ANTIMALARIAL DRUG RESISTANCE

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Abstract

Malaria is the most important parasite species of humans, affecting more than 2.2 billion people, and causing hundreds of millions of clinical cases and around one million deaths every year. Since the 1940s, mass-produced, inexpensive drugs have been available that could effectively treat individuals. However, the evolution of drug-resistance has repeatedly occurred, diminishing the therapeutic efficacy of drugs. The ability of the malaria parasite to quickly develop resistance to therapeutics is the result of its complex life-history. High mutation rates at the cellular level, which provide a means of continually evading the immune system, offer a mechanism for selection of resistance within a host, while interactions between other parasites and their hosts due to variation in transmission and host susceptibility, influence the probability of selection at the population level. A better understanding of the complex interactions between the parasite and its hosts can greatly improve our knowledge of how the evolution of resistance is related to, and impacts, particular life-history traits. This in turn may offer new strategies for controlling the disease. In this dissertation, immunity and within-host competition in malaria and how they can impact the evolution and spread of antimalarial drug resistance are examined. The dissertation is divided into three sections. Section I consists of an introduction to the biology of malaria and mathematical theories for control as well as a review of the ecology and epidemiology of antimalarial drug resistance. In Section II a within-host model of malaria is introduced to explore the mechanisms involved in generating chronic asymptomatic malaria infections. Section III explores the role of superinfection, or the simultaneous infection with multiple genetically distinct clones of the same parasite, and the impact of within-host competition on the evolution of antimalarial drug resistance. Lastly, the impact of heterogeneous
biting on antimalarial drug resistance as well as the coevolution of virulence and antimalarial
drug resistance are also explored in this section.
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Introduction

Malaria is the most important parasite species of humans, affecting more than 2.2 billion people, and causing hundreds of millions of clinical cases and around one million deaths every year. The discovery in the 1940s that the synthetic drug, chloroquine (CQ), could effectively treat individuals safely and cheaply helped spur malaria eradication efforts in the 1950s. However, the emergence of CQ resistance diminished its therapeutic efficacy and doomed initial efforts to eradicate the disease. The demise of initial eradication efforts led to a resurgence in the disease, and a significant change in the ecology, as CQ resistance spread from South-East Asia to Africa. In the ensuing years, CQ was replaced as a first-line drug by sulphadoxine-pyramethamine (SP), but resistance soon emerged to SP and spread widely. In recent years, artemisinin, has emerged as a new anti-malarial, and is being promoted as a new tool in the fight to eradicate the disease, though resistance is already reportedly spreading.

The rapid evolution of drug resistance is the result of interactions between the parasite and its hosts, but is facilitated by its complex life-history. Malaria parasites have evolved high mutation rates that help the parasite evade the immune system. This in turn provides a mechanism for the rapid emergence of de novo mutations that engender resistance within individuals. Selection for resistance at the population level, as well as the rate of spread, is influenced by the rate of drug treatment, which is the result of both social factors (such as access to treatment), as well as environmental factors (such as the rate of transmission) and host immunity. These factors also underlie many other facets of the parasite, such as the virulence of the parasite or its ability to generate chronic asymptomatic infections. Understanding the evolution of life-history traits and
how they impact factors such as parasite competition, can improve our knowledge of how drug resistance evolves, which is important for controlling the spread of resistance and may offer new strategies for controlling the disease.

In this dissertation, I examine how immunity and within-host competition in malaria can impact the evolution and spread of antimalarial drug resistance. The dissertation is divided into four sections. Section I consists of an introduction to the biology of malaria and mathematical theories for control as well as a review of the ecology and epidemiology of antimalarial drug resistance, with special emphasis placed on examining previous mathematical models for understanding and controlling the emergence and spread of the resistance.

Clinical immunity to malaria, which is manifested as reductions in the likelihood of clinical symptoms occurring, is the topic of Section II. Though protective of the host from severe disease, clinical immunity has significant ramifications for the development of drug resistance. In this section, the mechanisms involved in generating chronic asymptomatic malaria infections are explored. It is shown that antigenic variation, or continual variation of the antigens displayed on the surface of infected red blood cells, is the primary weapon the parasite uses to evade the immune system and prolong infection. Importantly, it is demonstrated that the initial response of the immune system is one of the keys to prolonging infection. By triggering a large response from the immune system to the initial antigen(s) displayed, the parasite is able to take advantage of cross-reactivity to keep the immune system from generating a significant response to the
additional antigens displayed, thus prolonging the duration of infection. This likely impacts the optimal level of virulence of the parasite—an issue that is explored further in Chapter 6.

Section III is devoted to exploring an issue that has been, and remains, a major question in ecology and is central to the development of antimalarial resistance: superinfection, or the simultaneous infection with multiple strains of the same parasite. Superinfection has been recognized since the early part of the 20th century as a major driver of malaria infection dynamics. Because superinfection is the rule, rather than the exception, parasite competition within the host is likely to impact several aspects of the parasite phenotype, including drug resistance. However, despite prior studies indicating that superinfection of other pathogens can be a significant evolutionary driver, particularly of pathogen virulence, a robust theory of the impact of superinfection on the evolution of drug resistance in malaria is still lacking. In the first chapter of the third section classical models of malaria are extended to consider the consequences of within-host competition among drug-sensitive and drug-resistant malaria parasites. The results from this model clearly demonstrate that within-host competition is a significant component in the emergence and spread of drug resistance even at lower transmission rates. Within-host competition significantly retards the ability of drug resistant parasites to invade, particularly as the transmission rate increases. This has a concomitant effect of slowing down the rate at which resistance spreads in a population and generating ranges of parameter space over which coexistence is likely. Significantly, it is shown that biological costs of resistance that reduce the ability of the drug-resistant parasite to transmit are less important to the evolutionary dynamics of resistance than reductions in the duration of drug-resistant infections. In addition, it is also
shown that random sampling of the population for resistant parasites is likely to significantly underestimate the frequency of resistