have an impact on resistance mechanisms that are believed to be resource dependent (Hardstone et al. 2010; Rivero et al., 2011). Reducing the expression of resistance would add another benefit to the biopesticide and suggest it could be used successfully in an integrated approach, with chemical insecticides, to tackle resistance and malaria transmission.

4.2 Materials and Methods

4.2.1 Study organisms

Two colonies of *Anopheles gambiae* used for the experiment; a DDT-resistant colony (ZANU) from Zanzibar with increased metabolism of the insecticide, catalyzed by members of the glutathione S-transferase (GST) enzyme family (Ranson, 2000) and a mildly pyrethroid-resistant (RSP) from western Kenya with elevated esterase and oxidase levels (Vulule et al., 1999) and possibly with knockdown resistance (kdr) (Ranson et al., 2000b; Christian et al., 2011)

As an environmental stressor the microsporidian *Vavraia culicis* was used. *V. culicis* is an obligate, intracellular parasite of several mosquito species (Becnel et al., 2005; Andreadis, 2007). Mosquito larvae are infected when they ingest the parasite's spores along with their food. Some infected larvae and pupae die and release a new

generation of the parasite's spores for horizontal transmission to other larvae. If the mosquitoes survive to emerge, the adults remain infected. The parasite has several effects on the adult, including a shorter lifespan (Koella et al., 2009a; Lorenz & Koella, 2011) and reduced susceptibility to malaria (Bargielowski & Koella, 2008). Although there is no transovarial vertical transmission, spores harbored by adult females can infest a new breeding site when they are released together with eggs (Andreadis, 2007). The prevalence of only a few microsporidian species in natural populations has been estimated; it is typically less than a few percent (Andreadis, 2007). While the prevalence of *V. culicis* in populations of *Aedes* mosquitoes ranges from 0% to about 50%, the only study on its prevalence in *Anopheles gambiae* found that 6.6% of the larvae in a West African population were infected (Andreadis, 2007).

4.2.2 Experimental procedures

Mosquitoes were reared individually in 12-well plates at a temperature of 26 (+/-2) °C and 70 (+/-10) % relative humidity with a 12 h:12 h light/dark cycle. Mosquitoes were fed with a standard amount of Tetramin fish food; 0.04 mg on day 2, 0.08 mg (day 3), 0.16 mg (day 4), 0.32 mg (day 5), 0.6 mg on day 6 and following days until pupation. Microsporidian spores were obtained by homogenizing infected adult mosquitoes, and then counting the spores at 400x magnification with a haemacytometer. The solution was diluted to 20000 spores / 100 µl. Each well obtained 100 µl of this solution when larvae were 2 days old. In earlier experiments, this infectious dose infected more than 95% of the larvae, but killed them only rarely. Controls received 100 µl of solution containing the same number of uninfected adults.

Resistance to DDT of the ZANU mosquitoes and resistance to permethrin of the RSP mosquitoes were measured in separate experiments according to World Health Organization guidelines with the standard WHO test-kit (WHO, 1998). Mosquitoes were exposed to the insecticide two or three days after emergence in groups of between 2 and 11. To measure DDT-resistance of ZANU, mosquitoes were exposed to DDT-treated filter paper (4%) for 0, 45, 90 or 135 minutes. For permethrin-resistance, mosquitoes were exposed to permethrin-treated filter paper (0.75%) for 0, 15 or 30 minutes. Exposure times were chosen based on earlier experiments. After

exposure, the mosquitoes were transferred to clean holding tubes and provided with cotton balls moistened with saturated sugar solution.

The number of dead mosquitoes in each tube was scored 24 h after exposure, and was analyzed with JMP 11.0 (SAS Institute, Cary, NC) with a glm (binomial distribution and logit link) that included replicate (for ZANU), infection status as a nominal factor, time of exposure as an ordinal factor and the interaction of infection and exposure. For DDT-resistance, 902 mosquitoes were tested in two replicates. For permethrin-resistance, one replicate of 152 mosquitoes was analysed. (Mosquitoes in several tubes were inadvertently not sugar-fed and were therefore left out of the analysis.). For the categorical variables that contained more than two levels, treatment contrasts (automatically given in JMP output) or *a posteriori* contrasts (Crawley, 2012) were used to determine the significance of each level. Contrasts were also used to explain any interactions observed with the multi-level categorical variables.

4.3 Results

The ZANU line showed considerable resistance to DDT. While WHO defines sensitive mosquitoes as those that die within 24 h after having been exposed 4% DDT for 60 minutes, only 45% of mosquitoes died after 135 minutes of exposure (Figure 4.1). Infection by the microsporidian increased the mortality in particular at intermediate exposures (Table 4.1): from 4% to 28% after 45 minutes of exposure (χ^2 = 25.0 , $p < 0.001$) and from 18% to 54% (χ^2 = 37.8 , $p < 0.001$) after 90 minutes of exposure. An increase in mortality due to microsporidia was also observed after an exposure of 135 mins however it was not quite significant (χ^2 = 3.0 , $p = 0.082$) and microsporidia did not increase the mortality of the 0 min group (χ^2 = 2.8 , $p = 0.093$), leading to a significant interaction between exposure time and infection status (Table 4.1; Figure 4.1).

As permethrin kills mosquitoes after a shorter exposure than DDT, exposures were shorter for RSP than for ZANU. RSP was moderately resistant to permethrin: after 30 minutes of exposure 50% died within 24 h (compared to 95% of a sensitive colony,

unpublished data) (Figure 4.2) Again, infection by the microsporidian increased mortality in particular at the intermediate exposure (Table 4.1): after 15 minutes mortality increased from 7% to 63% (χ^2 = 20.3, p < 0.001), while after 30 minutes mortality increased from 50% to 67% but was not significant (χ^2 = 1.16 , p = 0.28). Again microsporidia did not significantly increase the mortality of the 0 min exposure (χ^2 = 1.9 , p = 0.16), again leading to a significant interaction between exposure time and infection status (Table 4.1).

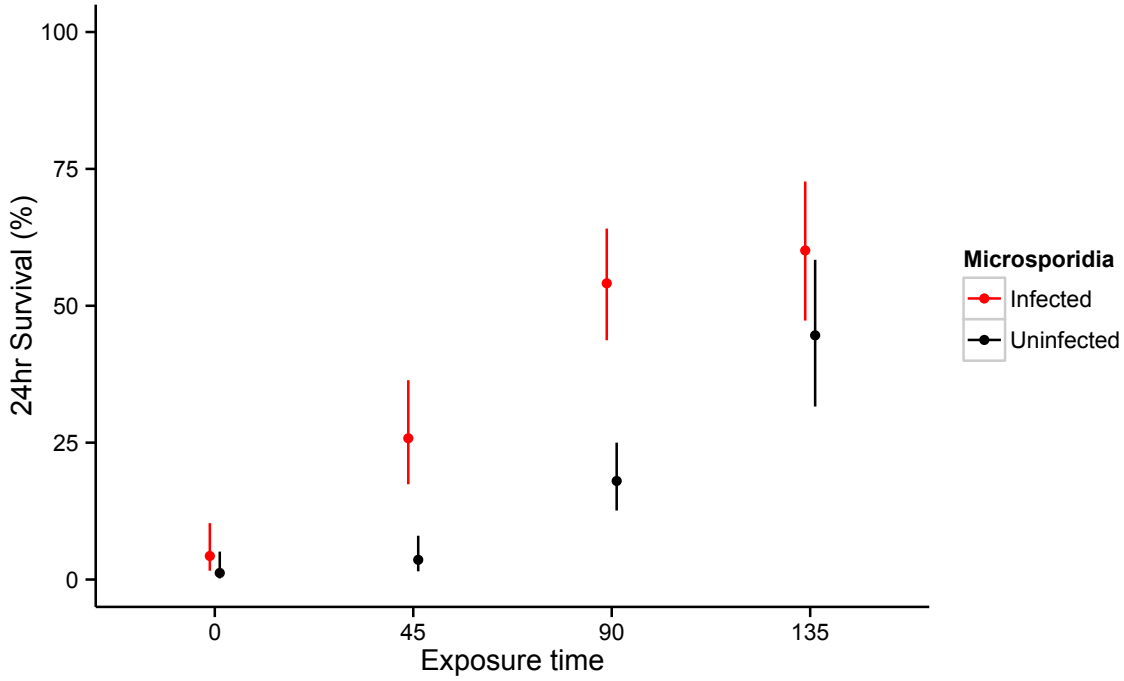


Figure 4.1. The proportion of insecticide-resistant mosquitoes (ZANU) dying within 24 hours DDT, as a function of *Vavraia*-infection. Red 95% confidence intervals (CIs) represent microsporidian-infected mosquitoes; black CIs represent uninfected mosquitoes. Exposure time is shown on the x-axis.

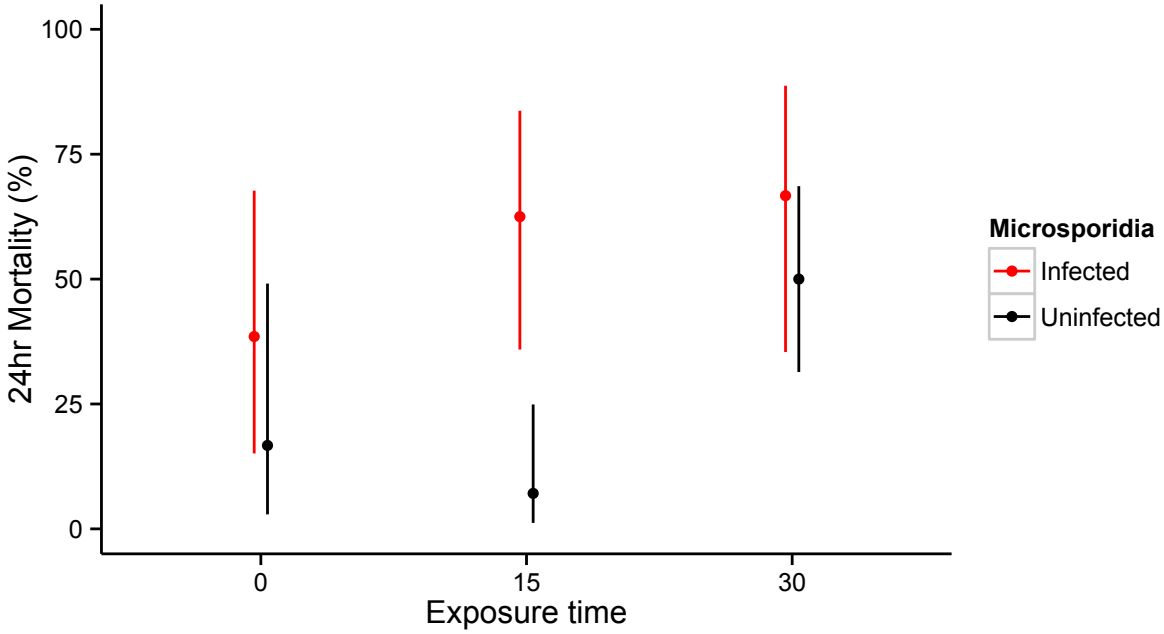


Figure 4.2. The proportion of insecticide-resistant mosquitoes (RSP) dying within 24 hours after an exposure to permethrin, as a function of *Vavraia*-infection. Red 95% confidence intervals (CIs) represent microsporidian-infected mosquitoes; black CIs represent uninfected mosquitoes. Exposure time is shown on the x-axis.

	ZANU: DDT			RSP: permethrin		
	df	χ^2	p	df	χ^2	p
Replicate	1	9.6	0.002			
Exposure time	3	178.7	< 0.001	2	10.7	0.005
Infection	1	2.8	0.093	1	1.9	0.166
Exposure time x infection	3	10.3	0.016	2	6.1	0.046

Table 4.1: GLM (with binomial distribution) of 24-hour mortality after exposure of uninfected and *Vavraia*-infected mosquitoes to insecticides.

4.4 Discussion

The infection of *V. culicis* increased the sensitivity of two resistant strains of *An.gambaie* to insecticide, suggesting environmental manipulation could at least partially restore the efficacy of traditional chemical control methods. Reducing the expression of resistance, thus, reducing the benefit of resistance to the mosquito, could also have important evolutionary consequences. A lower fitness benefit could reduce the speed at which a resistance gene spreads through a population. It is hypothesised that if *V.culicis* could also increase the fitness costs of resistance, by reducing the longevity of resistant mosquitoes more than sensitive ones (chapter 6), that the evolution of insecticide resistance could be blocked (Koella et al., 2012)

The mechanisms behind the increase in sensitivity with parasitism are unknown. They may be due to increased metabolic stress caused by the pathogens or, alternatively, the re-allocation of detoxification enzymes towards defence against the fungal pathogen rather than the insecticide (Koella et al., 2012, Farenhorst et al., 2009). *V. culicis* infects the malpighian tubule system, the fat body and the midgut epithelium of adult mosquitoes (Vávra and Becnel, 2007), damaging cells and depleting the lipid, glycogen and sugars reserves (Rivero et al., 2007). If sensitivity is linked to metabolic stress, higher parasitic loads or more virulent strains of *V. culicis* may further increase the sensitivity of mosquitoes to insecticide. *V. culicis* has also been shown to suppress aspects the larval immune system; the suppression of GST enzymes is of particular interest to this study (Biron et al., 2005; Duncan et al., 2012). GST enzymes mediate the resistance of ZANU mosquitoes so if suppressed by *V. culicis* may further explain the increase in sensitivity in mosquitoes infected with *V. culicis*. However, *V. culicis* also increased the sensitivity of RSP, which is associated with kdr point mutation and the overexpression of esterase but not GST, so the suppression of non-specific enzymes of the immune system may also increase sensitivity. A better understanding of the mechanisms leading to sensitivity is needed if we hope to fully restore sensitivity to genetically resistant mosquitoes.

Here, *V. culicis* increases the sensitivity of two resistance strains of mosquito yet there are several other resistance mechanisms described in mosquito populations (Hemingway & Ranson, 2000). For microsporidia to be truly successful at controlling resistance they will need to increase the sensitivity of mosquitos carrying any of the resistance mechanisms. Resistance mechanisms can be divided into two main categories; metabolic resistance (an increase in detoxification enzymes) and target-site resistance (alterations in neurological targets of insecticide rendering them less sensitive) (Ranson et al., 2000a; Hemingway & Ranson, 2000). Although RSP may have target-site resistance, metabolic resistance predominantly mediates the resistance of mosquitos used in this study. This is important as one might predict that parasitism may have a larger effect on metabolic resistance which is presumably more reliant on energetic reserves and will also be effected by the changing enzyme expression in parasitized mosquitoes (Biron et al., 2005; Duncan et al., 2012). However, neither this study nor the study with entomopathogenic fungi (Farenhorst et al., 2009) contained mosquitoes that only carried target site resistance, so further investigation is required to see if target-site resistance is also affected by parasitism.

The success of microsporidia in combating resistance will also rely on infecting as many resistant mosquitoes as possible. Successful larviciding campaigns such the eradication of *An. gambiae* from Brazil, using the larvicide Paris Green, involved significant manpower and detailed mapping of larval sites (Killeen, 2002). Such a programme would be needed if the microsporidian *V. culicis* were to be used to fight vector control and resistance management, however, microsporidia has further modes of dispersal which may make coverage of the biopesticide easier. Infected females can disperse the parasite when they are released into breeding sites during oviposition (Andreadis, 2007). Auto-dispersal of insecticides (using adult mosquitoes to disperse insecticides) is potentially a very effective way of reaching a large percentage of mosquito breeding sites and breeding sites that are difficult to identify. Studies with insect growth regulator pyriproxyfen (Chism & Apperson, 2003) and a juvenile hormone analogue (JHA) (Devine et al., 2009), in *Aedes albopictus* and *Aedes aegypti* respectively, have shown that these larvicides can be distributed to a high proportion of larval breeding sites even if the initial application is small. This is particularly useful for these vectors of dengue virus, where identification of breeding sites is

notoriously difficult (Moloney et al., 1998). Auto-dispersal of JHA and pyriproxyfen is achieved by exposing the adults to the insecticide which could also be achieved with *V. culicis* as they can also be fed to adult mosquitoes via sugar traps (Jacob C Koella unpublished results; Weiser and Zizka, 2004). Thus, a combination of distributing *V. culicis* spores to larval breeding sites, infecting the adults through sugar baits (Gu et al., 2011) and auto-dispersal by the adult mosquitoes may achieve high prevalence of *V. culicis* infection in natural mosquito populations.

The danger is that a high prevalence of microsporidia will lead to the selection for resistance against microsporidia itself. However, microsporidia impose little selection pressure on the mosquito as they kill older mosquitoes that have outlived the majority of the population. The late acting properties of microsporidia, and similar biopesticides, has lead to the suggestion that they could be used as evolution-proof methods of vector control (Koella et al., 2009a; Read et al. 2009).

In conclusion, manipulating the environment in a way that restores sensitivity to insecticides may help to manage the problem of insecticide-resistance. Much more needs to be done before manipulating the environment to control the evolution of insecticide-resistance becomes a reality. In particular, a better understanding is needed of the factors underlying sensitivity to insecticides. While science has made considerable progress at understanding some aspects of the genetic basis of resistance (Hemingway et al. 2004), much less in known about the impact of environmental variation (including infection by the biopesticide) on resistance in natural populations. Without this knowledge, it is difficult to predict how best to manage the environment to restore sensitivity to resistant mosquitoes. Nevertheless, the synergistic effect of a biopesticide and the chemical insecticide can enhance the efficiency and increase the effective life-span of the insecticide in malaria control programs.

Chapter 5

Modeling the impact of insecticide resistance and declining resistance with mosquito age on malaria transmission

5.1 Introduction

Chemical insecticides play a significant role in the fight against malaria and its mosquito vector. The current malaria eradication campaign, led by The Roll Back Malaria Partnership (RBM, 2008), has resulted in a dramatic increase in the use of insecticides for vector control (WHO, 2012a). Between 2005 and 2009 the number people protected by indoor residual spraying (IRS) of insecticides increased from 13 million to 75 million. In a period between 2008 and the end of 2010, 289 million insecticide-treated bed-nets (ITNs) were supplied to infectious areas (enough to cover 76% of the people at risk). Subsequently, and in combination with the use of anti-malarial drugs, a steady decline in malaria since 2004 has been achieved (Murray et al., 2012; WHO, 2012a). However, insecticides exert a strong selection pressure on the mosquito and resistance can evolve quickly. Insecticide-resistance is now widespread across malarious regions and has been reported against all classes of insecticides used for vector control, thus threatening to undermine control efforts (Corbel et al., 2011; Nauen, 2007). Yet despite the clear threat insecticide-resistance poses there is little evidence from the field that malaria control programs are failing due to insecticide resistance.

Greater mosquito survival and higher biting rates on humans are observed in areas where there is resistance in the local mosquito population (Asidi et al. 2012; N'Guessan et al. 2007a). However, indoor residual spraying (IRS) and insecticide treated bed-nets (ITNs) can decrease the prevalence of malaria, despite increasing

insecticide resistance (Protopopoff et al, 2008), even in areas with extremely high (80%) resistance (Henry et al., 2005). These results suggest that failure to control mosquito numbers does not necessarily mean failure to prevent malaria transmission. Here, mathematical models are use to determine if this phenomenon can be explained the phenotypic expression of resistance and how it may change with mosquito age.

Examples of insecticide resistance declining with mosquito age have been observed in multiple mosquito species, a variety of resistance mechanisms, in laboratory experiments but also to field interventions (Table 5.1). Malaria parasites go through a developmental period within the mosquito of at least ten days. Thus, declining resistance can lead to genetically resistant mosquitoes becoming almost fully sensitive to insecticide by the time they can transmit malaria. It is therefore possible that mosquito numbers could rise with resistance, because young mosquitoes can survive insecticide exposure, but malaria control could be maintained, because older malaria-transmitting mosquitoes are killed. It has been suggested that the decline in resistance with age is due to a decline in expression of detoxification genes and enzymes associated with resistance (Rajatileka et al., 2011), however, this relationship is not always observed (Christian et al., 2012; Hunt et al., 2005). Furthermore, phenotypic resistance can decline even when the resistance mechanism involves a permanent change to the mosquito nervous system, in what is known as a target site mutation (table 5.1), indicating the decline in resistance with age is not solely down to changes in detoxification genes. A decline in mosquito condition and energy reserves is therefore another possible explanation for the decline in resistance with mosqutio age. Hunt et al (2005) indicated that blood feeding may negate the decline in resistance with age, but this has not been repeated in other studies (Cristiensen, 2011).

Although resistance allows mosquitoes to survive an exposure to insecticide, little is know about how this translates to field interventions. Environmental factors, such as temperature (Hodjati & Curtis, 1999; Polson et al., 2012) and food (Bourguet et al., 2004; Oliver & Brooke, 2013), can also influence the expression of resistance in mosquitoes. It has even been suggested that the environment could be manipulated, through the use of biopesticides *Vavraia culicis* (Koella et al., 2012) *Metarhizium anisopliae* and *Beauveria bassiana* (Farenhorst et al., 2009), in order to restore

sensitivity to genetically resistant mosquitoes and combat resistance. Thus, environmental stresses may result in reduced phenotypic expression of resistance in the field.

Here the impact of phenotypic resistance on malaria transmission is assessed using mathematical modeling. Although there is substantial evidence that resistance decreases with age, the shape and speed of the decline varies significantly (Table 5.1). A flexible model is therefore developed to incorporate different decline scenarios. A model, adapted from Le Menach et al (2007), is used to calculate the mortality caused by insecticides at each gonotrophic cycle after adult mosquito emergence. This value is then introduced into a second model (Koella et al., 2009b) to calculate two entomological indicators of malaria transmission: (i) the probability that an emerging mosquito survives to become infectious (ii) the number of infectious bites expected from an emerged adult mosquito.

Study	Mosquito species (strain) and resistance mechanism	Insecticide	Age groups tested - % mortality	Estimated increase in mortality per gonotrophic cycle
Chouaibou et al 2012	Field collected *Anopheles gambiae* Unknown mechanism	Deltamethrin, permethrin, DDT, propoxur	Age groups - 1,2,3,5 and 10 days after emergence Mortality - 60%,19%, 17%, 72%, 90% (Deltamethrin) 3%,4%,3%, 40%, 81% (Permethrin) DDT-Low mortality for all age groups (<5%) 3%,3%,3%, 30%, 31% (Propoxur)	 40.6% 44.6% 0% 16.0%
Christian et al 2011	*Anopheles funestus* (FUMOZ-R) monooxygenase (P450) detoxification mechanism	Permethrin	Age groups - 3,5,10,14,20 and 30 Mortality - 21.9%, 28.3%,32.0%,62.3%, 48%, 62.5%	Day 14 – 14.7% Day 30 – 6.0%
Hodjati and Curtis 1999	*Anopheles stephensi* (DUB234) - mechanism not stated *An. gambiae* (RSP) - elevated esterase and oxidase levels	Permethrin treated net and treated paper	Age groups – newly emerged and 10 days after emergence *An. stephensi* mortality - 0% and 34.5% (netting) - 0% and 9.0% (paper) *An. gambiae* mortality - 86.4% and 98.7% (netting)	 13.8 % 3.6% 4.92%
Hunt et al., 2005	Wild caught *An.funestus* (FUMOZ) selected for pyrethroid resistance in lab Evidence of increases in GST levels and monooxygenase expression	Lambda-cyhalothrin	Ages groups - 1,2,3,4,10,14 and 20 days after emergence Mortality - 85%, 35%, 35%, 60%, 85%, 80%, 55%	Day 10 - 25.0% Day 14 - 15.0% Day 20 - 4.4%
Jones et al 2012	F1 generation from field caught *An.gambiae* S-form and M-form and *Anopheles arabiensis* 1014 F Kdr resistance	Deltamethrin, LLINs and bendiocarb	Age groups - 3-5 and 17-19 Mortality (2 test) - 28.2 - 56.1% and 97.1 - 98.1% mortality. Results also indicate resistance to LLINS and bendiocarb decrease with age however the details are not included as they split up in many different mosquito families.	 23.0% 14.0%

Kulma et al 2013 (chapter 3)	*An.gambiae* (ZAN/U) glutathione S-transferees enzyme family	DDT	Age groups - 5,10,15 Mortality - 7%,77%,90%	25.2%
Lines and Nassor, 1991	Wild caught *An.gambiae* from Zanzibar	DDT	Age groups - newly emerged and 12-14 days after emergence Mortality - 5% and 90%	26.1%
Rajatileka et al 2011	3 *An.gambiae* strains ZAN/U, RSP and Arkon. 3 *Aedes aegypti* strains Solidaridad, HCM2 and Merida All but ZAN/U have Kdr target site mutations Arkon also contains ace1-R target site mutation. ZAN/U and RSP express metabolic resistance with elevated GSTe2 with RSP also expressing CY6Z1	DDT Bendiocarb Deltamethrin	Age groups - 3 and 14 days after emergence ZAN/U - 55% and 80% (DDT) - 72% and 95% (Bendiocarb) RSP - 5% increase AKRON - 27% and 48% (DDT) - 8% and 37% (Bendiocarb) HCM2 - 3% to 39% (DDT) - 60% to 90% (Deltamethrin) Solidaridad - 65% to 75% (Deltamethrin) Merida - 5% to 20% (Deltamethrin)	 9.1% 7.6% - 13.1% 8.4% 10.5% 10.9% 3.6% 5.5%

Table 5.1: Studies indicating a decline in the phenotypic expression of insecticide resistance with mosquito age. The estimated reduction in resistance per gonotrophic cycle was calculated, first by calculating the decline per day and then multiplying that number by 4 (the length of the gonotrophic cycle in the model). Several studies showed an increase in resistance in the first couple of days of the mosquitoes life, so the highest resistance value was used. Studies indicating a levelling out of resistance were given multiple estimates depending on the day. As the estimates are based on a linear decline they have to be taken cautiously. In addition to the studies in the table, which focus on mortality, studies investigating the time to knockdown (another resistance test) provide further evidence of an age decline in resistance. (Rowland & Hemingway 1987; Rajatileka et al. 2011; Hodjati & Curtis 1999)

5.2 Methods

The effect of insecticide resistance on malaria transmission was modeled by assuming that insecticide resistance decreases the likelihood of an insecticide killing a mosquito, thus, increases the success of a mosquito feeding on an individual protected by insecticide. It is also assumed that the background mosquito survival is constant through time. All modeling and graphics were performed in R version 3.0.0 (R Core Team, 2013)

During each feeding cycle, or gonotrophic cycle, the probability of a mosquito being killed by an insecticide is termed 'effective coverage' c (Koella et al., 2012). The effective coverage is adapted from a feeding cycle model in (Le Menach et al., 2007) where, at each biting attempt, mosquitoes bite outdoors with the probability H, which is set at 0.1; if not, they encounter an insecticide-treated house with probability ϕ, the population coverage of insecticide. Mosquitoes are then either repelled and repeat the host searching cycle at a probability r, set to 0.6 (Le Menach et al., 2007), or they are not repelled and successfully feed with probability (1 - r)s or are killed by the insecticide with a probability (1 - r)(1 - s). Feeding success on a protected individual is therefore inversely proportional to the mortality caused by the insecticide, therefore, s, can be used synonymously with insecticide-resistance. A mosquito successfully feeds by biting outdoors, biting an unprotected human or successfully feeding on a protected human, with the probability:

$$H + (1-H)(1-\phi) + (1-H)\phi(1-r)s_i$$
$$= 1 - (1-H)\phi(1-(1-r)s_i) \qquad \text{(Equation 1)}$$

As resistance can change with age, and thus successful feeding on a protected individual, total feeding success is calculated for each gonotrophic cycle after emergence, i. Repelled mosquitoes repeat feeding attempts at the probability of $(1-H)\phi r$ and thus the number of attempts needed to complete the feeding cycle can be calculated as:

$$\frac{1}{1-(1-H)\phi r} \qquad \text{(Equation 2)}$$

The expected survival through the feeding stage is calculated by multiplying the probability of successful feeding at one attempt (equation 1) by the number of attempts needed (equation 2), thus by taking the reciprocal, effective coverage can be calculated as (Koella et al., 2012):

$$c_i = 1 - \frac{1-(1-H)\phi(1-(1-r)s_i)}{1-(1-H)\phi r} \qquad \text{(Equation 3)}$$

The effective coverage at gonotrophic cycle i is therefore determined by the success of feeding of mosquitoes in that gonotrophic cycle. Calculating the effective coverage at each gonotrophic cycle after adult mosquito emergence produces a very flexible model in which declines in resistance over a mosquitoes lifespan can be investigated. Note that according to the model in (Le Menach et al., 2007) repellency increases the duration of the gonotrophic cycle, and thus background mortality, due to an increase in host searching. However unless indoor biting, insecticide coverage and repellency are all close to 1, the gonotrophic cycle is close to that observed in the absence of insecticide so a time delay is not included in this model.

The effective coverage is then used to calculate the effectiveness of control with insecticides by adapting a second model from (Koella et al., 2009b). In this model the probability of surviving a complete gonotrophic cycle is (1- ϕ)(1- δ), where proportional to mortality caused by insecticides is equivalent to the insecticide coverage, ϕ; and the survival of unexposed mosquitoes for one gonotrophic cycle (1- δ) is equal to $(1 - \mu)^{\tau}$ where, μ, the daily background mortality, is set to 0.1 (Costantini et al. 1996; Charlwood et al. 1997; Takken et al. 1998; Killeen et al. 2000); Midega et al. 2007; Okech et al 2007) and τ, the length of the gonotrophic cycle in days, is set to 3, and is not influenced by insecticide use. The model was used to calculate the effectiveness of insecticides in sensitive populations; so to investigate the effectiveness of insecticides in resistant populations, insecticide coverage is substituted for effective coverage giving; $(1 - c_i)$ $(1 - \delta)$.

The probability of surviving one gonotrophic cycle can then be used to calculate the probability a mosquito survives to be infected by malaria at bite z:

$$(1-\delta)^z(1-\pi)^{z-1}\pi\prod_{i=1}^{z}(1-c_i) \qquad \text{(Equation 4)}$$

where π is the probability per bite that a mosquito is infected, and is set to 0.5 to represent a high transmission area. If effective coverage changes between gonotrophic cycles then the probability of surviving the parasite developmental period depends on when the mosquito was infected:

$$(1-\delta)^N\prod_{k=z+1}^{Z+N}(1-c_k) \qquad \text{(Equation 5)}$$

where N is the duration of the parasite's development in gonotrophic cycles, set at 4 and equivalent to a 12 day development period. The probability a mosquito is infected at bite z and then survives to become infectious is thus:

$$\sigma_Z = (1-\delta)^{z+N}(1-\pi)^z\pi\prod_{i=1}^{z+N}(1-c_i) \qquad \text{(Equation 6)}$$

and the probability an emerging mosquito survives to become infectious (being infected at any bite) is the sum of the survival terms.

$$\sum\sigma_Z \qquad \text{(Equation 7)}$$

Assuming a mosquito bites one individual per gonotrophic cycle, the total number of infectious bites it will give will be the probability of surviving further gonotrophic cycles once infectious, and this depends on, z, when they were infected:

$$b_z = \sum_{k=0}^{\infty} (1-\delta)^k \prod_{i=z+N}^{z+N+k} (1-c_i) \qquad \text{(Equation 8)}$$

The expected number of infectious bites from an emerged mosquito can therefore be calculated by multiplying the probability of survival to infectivity, if infected at bite z, (equation 6) by the number of expected bites once infective (equation 8), and taking the sum of these terms:

$$\sum \sigma_Z * b_Z \qquad \text{(Equation 9)}$$

5.3 Results

5.3.1 Resistance without a decline in age

A mosquito that finds a protected host and is not repelled either has a successful feed, or is killed. Thus, success of feeding on a protected individual, s, is inversely proportional to mortality and used as a proxy for the phenotypic expression of insecticide resistance. Figure 5.1 describes the impact of different levels of resistance on malaria transmission parameters when there is no change in resistance with mosquito age. The curves illustrate different population coverages of insecticide and indicate that unless insecticide-resistance results in 100% successful feeding, and thus 0% mortality, insecticides can still reduce the entomological parameters of malaria transmission.

The relationship between resistance and the malaria transmission parameters become increasingly non-linear with higher insecticide coverage. As a result, relatively small changes in resistance at the upper end of the scale can produce significant changes in the transmission parameters. For example, a 20% reduction in resistance, from 100% - 80%, results in the expected number of infectious bites from an emerged mosquito

dropping by 51.5% with 75% insecticide coverage, and 73% with 100% coverage, in comparison to non-intervention levels

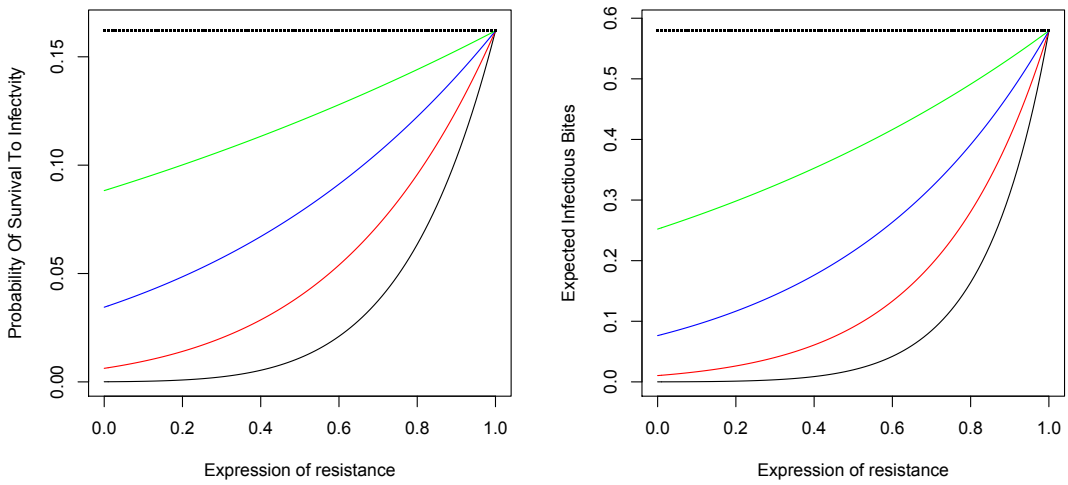


Figure 5.1; Probability of survival to infectivity and number of expected infectious bites from an emerged mosquito as a function of the expression of resistance. Expression of resistance is synonymous with the feeding success on a protected individual (s). The horizontal dashed line represents a situation where there are no insecticides, $\phi = 0\%$. Insecticide coverage increases from 25% (green), 50% (blue), 75% (red) and the maximum possible 100% (black).

The non-linear shape is a result of the probability that a mosquito will contact an insecticide more than once. For example, if resistance is expressed at a probability of 0.7, and a mosquito may contact insecticide four times before it can transmit malaria (the number of gonotrophic cycles for the mosquito to become infectious), the probability of surviving cumulative insecticide exposures before transmission is $0.7^{\wedge 4}$ or 0.24. Higher insecticide coverage increases the probability a mosquito contacts insecticide thus the curves are more pronounced. Repellency and outdoor biting reduce insecticide contact and so produce flatter curves when their values are increased. The two indicators of transmission, probability of survival to infectivity and expected infectious bites, are closely linked and produce similar curves, the remainder of the results focus on expected infectious bites from an emerged mosquito.

5.3.2 Resistance declining with age

Figure 5.2 describes a situation where resistance decreases with mosquito age and the impact it has on malaria transmission parameters. The resistance level at the first gonotrophic cycle is 1.0 and drops down to a lower limit of 0.1, which is the expected success rate of sensitive mosquitoes (Le Menach et al., 2007). An exponential decline in age (figure 5.2b) produces more conservative estimates than a linear decline (figure 5.2a), however, both indicate large reductions in the transmission parameters can be made when there is a decline in resistance with age. Estimates from table 5.1 indicate resistance can decline between each gonotrophic cycle at low levels of 2-4% right up to 40%. As with the previous example, small changes in resistance can have a dramatic effect on the expected number of infectious bites. Even if a mosquito is 100% resistant at its first bite, if resistance reduces by 10% per gonotrophic cycle, the expected number of infectious bites is reduced by around two thirds, with 75% insecticide coverage. As a guide the horizontal lines represent the expected bites from a sensitive mosquito at the corresponding insecticide coverage. They suggest that, if there is a decline in resistance with age, larger reductions in transmission can be made in resistant mosquitoes with high insecticide coverage than sensitive mosquitoes with lower insecticide coverage. For example, a decline in resistance of 10% with 100% coverage results in lower probability of malaria transmission than coverage of 50% with sensitive mosquitoes.

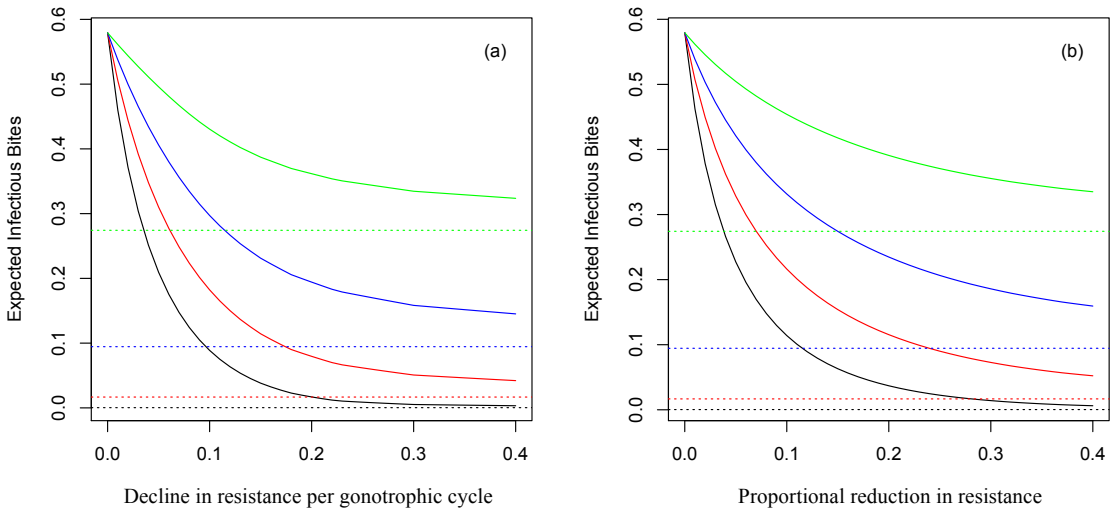


Figure 5.2 Expected infectious bites from an emerged mosquito with a linear decline (a) and exponential decline (b) in resistance. Insecticide coverage, ϕ, of 100% (black), 75% (red), 50% (blue) and 25% (green). Horizontal lines indicate expected infectious bites for a sensitive mosquito at the coverage of the same colour.

The graphs shown in figure 5.2 describe a situation where resistance at first gonotrophic cycle is 100%, however from the evidence in table 5.1, where there is only one example of 0% mortality in the younger ages, it might be unrealistic. On the other hand, few studies show that resistant mosquitoes become completely sensitive at older ages. Using WHO test procedures (WHO, 2008), mortality of over 98% with a discriminating dose of insecticide is considered insecticide-sensitive. Note, the studies in table 5.1 use several different resistance tests so the mortality levels are only used as a guide. Different "starting" resistance levels are investigated in figure 5.3, and figure 5.4 investigates when there is a limit to how far resistance can drop.

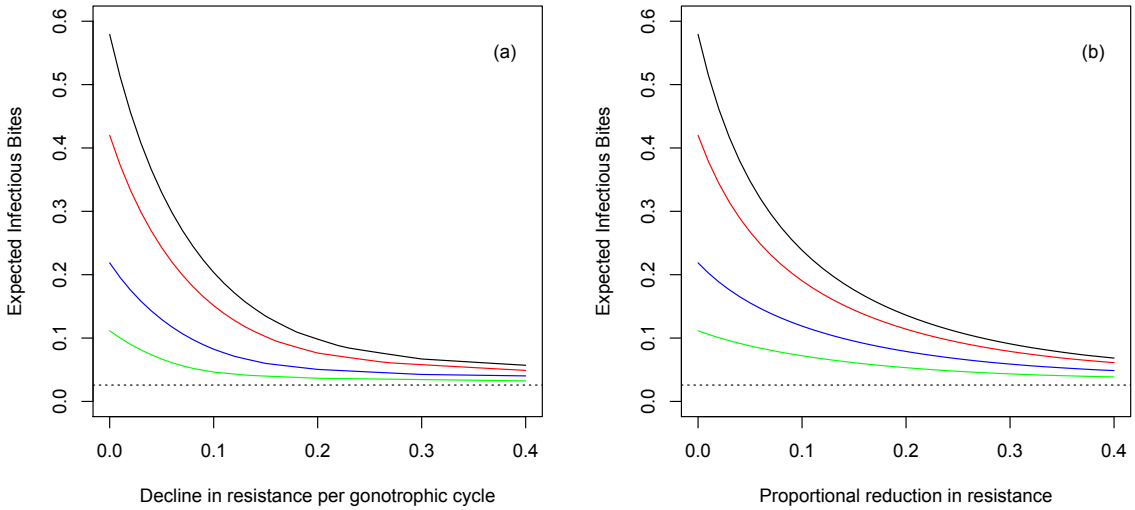


Figure 5.3: The number of infectious bites expected from an emerged mosquito as a function of declining resistance; linear decline (a) and exponential (b). Curves represent starting resistance values of 100% (black), 90% (red), 70% (blue) and 50% (green) and a lower limit of 0.1. The horizontal reference line represents the expected value for a sensitive mosquito. The value at 100% success with no decline is equivalent to expected values when there are no insecticides. Insecticide coverage is set to 0.7.

It is evident from figure 5.3 that the initial resistance level, at the mosquitoes' first bite, has a large impact on the expected number of infected bites, especially when decline is low. An initial resistance of 0.7, combined with realistic drop in success of 0.1-0.2 (table 5.1), can result in transmission predictions nearing that observed in sensitive mosquitoes. In contrast, a lower limit on how far success can drop (figure 5.4) has little effect when the decline is low but becomes more significant the faster the decline, with higher limits preventing estimates reaching sensitive levels. Even so, with a steep decline in resistance, transmission parameters can be reduced by over 50% despite a high starting resistance value of 1.0 and a lower limit of 0.5. Again the exponential decline results in more conservative predictions.

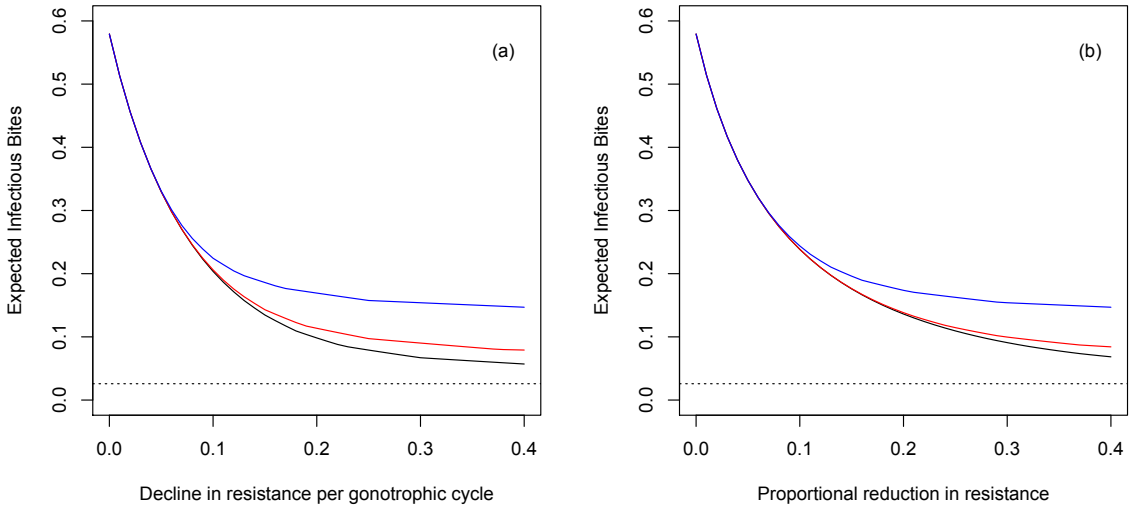


Figure 5.4: The number of infectious bites expected from an emerged mosquito as a function of declining resistance; linear decline (a) and exponential (b). The probability of success for a mosquito at their first gonotrophic cycle is 1.0. Curves represent lower limits of success of 0.5 (blue), 0.25 (red) and 0.1 (black). The horizontal reference line represents the expected value for a sensitive mosquito. The value at 100% success with no decline is equivalent to expected values when there are no insecticides. Repellency and insecticide coverage are set to 0.7.

5.4 Discussion

The models describe several realistic scenarios where insecticides can cause large reductions in transmission parameters, despite the presence of genetically resistant mosquitoes, thus confirming observations in the field that malaria transmission can be reduced in areas with resistance (Henry et al., 2005; Protopopoff et al, 2008). Key parameters to the threat of resistance are (i) how genetic-resistance relates to phenotypic resistance (ii) how resistance changes with mosquito age.

The mortality caused by insecticides on genetically resistant mosquitoes in the field is a key parameter when considering the threat of insecticide resistance. Resistance tests, such as the ones designed by the WHO and CDC (Aïzoun et al. 2013; CDC, 2013; WHO, 2008), are optimized to detect the presence of resistance mechanisms in a population but tell us little about how successful genetically resistant mosquitoes are

in the field. A study by Chandre et al (2000) investigated the feeding and mortality rates of resistant and sensitive strains of *Anopheles gambiae* with ITNs, containing holes, baited with guinea pigs. They found that the ITNs caused 60-80% mortality in the resistant strains despite the strain showing higher resistance in WHO resistance test with only 0-5%, thus highlighting the contrast between the standard resistance tests (primarily identifying genetic resistance) and the true effectiveness of field interventions on resistance mosquitoes. Overall the final blood feeding success of resistant mosquitos was 9-17% and for sensitive mosquitoes was 0% (they were all killed by the insecticide). Thus genetic resistance conferred a clear fitness advantage yet a significant proportion of the resistant mosquitoes were still killed by the insecticide. Applying the results of the Chandre et al (2000) study to Figure 5.1, 60-80% mortality (equivalent to 0.2-0.4 resistance) would produce low malaria transmission estimates. In contrast, transmission estimates would be much higher if based on mosquito genotype, or results form the WHO test.

Further evidence that insecticides can kill resistant mosquitos comes from field trial with ITNs, which have shown to be effective in resistant populations. However, the efficacy of the nets is greatly increased when used with a chemical synergist piperonyl butoxide, (N'Guessan et al., 2010; Koudou et al., 2011; Okia et al., 2013; Van Bortel et al., 2009). Determining the factors that affect the sensitivity of resistant mosquitoes may play an important role in future vector control strategies. As discussed, environmental factors can influence the expression of resistance. Does this mean the degree in which resistance threatens control varies throughout the year or from one ecological setting to the next? The models certainly suggest that biopesticides aimed at re-sensitizing resistant mosquitoes to insecticide could be very effective particularly if they can decrease resistance to less than 40%. In chapter 2, the impact of malaria itself on the expression of resistance is investigated. Although causing a relatively modest reduction in resistance, the model suggests small differences could have large impacts, particularly at high levels of resistance (figure 5.1). The phenotypic expression of resistance may also be affected by previous insecticide exposures, however the effect of exposure history on resistance has had mixed results. Curtis and Hodjati (1999) demonstrated, in two stains of *An.stephensi*, that a previous exposure to insecticide increased mortality when exposed again,

however, Glunt *et al* (2011) found no evidence of this when they conducted a similar experiment.

The models also indicate that changes in resistance with mosquito age can have a significant impact on the probability that a mosquito will survive to become infectious and thus the efficacy of insecticides. Although resistance is likely to increase the number of young mosquitoes, which may be reflected in increases biting rates in the field (Asidi et al., 2012; Rowland et al., 2012), unless the number of old malaria-transmitting mosquitoes also increases there, will be no change in malaria transmission. Table 5.1 shows the reductions in resistance with age vary significantly and are likely to be specific to the resistance mechanism and mosquito species. The ability of insecticides to control resistant mosquitoes is therefore likely to vary depending on these factors. However, small declines in resistance with age can cause dramatic declines in malaria transmission estimates, even if mosquitoes are fully resistant at the first bite or the mosquito population never reaches full sensitivity. Furthermore, environmental stressors such as parasitism may accumulate with mosquito age, enhancing the decline in resistance.

In summary, the models presented act as a guide to assess the threat of resistance. Any reductions in the phenotypic expression in resistance, whether it is due to the environment or changes with age, will reduce the probability a resistant mosquito survives to become infectious. Although the model suggests that the entomological parameters of malaria transmission can be reduced in a resistant population, estimates are still higher when compared to a sensitive population, so resistance is still undesirable. Much relies on the mortality caused by insecticides on genetically resistant mosquitoes in the field, However little is really know about this area. While WHO resistance tests can identify the presence of resistance in a population, studies identifying the mortality rate of resistant mosquitoes in the field are needed to assess the real threat resistance poses.

Chapter 6

Longevity of insecticide resistant and sensitive strains of *Anopheles gambiae* infected with microsporidian and malaria parasites

6.1 Background

Insecticides play a vital role in the fight against vector-borne diseases such as malaria. However, insecticide resistance has been reported against all classes of insecticides used for vector control, threatening to undermine control efforts (Corbel et al., 2011; Nauen, 2007). Resistance can lead to greater mosquito survival and thus more frequent biting on humans (N'Guessan et al., 2007a; Asidi et al., 2012). Yet indoor residual spraying (IRS) and insecticide treated bed nets (ITNs) can decrease the prevalence of malaria despite increasing insecticide resistance (Protopopoff et al, 2008), even in areas with extremely high (80%) resistance (Henry et al., 2005). Insecticides may also reduce the sporozoite rate (the percentage of mosquitoes with sporozoites in their salivary glands) without the reducing numbers of mosquitoes (Sharp et al., 2007).

Part of the reason underlying these seemingly contradictory results, that resistance increases mosquito numbers but has little effect on transmission, may be that the epidemiology of malaria is relatively insensitive to the number of mosquitoes, but rather depends strongly on their longevity (Macdonald, 1957). To reduce transmission, insecticides must not necessarily reduce the number of mosquitoes in a population, but the number of mosquitoes that live long enough to transmit malaria (Read et al. 2009; Koella et al. 2009). Therefore, to understand the impact of insecticide-resistance on malaria, we must focus on its effect on mosquito longevity.

One set of mechanisms that could maintain the efficacy of insecticides in old mosquitoes, despite widespread resistance, relies on demographic and environmental effects on the expression of resistance. Resistance becomes less effective as mosquitoes age (Chouaibou et al., 2012; Hunt et al., 2005; Jones et al., 2012; Kulma et al., 2013; Lines & Nassor, 1991; Rajatileka et al., 2011; Rowland & Hemingway, 1987), presumably in part because metabolic detoxification activity declines with age (Rowland & Hemingway, 1987; Rajatileka et al., 2011). Additional environment stresses such as parasitism (Farenhorst et al., 2010; Koella et al., 2012) and food availability can increase mosquito sensitivity to insecticides (Oliver & Brooke, 2013; Kulma et al., 2013). Moreover, environmental stresses, such as parasitism, can accumulate with age, further increasing the sensitivity of older mosquitoes to insecticide. As it is the older mosquitoes that transmit malaria, a reduction in resistance with age may maintain the efficacy of insecticides for malaria control (Chapter 5).

An additional set of mechanisms could be that insecticide-resistance itself, in the absence of insecticide pressure, decreases the longevity of mosquitoes and thus constrains the parasite's transmission. Such a cost of resistance is likely, for insecticide resistance affects the life-history of many species (Carriere et al., 1994; Janmaat & Myers, 2005; Kliot & Ghanim, 2012; Foster et al., 2003), including mosquitoes (Agnew et al., 2004; Gazave et al., 2001; Martins et al., 2012). It also affects, for example, energetic stores (Rivero et al., 2011) and the probability of parasitic infection (Alout et al., 2013; Berticat et al., 2002; Duron et al., 2006; Howard et al., 2010a) of mosquitoes, which in turn should affect longevity. In other words, the cost of resistance itself could prevent resistant mosquitoes from transmitting the parasite, complementing the insecticides' effect of killing older mosquitoes.

The cost, as well as the efficacy, of resistance can be affected by environmental conditions. Thus, the cost of resistance of *C. pipiens* to organophosphates is increased if the mosquitoes are infected by the microsporidian parasite *Vavraia culicis* (Agnew et al., 2004) or reared at high larval densities (Bourguet et al. 2004). The fitness cost of permethrin resistance of *C. pipiens* is enhanced if the mosquito is exposed to temephos, another insecticide (Hardstone et al., 2009). In the diamondback moth, the

cost of resistance to spinosad is low at the optimal temperature and increases at unfavourably low and high temperatures (Li et al., 2007), and the cost of resistance to *Bacillus thuringiensis* increases in harsh and competitive environments (Raymond et al., 2005). Finally, and most relevant to this study, the cost of resistance of *Anopheles gambaie* against DDT and permethrin is increased by the infection with a microsporidian parasite (Koella et al., 2012).

When dealing with insecticide-resistant vectors, an important aspect of the environment is, naturally, the vectored parasite. As with the infection by microsporidians, infection by malaria parasites may interact with insecticide resistance to shorten mosquito life-span, and thereby reduce its own transmission. This may be expected for two reasons: first, infection by malaria parasites increases mosquito mortality in unnatural parasite-mosquito interactions (Ferguson & Read, 2002), and insecticide-resistance has been suggested to provide an unnatural environment for malaria parasites (Rivero et al., 2010). Second, resistant mosquitoes store fewer lipids, sugars and energetic reserves than sensitive ones (Rivero et al., 2011), and the reduced energy reserves could influence the success of an energetically costly immune response (Schmid-Hempel, 2005) and thus the virulence of the parasite. Thus, parasitic infection has a greater effect on mortality in nutritionally compromised mosquitoes (Takken et al., 2013)

This study investigates how two parasites, the microsporidian *V. culicis* and the malaria parasite *Plasmodium berghei*, affect the survival of insecticide sensitive and insecticide resistant mosquitoes. Two questions are of particular interest. First, do insecticide-resistant mosquitoes infected by malaria parasites live less long than sensitive mosquitoes? If so, the evolution of resistance would have less severe effects on the epidemiology of malaria than feared. Second, does the additional stress of a second parasite enhance this effect? If so, this would give support for the idea that environmental management with parasites would help to manage the problem of insecticide-resistance for malaria control (Koella et al., 2012).

6.2 Methods

6.2.1 Study organisms

Three colonies of *Anopheles gambiae* were used: a DDT-resistant colony (ZANU) from Zanzibar with increased metabolism of the insecticide, catalysed by members of the glutathione S-transferase enzyme family (Ranson et al., 2000b); a mildly pyrethroid-resistant colony (RSP) from western Kenya with elevated esterase and oxidase levels (Vulule et al., 1999; David et al., 2005) and possibly with knockdown resistance (kdr) (Ranson et al., 2000b; Christian et al., 2011), and a sensitive colony (Kisumu), which was also colonized from western Kenya and is sensitive to all insecticides (Vulule et al., 1994).

The microsporidian *V. culicis* is an obligate, intracellular parasite of several mosquito species (Andreadis, 2007; Becnel et al., 2005), with a life cycle typical of microsporidians (Andreadis, 2007). Mosquito larvae are infected when they ingest the parasite spores along with their food. Some infected larvae and pupae die and release a new generation of the parasite's spores for horizontal transmission to other larvae. If the mosquitoes survive to emerge, the adults remain infected. The parasite has several effects on the adult, including a shorter life span (Koella et al., 2009a; Lorenz & Koella, 2011) and reduced susceptibility to malaria infection (Bargielowski & Koella, 2009). Although there is no transovarial vertical transmission, spores harboured by adult females can infest a new breeding site when they are released together with eggs (Andreadis, 2007). The prevalence of only a few microsporidian species in natural populations has been estimated; in populations of *Aedes* mosquitoes, it ranges from 0% to about 50%, while the only study on *An. gambiae* found 6.6% prevalence in larvae (Andreadis, 2007).

The malaria parasite, *P. berghei*, is often used as a model for the experimental study of mammalian malaria, because of its similarities in morphology, physiology and genetic make-up (genome structure and gene content) with human *Plasmodium* parasites (Sinden, 1978; Aikawa and Seed, 1980; Janse et al., 1994; van Lin et al.,

2000; Rich and Ayala, 2003). It is also simple and safe to manipulate and keep in the laboratory.

6.2.2 General design

Sensitive and resistant mosquitoes were reared individually. Half of each line were exposed to microsporidia as larvae at a dose that leads to very little larval mortality. After emergence the treatment groups were split again so half of the adult females were subsequently fed on malaria-infected blood (at a concentration that gives high infection rates), the other half on malaria-free blood. Thus, each line of mosquitoes contained four treatments; uninfected, infected with microsporidia, infected with malaria or infected with both parasites. The longevity of the blood-fed females was then measured.

This general design was run twice, once with the pyrethroid-resistant RSP and the sensitive Kisumu colonies, and once with eight lines we had artificially selected to be DDT-resistant or sensitive. The experiment with eight lines was run in two blocks, each with two lines selected for resistance and two lines selected for sensitivity.

6.2.3 Selection procedure

Four lines for DDT-resistance and for DDT-sensitivity were selected over five generations (at which point differences in resistance were clearly established). As the baseline colony for selection, the F2 generation was used after crossing females of the Kisumu colony with males of the ZANU colony and vice-versa. For the first generation of selection, blood-fed females were placed into individual cups. Once they had laid eggs, they were exposed to 4% DDT for 60mins with the standard World Health Organization test-kit (WHO, 1998). The eggs of the females that did not survive the exposure were used to establish the sensitive lines; the eggs of the females that did survive the exposure were used to establish the resistant lines. For each line, 300 newly emerged larvae were chosen haphazardly. Further selection for sensitivity was carried out in the same way (i.e. on individual mosquitoes). To select

for resistance, unfed mosquitoes were exposed to DDT in groups of 20 to 30, blood-fed the survivors and used 300 haphazardly chosen offspring for the next generation.

After five generations of selection the proportion of mosquitoes that survived a WHO assay were 55%, 59%, 64% and 77% for the four lines selected for resistance, and 15%, 16%, 19% and 50% in the four lines selected for sensitivity (mixed effect binomial GLM: resistance $z = -4.07$, $p < 0.001$; line within resistance: $z = -3.04$, $p=0.002$).

6.2.4 Mosquito rearing and microsporidia infection

Mosquitoes were maintained at 26 (+/-2) °C and 70 (+/-10) % relative humidity with a 12 h:12 h light/dark cycle. In each experiment 1008 larvae from each line (Kisumu, ZANU or the eight selected lines) were reared individually in 12-well plates and fed with Tetramin fish food (0.04 mg on day 2, 0.08 mg (day 3), 0.16 mg (day 4), 0.32 mg (day 5), 0.6 mg on day 6 and following days until pupation).

Half of the mosquitoes were exposed to about 20000 spores of the microsporidian *V. culicis* when they were two days old. The spores were obtained from adult mosquitoes that were previously infected with microsporidia. Dead adult mosquitoes were homogenized in 1ml of de-ionised water and the microsporidian spores were counted at 400x magnification with a haemacytometer. The solution was diluted to 20000 spores/100µl, and half of the mosquitoes obtained 100µl of this solution. The non-microsporidian larvae received 100µl of solution containing the same number of uninfected adults.

When larval development was completed, pupae were collected and transferred to holding cages. 24 hrs later after the adult mosquitoes emerged the females were collected and kept individually in 180ml cups. Females were given cotton wool soaked in 10% glucose solution to feed on, but this was removed 12 hrs before blood feeding to increase feeding success. Males were discarded.

6.2.5 Blood feeding and infection with *Plasmodium berghei*

Blood feeding was carried out in a climate-controlled room at 19 (+/-2) °C and 70 (+/-10) % humidity, and the mosquitoes subsequently kept in this room. The temperature was lowered from 26°C to 19°C to optimize *P.berghei* development. Temperatures of around 26°C cause deterioration in the parasite at each stage of the sporogonic cycle (Rastogi et al., 1987)

P.berghei ookinete culture was obtained from R.E. Sinden's lab at Imperial College London. Ookinete numbers were counted with a microscope at 400x magnification with a haemacytometer. The culture was centrifuged for 10 minutes at 19°C. Blood of uninfected mice was added to obtain a concentration of 600 ookinetes/µl. 400µl membrane feeders (kept at 37°C by a water bath) were filled with the ookinete mixture or with the blood of uninfected mice. Mosquitoes were moved to feeding cups (4 cups per treatment group), and allowed to feed on the malaria-infected or malaria-free membrane feeders for 1hr.

24 hrs after feeding, the mosquitoes that had taken a blood meal were transferred back into individual holding cups and supplied daily with fresh sugar water. Mortality was recorded every 12hrs (in the experiment with two-colonies) or every 24 hours (in the experiment with selected lines).

6.2.6 Statistical analysis

Analyses were carried out separately for the non-selected colonies and for the selected lines. Adult mosquito longevity, post blood-meal, was analysed with Cox Regression analysis. Mosquitoes that were still alive 42 days after blood-feeding were censored in the analysis. Infection status of malaria and microsporidia were also treated as binary factors in the analysis (exposed vs. unexposed), as were the resistance status for the unselected colonies (Kisumu and RSP) and the selection regime, (the direction in which the colonies were selected). A Mixed-Cox Regression was used to analyse the selected colonies with block as a random factor and selection line nested within selection regime.

The assumption of proportional hazards was violated for both analyses (Appendix B), so, following Sterne (2003) the time axis was split into two sections, within each of which the assumptions were met. As a splitting time 12 days after blood-feeding was chosen, based on inspection of the Schoenfeld residuals, which determine the proportionality of the model (Fox, 2002), and based on the biology of malaria parasites which start producing their transmissible stages at about that time.

Pupation rates and survival to feeding age were analysed with GLMs with binomial distribution. Again, for the selected lines block was included as a random factor and selection line was nested within selection regime. With the exception of a borderline significant result indicating line had an effect on survival of 0-12 day old mosquitoes (χ^2=6.00 , p= 0.050), our models found no significant effects of the random factors, so that we omit them from further discussions.

Analyses were performed with the 'lme4' (Bates et al., 2013), 'survival' (Therneau, 2013) and 'coxme' (Therneau, 2012) packages in R version 3.0.0 (R Core Team, 2013)

6.3 Results

6.3.1 Established colonies, Kisumu vs. RSP

Pupation

Microsporidian infection decreased the number of larvae reaching pupation from 913 pupae out of 1008 in the control group to 882 pupae out of 1008 larvae ($\chi^2 = 5.24$, df = 1, p = 0.02). There was no effect of resistance ($\chi^2 = 0.32$, df = 1, p = 0.57)

Adult survival pre-feeding

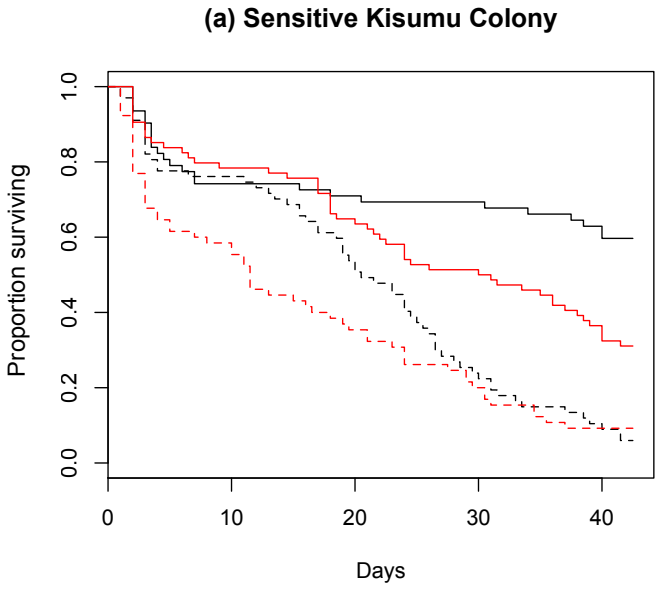
Resistant RSP mosquitoes were less likely (91.4%) to survive to feeding age (4-5 days after emergence) than sensitive Kisumu mosquitoes (97.4%) ($\chi^2 = 11.78$, df =1, p < 0.001). While microsporidian infection had no overall effect ($\chi^2 = 0.55$, df =1, p = 0.46), it decreased the longevity of RSP mosquitoes more than that of Kisumu mosquitoes (by 7.1%, and by 0.9%, respectively), leading to a close to significant interaction ($\chi^2 = 2.89$, df = 1, p = 0.089)

Survival post-feeding

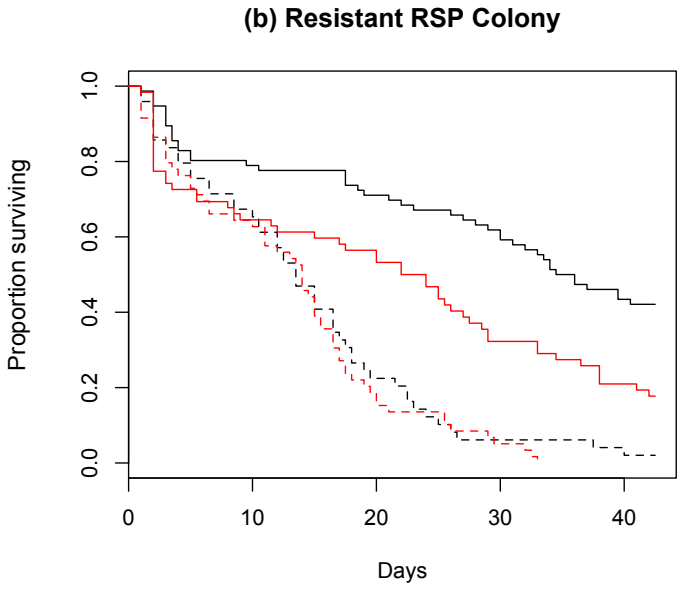
In the first time period (up to 12 days after blood-feeding) microsporidia, malaria and colony impacted survival through a 2-way interaction between microsporidia and malaria, and a 3-way interaction between microsporidia, malaria and resistance (table 6.1). Interactions in survival can be interpreted using the Wald statistic (z) and the p-value, which indicate when the effects of each parameter on survival are not additive. For example, a positive Wald statistic with a significant p-value indicates that the combination of parameters is increasing mortality more than expected. A two-way interaction is observed between malaria and microsporidia (Table 6.1), and a positive Wald statistic of 2.32, indicates that mortality in mosquitoes infected with both parasites was higher than expected if the effects of malaria and microsporidia were added together. A negative Wald statistic would indicate that mortality is lower than expected. To aid interpretation of the 3-way interaction data were analysed by colony.

Results indicate while a combination of microsporidia and malaria significantly increased the mortality of Kisumu mosquitoes ($z = 2.29$, $p = 0.022$), the interaction was the opposite but not significant in RSP ($z = -1.43$, $p = 0.151$). In contrast the main effects of microsporidia ($z = 0.20$, $p = 0.839$) and malaria ($z = -0.51$, $p = 0.608$) were not significant in Kisumu but were in RSP (microsporidia, $z = 2.26$, $p = 0.023$; malaria, ($z = 2.15$, $p = 0.031$). The statistics accurately reflect the observed survival; in the sensitive Kisumu colony only a double infection with both parasites reduced survival, with survival after the first period being 74.2%, 73.1%, 78.4% and 46.2% for control, microsporidia-infected, malaria-infected and doubly infected mosquitoes, respectively (Figure 6.1a). In contrast, the three types of parasitic infection reduced the survival of resistant RSP mosquitoes to a similar extent, with survivals of 77.6%, 57.1%, 61.3% and 56.0% for control, microsporidia-infected, malaria-infected and doubly infected mosquitoes, respectively (Figure 6.1b)

In the second time period (from 13 days after blood-feeding onward) microsporidian-infection increased the risk of death by 9.6 (Table 6.1). Malaria infection also increased the risk of death by 3.9 and RSP mosquitoes had a higher risk of death than Kisumu mosquitoes. Again there was a significant 2-way interaction between microsporidia and malaria, and a significant 3-way interaction between microsporidia, malaria and colony. To aid interpretation of the 3-way interaction data were analysed by colony. The results indicate that a double infection form microsporidia and malaria caused was significantly lower mortality than expected if the individual effects were added together ($z = -3.77$, $p < 0.001$), whereas the interaction was not significant for RSP ($z = -1.02$ $p = 0.307$). The main effects for malaria and microsporidia were significant in both Kisumu (microsporidia, $z = 6.40$, $p < 0.001$; malaria, $z = 3.82$, $p < 0.001$) and RSP (microsporidia, $z = 7.06$, $p < 0.001$; malaria, $z = 2.46$, $p = 0.014$). Mortality caused by malaria alone was higher in Kisumu than in RSP mosquitoes, but mortality in doubly infected mosquitoes was higher in RSP than in Kisumu mosquitoes.



Uninfected	62	48	45	44	40
Malaria	74	59	49	39	28
Microsporidia	67	52	41	18	8
Double	65	38	26	16	7



Uninfected	76	61	56	48	35
Malaria	62	41	36	22	16
Microsporidia	49	33	15	4	2
Double	59	38	14	5	0

Figure 6.1: Kaplan-meier survival curves of (a) sensitive Kisumu mosquitoes and (b) Pyrethroid resistant RSP mosquitoes; infected with malaria (solid red), microsporidia (black dashed), double infection (red dashed) or no infection (solid black). Tables represent the number of mosquitoes at risk (alive) at the time points indicated on the x-axis in each treatment group.

0-12day survival (n=505)	HR (95% CI)	z	P
Resistance	0.85 (0.43-1.69)	-0.46	0.647
Microsporidia	1.07 (0.54-2.09)	0.19	0.848
Malaria	0.83 (0.41-1.66)	-0.53	0.598
Resistance*Microsporidia	1.96 (0.77-4.95)	1.416	0.157
Resistance*Malaria	2.39 (0.94-6.06)	1.83	0.066
Microsporidia*Malaria	2.89 (1.18-7.07)	2.32	0.030
Resistance*Microsporidia*Malaria	0.18 (0.05-0.64)	-2.67	0.008
13-42 day survival (n=332)			
Resistance	2.53 (1.22-5.23)	2.51	0.012
Microsporidia	9.62 (4.82-19.19)	6.42	<0.001
Malaria	3.92 (1.93-7.92)	3.81	0.001
Resistance*microsporidia	0.78 (0.32-1.86)	-0.56	0.575
Resistance*Malaria	0.50 (0.21-1.21)	-1.53	0.126
Microsporidia*Malaria	0.19 (0.08-0.45)	-3.77	0.002
Resistance*Microsporidia*Malaria	3.55(1.14-11.07)	2.19	0.028

Table 6.1 – Cox regression survival analysis for the sensitive Kisumu and resistant RSP colonies infected with malaria and microsporidian parasites

6.3.2 Selected lines

Pupation and survival pre-feeding

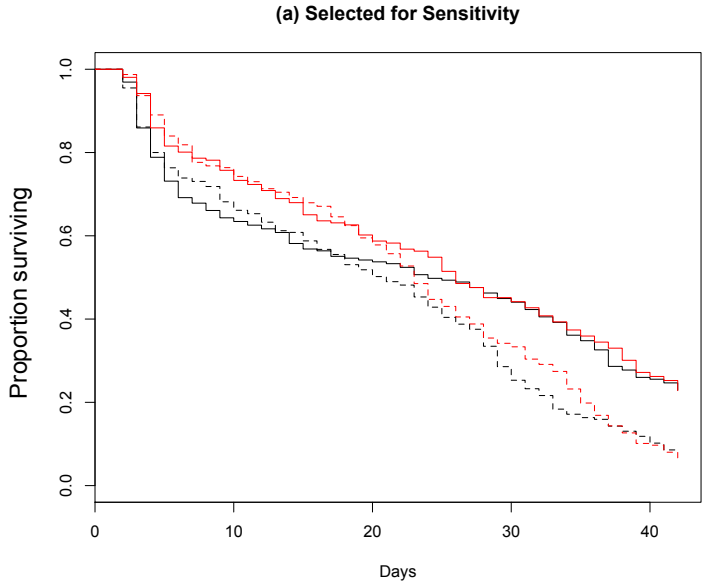
With an average survival of 91.6% in resistant lines and 91.4% survival in sensitive lines there was no effect of selection for resistance on survival to pupation (z=-0.07, p=0.93). There was no significant effect of microsporidian infection on survival to pupation (z=0.56, p=0.57), with 92.3% survival in exposed mosquitoes and 90.7% in unexposed.

There was also no difference of the survival up to feeding between mosquitoes selected for sensitivity (97.1%) and for resistance (98.3%)($t = 1.41$, $p=0.18$), or between mosquitoes exposed or unexposed to microsporidia (both with survival of 97.7%) ($t = 0.04$, $p=0.97$).

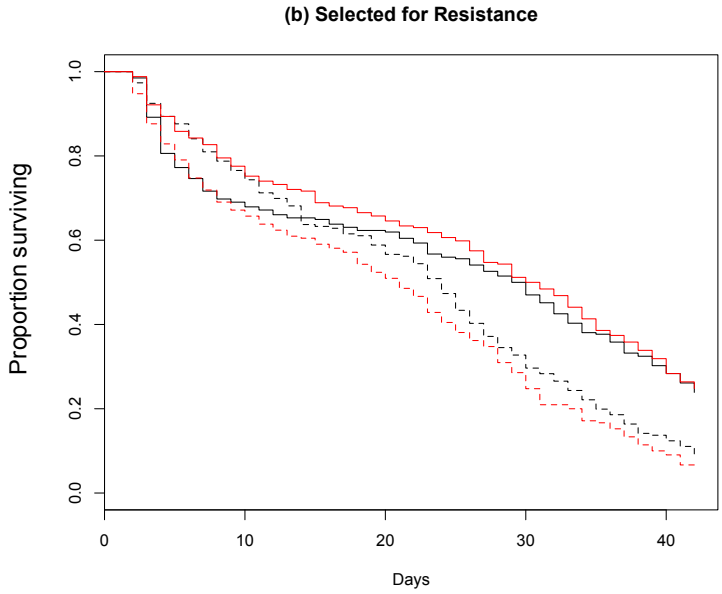
Survival post-feeding

In the first time period (up to 12 days after blood-feeding) infection with *P. berghei* increased the survival of mosquitoes (table 6.2, fig 6.2); however the extent of this benefit was significantly reduced in mosquitoes selected for resistance than those selected for sensitivity, as indicated by the interaction between malaria and resistance and the positive Wald statistic (table 6.2, fig 6.2). A significant interaction between microsporidia and malaria, and the positive Wald statistic, indicated that survival of mosquitoes doubly infected was lower than expected than if the effects were additive. Selection line was borderline significant; no other variables were significant.

In the second time period (from 13 days after blood-feeding onwards) microsporidia infection doubled the hazard of death; no other variables were significant (table 6.2).



Uninfected	227	146	124	105	63
Malaria	206	156	129	98	62
Microsporidia	245	167	130	82	32
Double	237	181	148	84	30



Uninfected	268	185	169	138	87
Malaria	254	197	169	138	86
Microsporidia	226	173	138	78	32
Double	210	141	114	64	24

Figure 6.2: Kaplan-meier survival curves of (a) mosquitoes selected for sensitivity (b) mosquitoes selected for resistance. Mosquitoes infected with malaria are indicated by red and dashed lines indicate infected with microsporidia. The solid black line therefore represent mosquitoes not infected with parasites and the red dashed line mosquitoes infected with both parasites. Tables represent the number of mosquitoes at risk (alive) at the time points indicated on the x-axis in each treatment group.

0-12day survival (n=1822)	**HR (se)**	**z**	**P**
Resistance	0.84 (0.17)	-1.35	0.320
Microsporidia	0.88 (0.11)	-1.19	0.230
Malaria	0.60 (0.15)	-3.43	<0.001
Microsporidia*Malaria	1.39 (0.16)	2.02	0.044
Malaria* Resistance	1.41 (0.16)	2.09	0.036
*Microsporidia * Resistance*	*1.19 (0.16)*	*1.08*	*0.280*
*Microsporidia*Malaria*Resistance*	*1.86 (0.33)*	*1.89*	*0.059*
13-42 day survival (n=1226)			
Microsporidia	2.07 (0.07)	11.07	<0.001
Malaria	*1.11 (0.06)*	*1.57*	*0.120*
Resistance	*0.96 (0.08)*	*-0.55*	*0.610*
*Microsporidia*Malaria*	*0.96 (0.13)*	*-0.31*	*0.760*
Microsporidia Resistance*	*1.07 (0.13)*	*0.51*	*0.610*
Malaria Resistance*	*0.98 (0.13)*	*-0.15*	*0.880*
*Microsporidia*Malaria*Resistance*	*1.16(0.26)*	*0.58*	*0.560*

Table 6.2 – Cox regression survival analysis for lines selected for sensitivity and resistance infected with malaria and microsporidian parasites. Parameters in italics were removed from the final model by stepwise selection in order on the table (bottom-up). Statistics quoted represent statistical estimates before parameters were removed from the model.

6.4 Discussion

It was hypothesised that costs of resistance, in particular to mosquitoes stressed by malaria or other parasites, may reduce the vector capacity of resistant mosquitoes. Reduced vector capacity in resistant mosquitoes would help to maintain a low prevalence of malaria in areas where wide-spread resistance has decreased the efficacy of killing mosquitoes. The experiment comparing an insecticide-sensitive colony of *An. gambiae* (Kisumu) with a permethrin-resistant one (RSP) showed evidence for this idea; in the first time period, before mosquitoes would be expected to be infectious, malaria decreased the survival of resistant mosquitoes but not sensitive ones. 78.4% of malaria infected Kisumu survived to the time point were they

would be expected to start producing sporozoites, compared to 61.3% of RSP – indicating the resistant mosquitos have lower vector capacity. Evidence from the experiment with DDT-resistant mosquitoes was less convincing. Overall malaria increased survival of mosquitoes in the first time period, yet the survival of resistant lines was significantly lower than the sensitive lines when infected with malaria. Again this would suggest that the resistant mosquitoes have a lower vector capacity, however, the effect size was small and so in reality there is not much difference in vector capacity of the resistant and sensitive lines (Figure 6.2).

Mosquitoes were infected with microsporidian parasites to see if a double infection would further reduce survival and increase the resistance costs. Over the full length of the study, all mosquitoes with a double infection had lower survival than mosquitoes infected with malaria alone, thus reducing their ability to transmit malaria. In particular a double infection in RSP was the only treatment to reach 100% mortality suggesting microsporidia could be used successfully in areas of insecticide resistance. Confirming current evidence that suggest microsporidia could be for malaria control (Bargielowski & Koella, 2009; Koella et al., 2009a; Lorenz & Koella, 2011). However to increase the resistance costs the parasites would have to reduce the survival of resistant mosquitoes to a greater extent than sensitive mosquitoes. A comparison between survival of Kisumu and RSP in the first time period suggested a double infection negated the costs exposed by a single infection (the survival of RSP was lower than Kisumu when infected with a single parasite, but not when infected with both). Again results from the selected lines indicated little; a close to significant 3-way interaction would suggest the double infection was having a greater impact on the survival of the resistant lines compared to the sensitive lines, thus increasing the costs of resistance. However, the interaction was not significant and again the effect size was small.

Several reasons may explain why an apparent cost of resistance was observed in the initial experiment but not the experiment with the selected lines. Firstly, the resistance mechanisms of the RSP and the selected lines (originally form ZANU) differ, and so could have different costs associated with them. The fitness cost of each mechanism may also be manipulated differently by parasitism. Agnew et al (2004) show that *V.culicis* infection increases the cost of resistance in *Culex pipiens* with esterase

resistance but decrease the relative fitness cost of acetylcholinesterase target site resistance. Here, the cost of esterase resistance, in RSP, was also increased by *V.culicis* infection but GST resistance in the selected lines was not. However, the original ZANU colony, with overexpression GST enzymes, has previously shown to have costs to longevity when infected with microsporidia (Koella et al., 2012).

Secondly, the selection process may not have established a large enough difference between the mosquitoes selected for resistance and sensitivity to exposes costs of the resistance mechanism. WHO resistance tests indicated survival after exposure to DDT ranged from 15-50% in the lines selected for sensitivity and 55-77% in lines selected for resistance. Resistance cost may therefore be diluted due to heterogeneity of resistance in the populations. On the other hand, the colonies were crossed so that the resistant and sensitive mosquitos would have similar genetic background, in order to eliminate differences that are not due to insecticide resistance. The differences in survival between RSP and Kisumu may therefore not be related to insecticide resistance at all. The alternative method of selection would to involve backcrossing of the colonies so that eventually they only differed by the resistant gene (Agnew et al. 2004), however this was not possible due to time constraints of the study. Finally, as the experiments were run over different time periods there may be differences in infection rates or virulence of the particular parasites used. Virulence of the malaria and microsporidian parasites in the second experiment appear to be much lower and may not have caused enough stress to expose costs of resistance.

It was expected that a double infection would exert extra stress on the mosquito and reduce survival to a greater extent to one parasite. However in RSP a double infection with both microsporidia and malaria closely resembled the survival of mosquitoes with microsporidia alone (Figure 6.1). Microsporidia infect mosquitoes at the larval stage so are already present when the adult mosquito takes a blood meal containing malaria parasites. Microsporidian infection has been shown to reduce malaria infection in the mosquito (Bargielowski & Koella 2009). Microsporidia infection is associated with changes in mosquito immunity (Biron et al., 2005; Duncan et al., 2012) and may prime the mosquitoes immune system ready for a malaria infection. The bacteria *Wolbachia pipientis* also reduces filarial nematode and *Plasmodium* numbers in mosquito vectors by priming the immune system in a similar fashion

(Kambris et al. 2010; Kambris et al. 2009). Thus, a reduction in malaria infection could explain why malaria does not further decrease the survival of RSP mosquitoes infected with microsporidia, yet the opposite trend is seen in Kisumu. Do microsporidia prime the immune system of Kisumu and RSP mosquitoes differently? Resistance in RSP is mediated by the overexpression on esterase and esterases may also been linked with mosquito immunity to malaria infection (Vernick & Collins, 1989). Moreover, genes encoding for resistance to pyrethroids, are up-regulated in the mosquito mid-gut when infected with malaria parasites (Félix et al., 2010; Stevenson et al., 2011) suggesting another possible link between resistance and mosquito immunity. RSP mosquitoes may therefore be predisposed to be immune to malaria infection. Although the expression of esterase does not reduce the malaria infection rate or oocyte count in resistant *Culex pipiens* mosquitoes (Vézilier et al., 2010), infection by microsporidia may trigger the immune response and enhanced the effect. Identifying malaria parasite loads may be an interesting extension to this study and could shed light on the relationships seen.

By splitting up the survival analysis a marked difference in the effect size of microsporidia and malaria is seen pre and post 12-day survival. Microsporidia have been proposed as a late acting pesticide (Koella et al., 2009a; Lorenz & Koella, 2011) so it comes as no surprise that they had a greater effect in the second time period. The malaria parasite also had different effects in the different time periods, with higher mortality in the later period especially evident in Kisumu, and may be explained by the different life stages of the malaria parasite. A meta-analysis by Ferguson & Read (2002) also showed that mosquito mortality was higher when malaria parasites had reached the sporozoite stage and an effect of malaria was more likely in longer studies. Malaria is not transmitted until the parasites survive the incubation period so natural selection will act against virulence in the non-transmissible oocyte stage. In contrast, once sporozoites are produced the parasites will optimise their transmission, which could come at a cost to the mosquito. This is evident in the parasites manipulation of mosquito behaviour. Blood feeding is risky for mosquitoes and it has been shown that while mosquitoes are infected with oocyts host-seeking and biting are reduced, thus increasing the mosquitoes survival, but if the mosquitoes are infected with sporozoites these risky behaviours increased, increasing parasite transmission (Anderson et al., 2000; Koella, 1999; Schwartz & Koella 2001).

In the selected lines malaria actually increased survival in the first time period, but neither increased or decreased survival in the second. Previous examples of malaria increasing survival have been shown in mosquitoes with limited access to sugar as adults (Vézilier et al., 2012; Zhao et al., 2012). Malaria parasites may manipulate carbohydrate catabolism in order to increase mosquito survival and their own transmission (Zhao et al., 2012). Alternatively mosquito survival is increased by malaria at the cost of mosquito fecundity. A trade-off between fecundity and survival was demonstrated by Vézilier et al (2012), they also hypothesis that increased survival in malaria-infected mosquitoes may have been masked in previous studies due to egg reabsorption. Not allowing mosquitoes to lay eggs, thus forcing egg reabsorption, could prevent a trade-off between fecundity and survival (because fecundity is essentially zero) and in turn the manipulation of this trade-off by malaria cannot be observed. In both experiments the effect of malaria was different in the early and late time periods, suggesting stage specific effects on mosquito survival, thus, future studies into the effects of malaria infection on mosquito survival may benefit from analysing the life stages separately.

Costs of resistance such as reduced longevity, will not only affect the vector capacity of the mosquito but also the evolution of resistance itself. Increasing the cost of resistance will likely slow the evolution and spread of resistance. Furthermore, microsporidian parasites have also been shown to reduce the benefit of resistance, by increasing the sensitivity of resistant mosquitoes to the lethal effects of insecticides (Chapter 4). It has been hypothesised that a combination of these two effects, increasing the cost and decreasing the benefit, could even block the evolution of resistance (Koella et al., 2012)

It was hypothesised that resistance costs, such as longevity, could be exacerbated by environmental stresses thereby reducing the vector capacity of resistant mosquitoes, in turn allowing insecticides to remain effective despite the evolution of resistance. However resistance costs are not the only mechanism in which insecticides may remain effective in areas of high resistance. The reduction of phenotypic expression of resistance with age (Chouaibou et al., 2012; Hunt et al., 2005; Jones et al., 2012; Kulma et al., 2013; Lines & Nassor, 1991; Rajatileka et al., 2011), which will also

reduce the number of resistant mosquitoes surviving to transmission, could also explain why vector control strategies remain effective. A third mechanism could be that resistant mosquitoes are still repelled by insecticides, so personal protection remains, to a degree (Corbel et al., 2004). Of course, all three mechanisms may be acting at the same time, which would suggest the evolution of resistance may have less dramatic effects on the epidemiology of malaria than feared.

In conclusion, a key parameter for malaria transmission is the number of infectious mosquitoes. This can be (but is not always) associated with total mosquito numbers, however it is closely linked with mosquito longevity. Any reduction in longevity, due to costs of resistance, will therefore reduce numbers of mosquitoes surviving to transmit malaria. Thus resistance may not increase malaria transmission despite the loss in efficacy of insecticides at mosquito killing.

Chapter 7

The effects of mosquito age and microsporidian infection on the repellency and irritancy of DDT and permethrin to *Anopheles gambiae* mosquitoes

7.1 Introduction

Insecticides are one of the main weapons in the control of mosquito borne diseases such as malaria (WHO, 2012a), and are generally believed to attain their high efficacy because of a combination of two effects. First, insecticides, by definition, kill insects and thus reduce the number of mosquitoes if coverage is high enough. Second, used as indoor-residual sprays or on bed nets they some insecticides deter mosquitoes from houses before the insecticide can have a killing effect (Achee et al., 2012; Grieco et al., 2007), and thus offer personal protection against indoor biting mosquitoes. This protection is achieved by non contact repellency, which reduces the number of mosquitoes entering houses, and contact irritancy, which increases the rate of exiting mosquitoes from houses before they have bitten (Manda et al., 2013); both reduce human-mosquito contact. The synergy between deterrence and killing is, however, weakened by an intrinsic trade-off between the two. Mosquitoes that are deterred by the insecticide are less likely to contact it long enough to be killed. Deterred, infectious mosquitoes can thus continue to transmit their parasite to unprotected hosts, making repellency and irritancy undesired traits of insecticides (Siegert et al., 2009).

Understanding repellency and irritancy of insecticides, and how they interact with the insecticides lethal effects, is therefore indispensable to understand the efficacy of insecticides used for the control of vector borne diseases. Repellency and irritancy are generally considered to be determined by the type of insecticide and dose used (Corbel et al., 2004; Grieco et al., 2007). Indeed, for *Aedes aegypti* mosquitoes the

insecticide DDT is highly repellent, permethrin is primarily an irritant and dieldrin has no deterrence effects at all (Grieco et al., 2007). In contrast *Anopheles gambiae* and *Anopheles funestus* are deterred by dieldrin (Zulueta & Cullen, 1963). Environmental factors and attributes of the mosquito can also affect the degree to which the mosquitoes are deterred. Temperature, light intensity, time of day and mosquito density can affect mosquito behaviour and the results of excito-repellency tests (Busvine, 1964). Deterrence effects of insecticides also varies among mosquito species (Cooperband & Allan, 2009; Smith & Nebley, 1968) and insecticide resistant mosquitoes are less likely to be repelled than sensitive mosquitoes (Chandre et al., 2000; Elliott, 1964; Thanispong et al., 2009). The physiological state of the mosquito can also affect deterrence; blood-fed and sugar-fed females are less repelled by insecticides than unfed mosquitoes (Polsomboon et al., 2008; Sungvornyothin et al., 2001). Finally, repellency may decrease with the mosquito's age (Busvine, 1964). Such a demographic effect would influence the efficacy of indoor-used insecticides for malaria control, only the oldest mosquitoes that become infectious and can transmit the parasite. Nevertheless, the effect of mosquito age on repellency has not been discussed in the context of malaria control.

Such aspects are of more than academic interest. Indeed, it may be possible to manipulate the mosquito's environment and condition in a way that changes the degree to which mosquitoes are deterred by insecticides, thereby enhancing personal protection (by increasing deterrence) or the insecticide's lethal action (by decreasing deterrence). A possible way to manipulate the mosquitoes' physical condition, and response to insecticides, is by infecting them with parasites. The microsporidian *Vavraia culicis* infects the malpighian tubule system, the fat body and the midgut epithelium of adult mosquitoes (Vávra and Becnel, 2007), damaging cells and depleting the lipid, glycogen and sugars reserves (Rivero et al., 2007). Infected mosquitoes may also have different behavioural responses to insecticides, as fungal infection can desensitize neurons involved in host seeking (George et al., 2011). Mosquitoes infected with the microsporidian *Edhazardia aedis* show increased repellency response to the chemical DEET (Barnard et al., 2007) but the behavioural response of parasitized mosquitoes to insecticides used for vector control has yet to be investigated.

Mosquitoes that are not deterred and are not killed, may achieve a successful bite. How successful these mosquitoes are has, however, received little attention. Insecticides may still impact the mosquitoes feeding success; exposure to insecticides can reduce host seeking in mosquitoes (Cohnstaedt & Allan, 2011), whereas in ticks insecticide exposure can actually increase host feeding (Mohamed et al., 2000). A change in feeding success in either direction will impact the probability of a mosquito transmitting malaria.

This study investigates the effects of mosquito age and microsporidian-infection on the deterrence (repellence and irritance) of *Anopheles gambiae* mosquitoes by the insecticides permethrin and dichlorodiphenyltrichloroethane (DDT). The blood-feeding success of mosquitoes that are not deterred by the insecticide is also assayed.

7.2 Methods

7.2.1 Study organisms

Repellency and irritancy tests were carried out on a colony of *Anopheles gambiae* from western Kenya (Kisumu), which is sensitive to all insecticides (Vulule et al., 1994).

The microsporidian *Vavria culicis* is an obligate, intracellular parasite of several mosquito species (Becnel et al., 2005; Andreadis, 2007), with a life cycle typical of microsporidians (Andreadis, 2007). Mosquito larvae are infected when they ingest the parasites' spores along with their food. Some infected larvae and pupae die and release a new generation of the parasites' spores for horizontal transmission to other larvae. If the mosquitoes survive to emerge, the adults remain infected. The parasite has several effects on the adult, including a shorter life span (Koella et al., 2009a; Lorenz & Koella, 2011), reduced susceptibility to malaria (Bargielowski and Koella 2008), and increased sensitivity to insecticides (Koella et al., 2012)

7.2.2 Experimental design

The experiments were carried out at 26 (+/-2) °C and 70 (+/-10) % relative humidity with a 12 h:12 h light/dark cycle.

Mosquitoes were reared individually in 12-well plates and fed with Tetramin fish food (0.04 mg on day 2, 0.08 mg (day 3), 0.16 mg (day 4), 0.32 mg (day 5), 0.6 mg on day 6 and following days until pupation). As 2-day larvae, half of the mosquitoes were exposed to 20,000 microsporidian spores, a dose that leads to very little larval mortality. Adults emerged from pupae 9 or 10 days after hatching.

Repellency and irritancy test to DDT, an organochlorine insecticide, and permethrin, a pyrethroid insecticide, were carried out on young (3 to 4 day-old) and on old (13 to 14 day-old) adult females. As mosquito activity is influenced by circadian rhythms (Bowen, 1991) and repellency tests can be influenced by time of day and light intensity (Busvine, 1964) all repellency and irritancy tests were conducted between 10am and 2pm with the light intensity kept constant at 30 lux.

To measure irritancy, mosquitoes were kept individually in 180ml cups. For each treatment group (microsporidian infection status and age at exposure) between 25 and 30 mosquitoes were exposed, again individually, to filter paper coated with 4% DDT, 0.75% Permethrin or a control with no insecticide, using exposure cylinders (12cm tall, 5cm in diameter). Mosquito activity was recorded with several digital cameras. Irritancy was measured as the number times a mosquito would take-off (assayed by visual inspection of the videos) within a 5-minute period.

To measure repellency, a set-up consisting of three compartments was used; a release chamber, an insecticide chamber and a control chamber (dimension for each chamber: 20 x 20 x 20 cm) (Fig. 7.1). The insecticide chamber was separated from the release chamber with a sheet of insecticide treated paper, 4% DDT or 0.75% permethrin, (dimensions: 12cmx15cm) and contained 9 evenly spaced holes (1cm in diameter) for the mosquitoes to pass through. The control chamber was separated from the release chamber with control paper containing the same number and spacing of holes. The

researcher placed a hand into the insecticide chamber and the control chamber as stimulus for the mosquitoes. At each replicate, 25 to 30 female mosquitoes were released into the middle chamber. Ten minutes later the number of mosquitoes in each chamber and their blood feeding status were recorded. The experiment was run in two identical blocks, 14 days apart, each with 5 replicates per treatment (microsporidian infection status and age at exposure) for each insecticide.

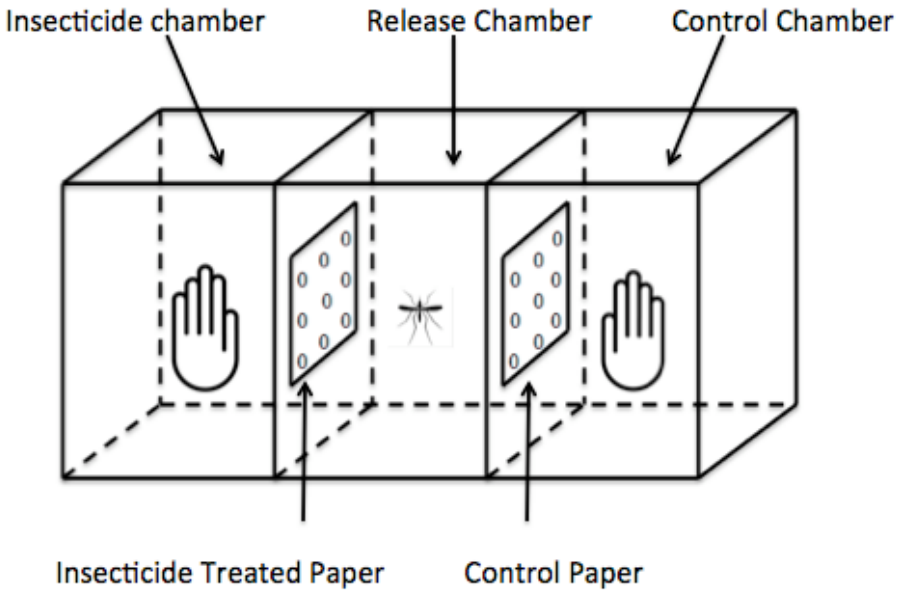


Figure 7.1: Experimental chamber to assay repellency. Mosquitoes were released into the middle chamber, hands of the researcher were placed into the insecticide and control chambers, separated from the release chamber by insecticide treated paper and control paper respectively. The control and treated paper were 12 x 15cm and contained 9 evenly spaces holes, of 1cm in diameter, for the mosquitoes to pass through. Chamber dimensions were 20 x 20 x 20 cm. The final position and feeding success of the mosquitoes was recorded after ten minutes.

7.2.3 Statistical analysis

The DDT and permethrin tests were analysed separately in R version 3.0.0 (R Core Team, 2013). Irritancy tests were analysed with a GLM, with the number of take-offs as the dependent variable and insecticide, infection status, age at exposure and their interactions as nominal factors. To take account of the overdispersion observed in a Poisson model, a negative binomial distribution was used.

For the repellency tests, the proportion of mosquitoes entering the insecticide and control chambers were analysed with a GLM (binomial distribution and logit link). Age at exposure, infection status and their interactions all entered the model as nominal factors. Block and replicate were included as random factors. The feeding success (fed/unfed) of the mosquitoes entering the control or insecticide chamber was also analysed with a GLM (binomial distribution and logit link). To account for overdisspersion in the mixed-models an observation level random factor was included. *A posteriori* interaction contrasts were conducted with the "phia" package (Rosario-Martinez, 2013) to interpret any interactions observed in the models.

7.3 Results

7.3.1 Irritancy

Mosquitoes exposed to DDT-paper had a higher number of take-offs than the controls ($z=8.02$, $p<0.001$), and old mosquitoes had a higher number of take-offs than young ones ($z=2.32$, $p=0.02$) (Figure 7.2). Although the main effect of microsporidia was not quite significant ($z = -1.90$, $p = 0.057$) there was a significant interaction between microsporidian and age at exposure ($z=4.26$, $p<0.001$). Contrasts indicated that although microsporidia infection reduced the number of take-offs in young mosquitoes it was not significant ($\chi^2 = 3.63$, $p = 0.057$), whereas microsporidia significantly increased the number of jumps in old mosquitoes ($\chi^2 = 19.92$, $p < 0.001$). All other interactions were insignificant.

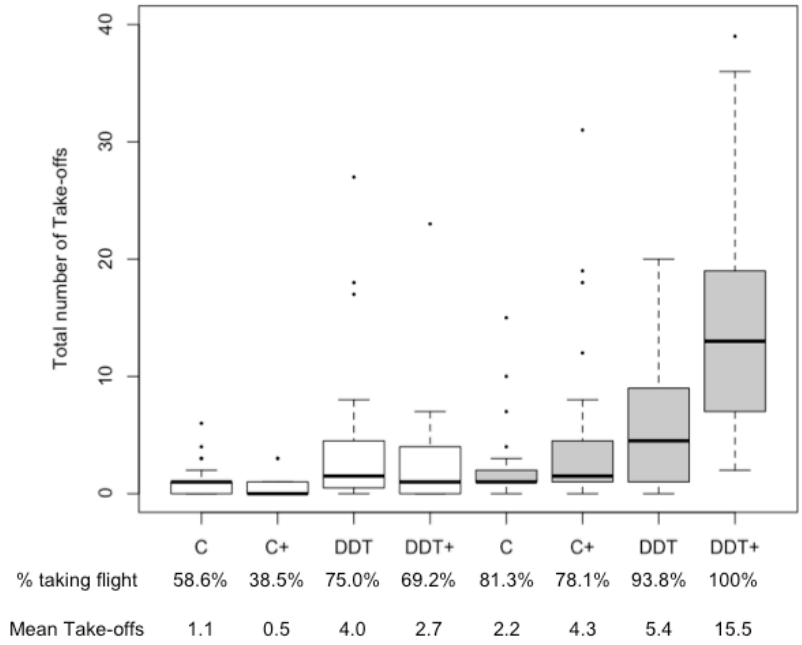


Figure 7.2: Irritancy of DDT measured as the number of take-offs in a 5 minute exposure. Boxplots represent the distribution of take-off counts for each treatment group: controls (C) and DDT tests, with or without microsporidia (microsporidia indicated +), at 2 ages: white boxplots 3-4 day old mosquitoes, grey boxplots 13-14 day old mosquitoes.

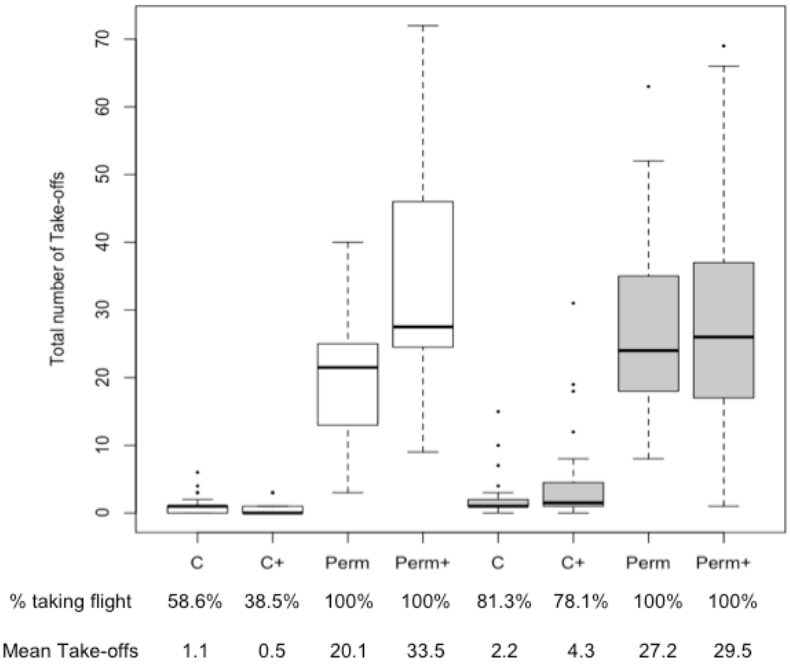


Figure 7.3: Irritancy of permethrin measured as the number of take-offs in a 5 minute exposure. Boxplots represent the distribution of take-off counts for each treatment group: controls (C) and permethrin tests, with or without microsporidia (microsporidia indicated +), at 2 ages: white boxplots 3-4 day old mosquitoes, grey boxplots 13-14 day old mosquitoes.

Mosquitoes exposed to permethrin-paper had a higher number of take-offs than controls (z=11.4, $p < 0.001$), and old mosquitoes had a higher number of take-offs than young ones (z=2.72, p=0.007)(Figure 7.3). The main effect of microsporidian infection was not quite significant (z=-1.94, p=0.053) but microsporidia infection was involved in a significant 3-way interaction between age at exposure and exposure to insecticide (z=-3.61, $p<0.001$). Interaction contrasts indicate that microsporidia increased the number of take-offs in young mosquitoes exposed to permethrin (χ^2 = 6.88, p = 0.026) but not old mosquitoes (χ^2 = 0.262, p = 0.608); the opposite was true for exposures to control paper, with take-offs due to microsporidia infection increasing in old mosquitoes (χ^2 = 9.19, p = 0.010) but not young mosquitoes (χ^2 = 3.75, p = 0.105) (Figure 7.3)

7.3.2 Repellency

Out of the 746 mosquitoes tested for repellency by DDT, 611 moved from the release chamber to the insecticide or control chamber and were used in the analysis. DDT indicated repellency with 80% of the mosquitoes entering the insecticide-free control chamber (Figure 7.4) (z = -5.46, $p < 0.001$), independently of the microsporidian infection (z = 0.153, p = 0.878). In contrast to irritancy, repellency was lower in the mosquitoes tested 13-14 days after emergence than in those tested 3-4 days after emergence (z = 2.00, p = 0.045) (Figure 7.4). The interaction between age and microsporidia was not significant so dropped from the model.

Despite a trend for older mosquitoes feeding less in DDT chambers than in control chambers, there was no significant effect of age (z=-0.032, p=0.97), microsporida (z=0.011, p=0.99), chamber (z=-0.039, p=0.969) or any interactions on the probability of mosquitoes feeding in the DDT repellency tests (Figure 7.5).

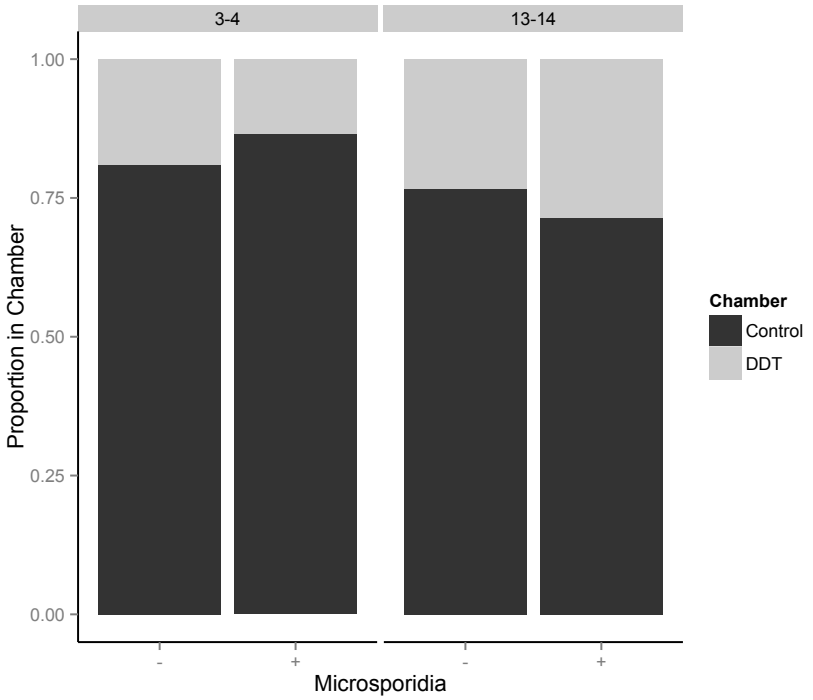


Figure 7.4: Proportion of mosquitoes in the control (black) and DDT (grey) chambers after 10 minutes in the experimental chamber, by microsporidia infection (+ indicates microsporidia infection) and age of exposure (indicated by top bar). Note, the barplots represent the proportions of mosquitoes that entered the stimulus chambers, thus, mosquitoes that remained in the middle chamber not included.

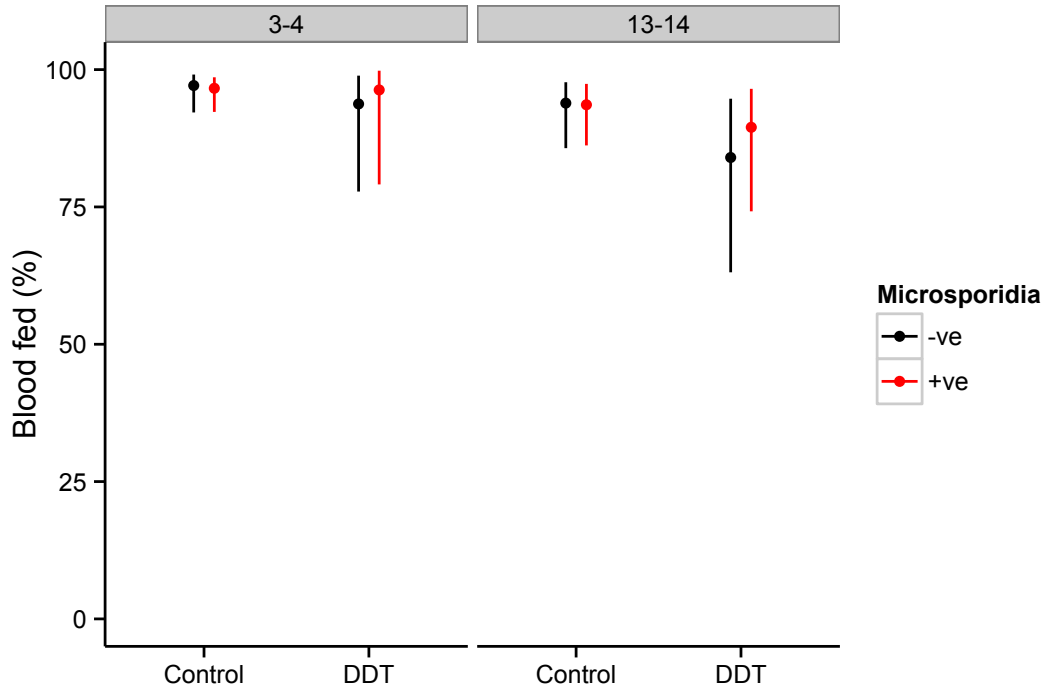


Figure 7.5: Percentage of mosquitoes that blood fed after entering the control and DDT chambers, by mosquito age (indicated by the top bar) and microsporidia infection (Red CIs represent microsporidia-infected, black CIs represent uninfected mosquitoes)

Out of the 699 mosquitoes tested for repellency by permethrin, 383 entered the insecticide or control chamber and were used in the analysis. Although 71% of the mosquitoes moved to the permethrin-free control chamber, the repellency of permethrin was not significant (z=-0.19, p=0.85) (Figure 7.6). Microsporidian infection did not alter repellency (z=-1.15, p=0.25) however age at testing increased repellency (z=-2.032, p=0.042). Despite the appearance of highest repellency in old microsporidian infected mosquitoes the interaction between age and microsporidia was not significant (z=-0.88, p=0.38)

Microsporidian infection (z=0.75, p=0.46) did not affect feeding behaviour. Interaction contrasts indicated that feeding increased with mosquito age in control chambers (χ^2 = 7.07, p = 0.016) but not permethrin chambers (χ^2 = 1.38 , p = 0.240). Furthermore, the probability of blood feeding was lower in the permethrin chamber than the control chamber (z=-7.04, p<0.001) (figure 7.7). No other interactions were significant.

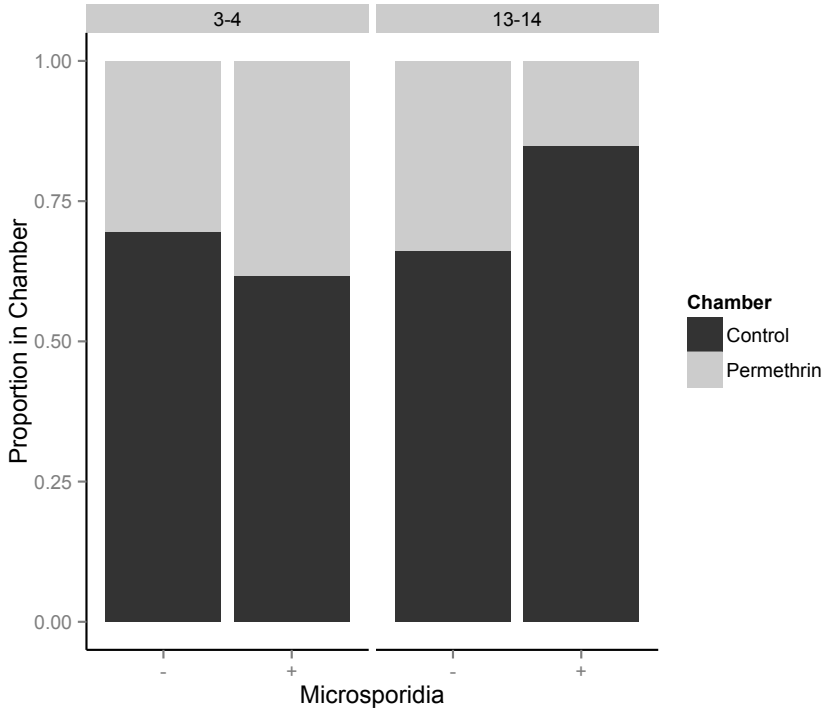


Figure 7.6: Proportion of mosquitoes in the control (black) and permethrin (grey) chambers after 10 minutes in the experimental chamber, by microsporidia infection (+ indicates microsporidia infection) and age of exposure (indicated by top bar). Note, the barplots represent the proportions of mosquitoes that entered the stimulus chambers, thus, mosquitoes that remained in the middle chamber not included.

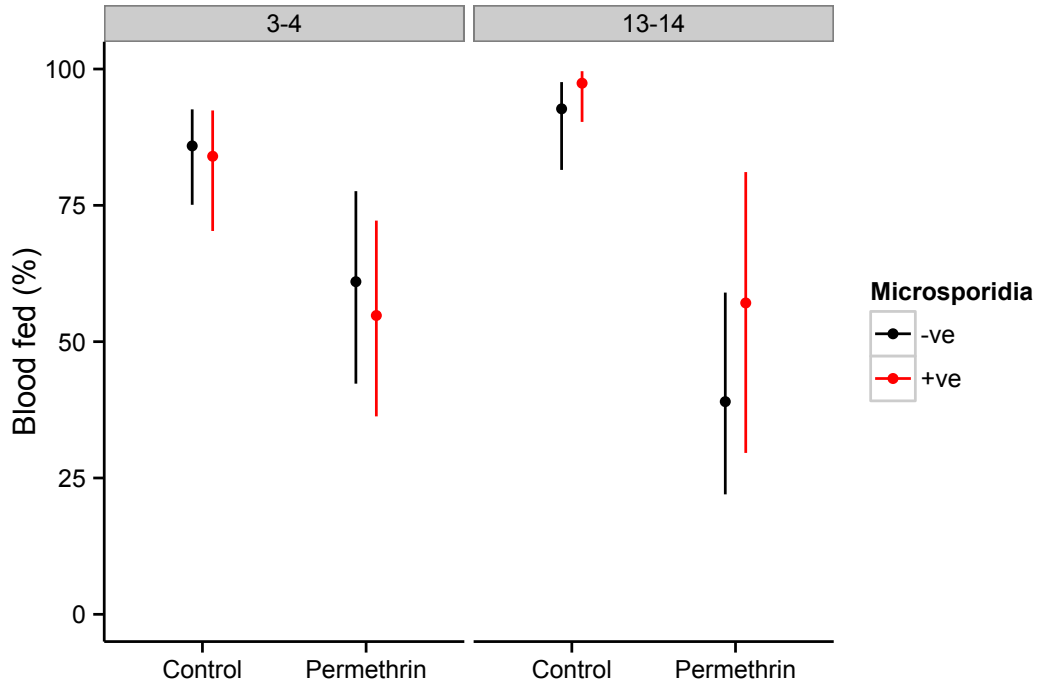


Figure 7.7: Percentage of mosquitoes that blood fed after entering the control and permethrin chambers, by mosquito age (indicated by the top bar) and microsporidia infection (Red CIs represent microsporidia-infected, black CIs represent uninfected mosquitoes)

7.4 Discussion

Deterrence effects of insecticides could have a significant impact on the efficacy of insecticides in controlling mosquito borne diseases. On one hand deterrence reduces human-mosquito contact, thus providing personal protection, but on the other hand deterred mosquitoes are not killed and can continue to transmit disease. Yet, significant reductions in malaria have been achieved using highly repellent insecticides such as DDT (Nájera et al. 2011). DDT was initially hailed for its toxic effects, however, the deterrence effects were identified soon after it was being used for vector control (Kennedy, 1947) and further studies indicated that the primary action of DDT was indeed repellency, with irritancy the secondary action and finally toxicity (Roberts et al., 2000; Smith & Webley, 1969; Zulueta & Cullen, 1963). Deterrence effects are however not solely determined by the insecticide used, and understanding how environmental effects and mosquito demographics may influence deterrence is important if we are to use them to our advantage.

This study indicates that both mosquito age and infection with microsporidia can affect mosquito deterrence to insecticides. Irritancy to both DDT and permethrin was higher in older mosquitoes, as was repellency to permethrin, however, this effect was not seen in the repellency to DDT. Microsporidia increased the irritancy of young mosquitoes exposed to permethrin and older mosquitoes exposed to DDT; but they did not significantly affect spatial repellency or blood feeding rates. Mosquitoes in permethrin chambers had reduced blood feeding, especially in older mosquitoes, which suggests an additional advantage of the insecticide in the use for malaria control.

An important question is whether the observed effect sizes are large enough to be important in malaria transmission? It is difficult to predict how numbers of take-offs and the mosquitoes choice between two chambers in the laboratory would affect deterrence of mosquitoes in reality, however theoretical work can give us an idea on the effect size needed to impact disease transmission and mosquito mortality. Koella

et al. (2012) indicate that a repellency of 90% results in far fewer mosquito deaths, compared to a repellency of 60%, and therefore repellency may be detrimental to disease control. Killeen et al. (2011) also demonstrate that increases in repellency may be detrimental to disease control. Here, the general trend was for deterrence to increase with age. Though perhaps different in natural populations, it suggests that deterrence, and thus personal protection, is highest when the mosquitoes are most dangerous (at the age when they could transmit malaria). Furthermore lower repellency, and thus higher mortality, when the mosquitoes are young, could benefit the community without the risk of the individual being infected with malaria. Recent work with the repellent DEET suggests that the mosquitoes' repellency response is plastic, with repellency decreasing with re-exposure (Stanczyk et al., 2013). If the behavioural response to insecticides also diminishes with re-expose it could potentially mean that older malaria-transmitting mosquitoes are less repellent, thus threatening the efficacy of repellency in malaria control interventions.

The reasons why we observe changes in repellency and irritancy are unknown, and better insight would be needed if we were to manipulate repellency to our advantage. By definition repellency is non-contact so will likely be determined by the olfactory detection of insecticide and the mosquitoes subsequent response. On the other hand irritancy requires contact so will possibly be a reaction to the neurotoxic effects of the invading insecticides (Haynes, 1988). It appears that microsporidia affect these two mechanisms of insecticide deterrence in different ways. Microsporidia had no effect on repellency – suggesting they are not affecting the olfactory system, but there is evidence that they increased irritancy suggesting they can influence the sensitivity of mosquitoes to insecticides. Indeed, infection by microsporidian increases a mosquitoes sensitivity to the lethal of effects of insecticide (Koella et al., 2012). Furthermore a mosquitoes' tolerance to the lethal effects of insecticides decreases with age (Glunt et al., 2011) mirroring the increase in irritancy with age to DDT and permethrin. As DDT is generally believed to be much slower acting than permethrin (Grieco et al., 2007) it may explain why irritancy is also lower in DDT. Overall, the results corroborate with previous studies showing DDT is a highly repellent insecticide whereas pyrethroids (in this case permethrin) are highly irritant (Achee et al., 2009).

In agreement with previous findings (Busvine 1964), the repellency of DDT decreased with mosquito age, displaying the opposite effect to irritancy. It is possible that the olfactory receptor sensitivity may decrease with age, although most of the evidence comes from work with *Drosophila* not mosquitoes (Bowen, 1991). It also does not explain the increase in repellency to permethrin with age. As the mosquitos have to pass through the treated paper, contact is made and therefore irritant effects may still be involved, this will more likely effect the results the permethrin test as it has high levels of irritancy. For malaria control, repellency of insecticides is only important if it overrides mosquito host seeking behaviour. For that reason, unlike some repellency tests (Grieco et al., 2007), bait was provided to see if the insecticides would prevent the mosquitoes from biting. The limitations of this method was that only one researcher was used as bait. As human attractiveness to mosquitoes can vary (Lindsay et al., 1993), so might the results of the repellency test, so using several researches may more accurately reflect the effects of repellency.

Combining insecticides with chemical repellents would be a straightforward way of increasing repellency for personal protection (Faulde & Nehring, 2012). It may prove more difficult to reduce deterrence to current insecticides and increase community protection. There are however newly proposed insecticides that show low deterrence effects (N'Guessan et al., 2007b; N'Guessan et al., 2009). Furthermore, although deterrence can be seen as a negative effect of insecticides it could be used combination with outdoor traps in a push-pull strategy thus maintaining the personal protection of repellency but still achieving mosquito killing (Cook et al., 2007; Manda et al., 2013).

Mosquitoes in permethrin chambers had reduced blood-feeding rates compared to the control chambers (Figure 7.7). Insecticide exposure can reduce host seeking (Cohnstaedt & Allan, 2011), however here the mosquitoes enter the bait chamber, indicating host seeking has taken place, but had reduced blood feeding when inside. Host-seeking and biting are however two distinct behaviours (Bowen, 1991) and so reduced biting could be another advantage of permethrin in reducing the disease transmission. Mosquitoes in permethrin chambers were observed rubbing their proboscis; this behaviour is a sign of irritancy and suggests the mosquitoes were

exposed to the insecticide when passing through the paper. The reduced biting rate may therefore be an artefact of the experimental set-up, but a similar situation may arise if mosquitoes are passing through holes in ITNs.

In conclusion, with increasing acknowledgment that deterrent effects of insecticides may have a large role to play in disease transmission it is important to investigate the environmental effects on repellency. Repellency and irritancy to insecticides may vary from mosquito to mosquito, but also within a single mosquito's lifetime. Studying the differences between mosquitoes that are deterred by insecticides and mosquitoes that are killed will give us a better insight into how these effects impact disease transmission and how to use repellency and irritancy to our advantage through environmental manipulation.

Chapter 8

General Discussion

Biochemical mechanisms allow a mosquito to survive insecticide exposure and thus threaten malaria control. However, the threat of resistance may be more complicated than it first appears; genetic resistance may not always confer phenotypic resistance and the evolution of resistance mechanisms may alter the ability of the mosquito to transmit malaria. The aim of this thesis was therefore to identify important factors in determining the extent of the resistance threat.

8.1 Summary of results

Several environmental and demographic effects on the phenotypic expression of resistance were investigated in chapters 2 to 4; mathematical modeling in chapter 5 brings these ideas together. In chapter 2, mosquitoes that fed on mice infected with *Plasmodium berghei* were more sensitive to insecticide than mosquitoes that fed on control mice. Unexpectedly, it was the mosquitoes that fed on the infected mice, but were not subsequently infected themselves, that had the highest sensitivity to insecticide. This might suggest that mounting an immune response to plasmodium infection may be more costly to the expression of resistance than infection of the parasite itself, however results have to be viewed cautiously due to the small sample size involved. In chapter 3, larval food treatment had only a small impacted on the phenotypic expression of resistance. As resistance mechanisms can involve energetic costs (Hardstone et al. 2010; Rivero et al. 2011), the results may be surprising. However, the energetic costs in the ZANU strain are unknown. Another reason may be due to the importance of resistance for the fitness of mosquitoes, so that resource allocation to resistance may be maintained even in face of low resource availability. Finally, increased feeding in the adult stage may occur to compensate for energy not acquired as larvae. In chapter 4, the sensitivity of genetically resistant mosquitoes was

partially restored, by infection with the microsporidan *Vavria culicis*, suggesting it may be possible to manipulate the expression of resistance to reduce its threat to malaria control. Overall, several environmental impacts on the expression of resistance were identified but none seemed to be as dramatic as the decline in resistance with age (chapters 2 & 4). There are now several studies confirming the phenomenon (Table 5.1) and the models in chapter 5 indicate resistant mosquitoes that show this decline will pose a significantly lower threat to malaria control. Genetic resistance has to lead to complete phenotypic resistance, throughout the entire mosquito's life, for ITNs or IRS not to have an effect on malaria transmission. The relatively small declines in resistance observed for the environmental factors, in chapter 2 and 3, also appeared more significant, as the modelling showed only small reductions in resistance could have large impacts on the probability that a mosquito will transmit malaria.

In chapter 6, costs of resistance were assessed under the environmental stress of parasitic infection. While resistance costs can take various forms (Agnew et al., 2004; Gazave et al., 2001; Martins et al., 2012), the cost to longevity was investigated for its significance in malaria transmission. There were mixed results in the two arms of the study, however, there was evidence that both *V.culicis* and *P.berghei* could increase the costs of resistance thus decrease the probability an infected mosquito surviving to transmit malaria. Finally, in chapter 7, the effect of *V.culicis* infection and mosquito age on the deterrence effects of insecticides were both investigated. There was a trend for deterrence to increase with mosquito age and microsporidia increased irritancy but did not have an effect on spatial repellency. Blood feeding rates due to permethrin exposure, indicating another advantage of the insecticide. With increasing acknowledgment that deterrent effects of insecticides may have a large role to play in disease transmission it is important to investigate the environmental effects on repellency.

8.2 Results in the context of malaria control & further research

As with all laboratory studies, how the results transfer to the field is vitally important. In terms of the model system used, the *An.gambaie* colonies were sourced from Africa and the resistance mechanisms originated in the field. However, the colonies have gone through many generations in the lab, and resistance was maintained through laboratory selection (Appendix A), both factors may lead to different results in field mosquitoes. In chapters 2 and 6, *P.berghei* provided a safe and easy alternative to *P.falciparum,* but the unnatural combination with the vector *An.gambaie* may have lead to higher virulence rates of the parasite (Ferguson & Read, 2002). Therefore, further experiments would be needed to see if the same results are replicated in human malaria parasites and in natural combinations of vector and parasite. As discussed in chapter 5 relatively little is know about the success and mortality of genetically resistant mosquitoes in the field, and this is a vital component to the threat of resistance. Resistance tests, such as the ones designed by the WHO and CDC (CDC, 2013; WHO, 2008), are optimized to detect the presence of resistance in a population but tell us little about how successful genetically resistant mosquitoes are in the field. It is this information that is vital to assess the threat of insecticide resistance.

This thesis investigated the impact of environmental stressors on the phenotypic expression of resistance and on the costs of resistance. Like many laboratory studies environmental stressors were assessed mainly on an individual basis, but in the field stressors may be more intense, more in number and come in a various number of combinations. Furthermore, mosquitoes in the lab are kept in a stable environment with an unlimited supply of sugar; the effect of any stressors, such as parasitism, on resistance may therefore be greater in more variable field conditions and where food may not be instantly accessible to replenish resources. It seems increasingly unlikely that all genetically resistant mosquitoes will express resistance and threaten the effectiveness of control measures.

The scope for further study is vast. The phenotypic expression and costs of resistance are likely to depend on the resistance mechanism involved, the mosquito species,

multiple environmental factors and all the different permutations. While we are beginning to understand that the phenotypic expression of resistance can vary significantly depending on environmental and demographic factors an area that has been studied very little is the ability of resistant mosquitoes to transmit malaria. Resistant mosquitoes may be shorter lived, less infectious and have behavioural differences that mean that their vector capacity is reduced (Rivero et al., 2010). Current understanding of the costs of resistance in malaria vectors (Djogbenou et al., 2010) and the impact of the resistance mechanism have on the malaria parasite (Alout et al., 2013; Vézilier et al., 2010) is sparse. A better understanding of the costs of resistance, and how the resistance mechanisms may impact the malaria parasites, is vital in assessing the threat of resistance.

Outside of the lab, the WHO have recommended linking public heath studies with rigorous entomological data, to properly assess the impact insecticide resistance has on malaria transmission (WHO, 2012b). The thesis describes two mechanisms, the decrease in resistance with mosquito age and the cost of resistance to longevity, which would result in mosquito numbers increasing with resistance without an increase in malaria transmission. A potential recommendation therefore, would be to use the sporozoite rate (the percentage of mosquitoes with sporozoites in their salivary glands) as an entomological indicator of malaria transmission, as an alternative to mosquito numbers and biting rates.

The thesis may also act as a warning, not to abandon or relax interventions if resistance is found in a population. 91% of malaria resurgence events since 1930s have been linked to relaxing of interventions through war or lack of funding (Cohen et al., 2012). We need a better understanding of the true threat of resistance and how best to deal with it, but currently it appears that abandoning or relaxing an intervention would do more harm than the presence of the resistance mechanism. Evidence from the field (Henry et al., 2005;Protopopoff et al, 2008) and the models in chapter 5 indicate increasing interventions can still reduce transmission even in a resistant mosquito population.

Despite these encouraging claims that resistance may not threaten malaria control as much as imagined, it would be foolish to rule out resistance as a threat. Gene

replacement (Guillemaud et al., 1998, Labbe et al., 2005) and modifier genes (Mckenzie and Ofarrell, 1993) can reduce the costs of resistance and it is highly possible, maybe inevitable, that mosquitoes evolve with increased phenotypic expression of resistance. Research into new chemical insecticides, resistance management techniques and new methods of vector control are still vital in the fight against malaria. For this reason the secondary theme running through this thesis was to evaluate the effectiveness of microsporidia as a biopesticide agent against insecticide resistant mosquitoes.

8.3 *Vavria culicis* as biopesticide agent

The microsporidian *V.culicis* has been proposed as an alternative to chemical insecticides. Termed evolution-proof because it is late acting, and so kills older mosquitoes, thus exerting little evolutionary pressure. This thesis presents further advantages of *V.culicis* as a biopesticide, particularly in mosquito populations resistant to chemical insecticides. First, it partially restores the sensitivity of resistant mosquitoes to chemical insecticide. As seen by the modeling in chapter 5 reductions in phenotypic resistance to below 40% can have dramatic effects on the probability a mosquito will survive until it becomes infectious. Secondly it reduces the survival of resistant mosquitoes more than sensitive mosquitoes, thus increasing the cost and reducing the threat of insecticide resistance. The combination of the two processes, decreasing the benefit but increasing to costs of resistance, could even block the evolution of resistance (Koella, 2012).

Increasing parasitic loads or using more virulent *V.culicis* strains could potentially restore sensitivity of resistant mosquitoes further than already achieved in chapter 4. Of course other parasites, such as the entomopathogenic fungi *Metarhizium anisopliae* and *Beauveria bassiana*, may also be used to restore sensitivity (Farenhorst et al., 2010). However, a better understanding of the mechanisms leading to sensitivity is needed if we hope to fully restore sensitivity to genetically resistant mosquitoes.

8.4 Conclusions

In conclusion, the phenotypic expression of resistance and the costs of resistance are two factors that will determine the threat insecticide-resistance has to malaria control. It was demonstrated in the laboratory that these two factors can vary due to environmental and demographic factors, but to fully understand the threat of resistance these ideas have to be investigated in the field.

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Appendix A

Study organisms and colony maintenance

***Anopheles gambiae* sensu stricto (Diptera: Culicidea)**

Three different colonies of *An.gambiae*, were used through the thesis. A dichlorodiphenyltrichloroethane (DDT) resistant colony (ZANU) from Zanzibar with increased metabolism of the insecticide, catalyzed by members of the glutathione S-transferase enzyme family (Ranson, 2000). A mildly pyrethroid-resistant colony of *Anopheles gambiae* (RSP) from western Kenya with elevated esterase and oxidase levels (Vulule et al. 1999). And a sensitive colony (Kisumu), which was also colonized from western Kenya and is sensitive to all insecticides (Vulule et al. 1994).

The follow rearing procedure was carried out for each of the three colonies. Mosquitoes were reared at a temperature of 26 (+/-2) °C and 70 (+/-10) % relative humidity with a 12 h:12 h light/dark cycle. Male and female adults were kept in three to five cages (dimensions: 20 x 20 x 20 cm) at a density of around 300 per cage, at a sex ratio of 1:1. Mosquitoes were provided with *ad libitum* de-ionised water and 10% glucose solution. Bloodmeals were provided by Jacob C Koella or Adam Saddler once a week. Eggs were laid three days later in cups filled with water and lined with filter paper. Eggs were then transferred to Petri dishes, provided with a pinch of Tetramin fish food, and allowed to hatch. One to two days later, 600 larvae were randomly selected from the Petri dish and divided into four trays (150 larvae per tray, dimensions 33.9 x 23.7 x 5cm) containing at least 1L of de-ionised water per tray. Larvae were provided with Tetramin fish food daily at increasing quantities to compensate for larval growth. Pupae from the trays were transferred into two new cages and adults were allowed to emerge completing the mosquito life cycle.

The resistant colonies, ZANU and RSP, were exposed to insecticide every two to three months to maintain resistance and selection was carried out 2 generations before

each experiment. Adult ZANU mosquitoes were taken from the colony cages and exposed to 4% DDT for 60mins with the standard World Health Organization test-kit (WHO, 1998), in groups of 20-30 per insecticide exposure tube. Thirty tubes allowed over 600 mosquitoes to be exposed at one time. Exposed mosquitoes were transferred into fresh cages. The surviving females were given a blood meal 24 hours later and their eggs used for colony maintenance as described above. This procedure selected out any sensitive mosquitoes and maintained resistance in the colony. The selection regime maintained >85% resistance in 3-4 day old adult ZANU mosquitoes when exposed to 4% DDT. The same procedure was carried out for RSP mosquitoes using 0.75% Permethrin, however, due to milder resistance, an exposure time of 20-30 minutes was used.

***Vavraia culicis* (floridensis) (Microsporidia: Pleistophoridae)**

The microsporidian *Vavraia culicis*, used in chapters 4,6 and 7 was maintained in the laboratory at Imperial College London. *V. culicis* is an obligate, intracellular parasite of several mosquito species (Becnel et al., 2005; Andreadis, 2007), with a life-cycle typical of microsporidians (Andreadis, 2007). Mosquito larvae are infected when they ingest the parasite's spores along with their food. Some infected larvae and pupae die and release a new generation of the parasite's spores for horizontal transmission to other larvae. If the mosquitoes survive to emerge, the adults remain infected. The parasite has several effects on the adult, including a shorter lifespan (Koella et al., 2009a; Lorenz & Koella, 2011) and reduced susceptibility to malaria (Bargielowski and Koella 2008). Although there is no transovarial vertical transmission, spores harbored by adult females can infest a new breeding site when they are released together with eggs (Andreadis, 2007). The prevalence of only a few microsporidian species in natural populations has been estimated; it is typically less than a few percent (Andreadis, 2007). While the prevalence of *V. culicis* in populations of *Aedes* mosquitoes ranges from 0% to about 50%, the only study on its prevalence in *Anopheles gambiae* found that 6.6% of the larvae in a West African population were infected (Andreadis, 2007).

Vavraia culicis spores were obtained from J.J. Becnel (USDA Gainesville,

USA). In Gainesville, V. culicis spores were produced by alternating spore propagation between the lepidopteran host *Helicoverpa zea* and the mosquito *An. quadrimaculatus* as described in Vávra and Becnel (2007). At Imperial College London the *V.culicis* was propagated in *An.gambiae* mosquitoes. Ten Petri dishes of twenty mosquito larvae were set up with de-ionised water and treated with a dose of 20,000 microsporidan spores per larvae. Larvae were reared under standard laboratory conditions, outlined above, and adults provided with 10% once they had emerged from pupae. Adult mosquitoes were left to die naturally and were collected in Eppendorf tubes in groups of ten. The dead mosquitoes were homogenized in 1ml of de-ionised water and the *V.culicis,* spores were counted under a phase-contrast microscope (400x magnification) with a haemocytometer. The spore concentration was noted and the Eppendorf tubes containing spores kept at 4°C until used in an experiment or for further propagation.

Appendix B

Proportional hazard testing for Cox-regression model

The survival analyses in chapter 6 were carried out using a Cox proportional-hazards model. As the name suggests, the assumption of proportional hazards is key to Cox regression analysis. Analysis of *scaled Schoenfeld residuals* allow us to determine the assumption of proportional hazards (Fox, 2002). Using the statistical program R version 3.0.0 (R Core Team, 2013), models can be tested for proportionality with the command: cox.zph(model). Table B.1 and B.4 indicate, by significant p-values, that the assumption of proportional hazards was violated for the survival analysis on both the established colonies (Table B.1) and selected lines (Table B.4). Following Sterne (2003) the time axis was split into two sections, within each of which the assumptions were met (Tables B.2, B.3, B.5 & B.6). As a splitting time 12 days after blood-feeding was chosen, based on inspection of the Schoenfeld residuals (Figure B.1), which determine the proportionality of the model (Fox, 2002), and based on the biology of malaria parasites which start producing their transmissible stages at about that time.

Established Colonies

	χ^2	P
Colony	5.51	0.018
Microsporidia	18.22	<0.001
Malaria	7.47	0.006

Table B.1: Schoenfeld residual test in the statistical package R, for the survival analysis of the established colonies. Significant p-values for each of the variable indicate that they have violated the assumption of proportional hazards.

	χ^2	P
Colony	0.05	0.827
Microsporidia	0.58	0.446
Malaria	0.72	0.395

Table B.2: Schoenfeld residual test for the survival analysis of the established colonies in the time period 1 to 12 days after blood feeding. P-values for each of the variable indicate that the assumption of proportional hazards is appropriate.

	χ^2	P
Colony	0.03	0.870
Microsporidia	0.06	0.813
Malaria	0.74	0.389

Table B.3: Schoenfeld residual test for the survival analysis of the established colonies in the time period 13 to 42 days after blood feeding. P-values for each of the variable indicate that the assumption of proportional hazards is appropriate.

Selected Lines

	χ^2	P
Resistance	1.18	0.112
Microsporidia	41.94	<0.001
Malaria	8.36	0.004

Table B.1: Schoenfeld residual test in the statistical package R, for the survival analysis of the selected lines. Significant p-values for malaria and microsporidia indicate that they have violated the assumption of proportional hazards.

	χ^2	P
Resistance	0.10	0.747
Microsporidia	2.23	0.135
Malaria	0.24	0.626

Table B.2: Schoenfeld residual test for the survival analysis of the selected lines in the time period 1 to 12 days after blood feeding. P-values for each of the variable indicate that the assumption of proportional hazards is appropriate.

	χ^2	P
Resistance	0.84	0.360
Microsporidia	0.33	0.563
Malaria	0.16	0.690

Table B.3: Schoenfeld residual test for the survival analysis of the selected lines in the time period 13 to 42 days after blood feeding. P-values for each of the variable indicate that the assumption of propotional hazards is appropriate.

Plots for established colonies

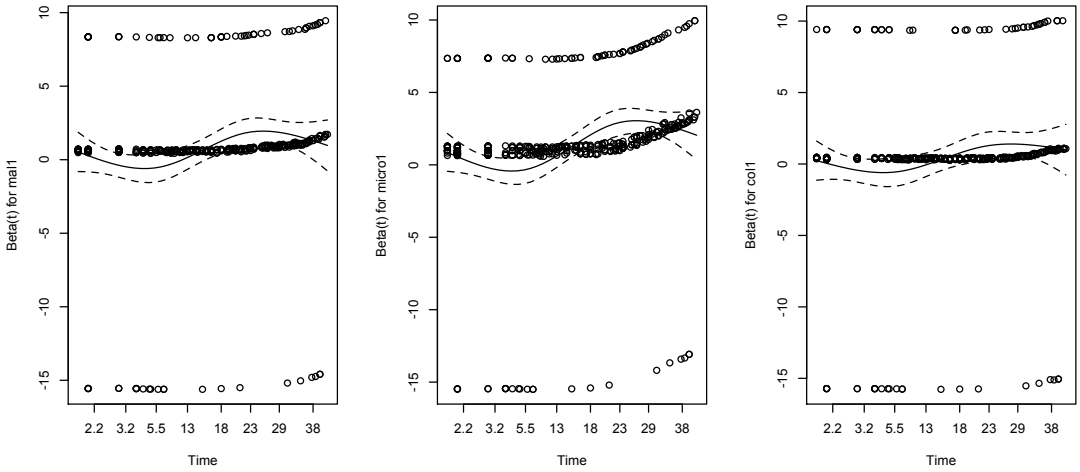

Figure B.1: Schoenfeld residual plots for the established colonies to assess the assumptions of proportinal hazards for a Cox-regression and to identify a appropriate split time. To facilitate interpretation of the graphs a smoothing-spline is fit to the plots (solid line), with the dashed lines representing a +/- 2 standard error band around the fit. Deviations from horizontal represent violations in the proportional hazards assumption. In agreement with the statistical report malaria status (left) and micrsporidia (right) violate the assumption of preoportional hazards, but resistance appers to be fine (middle).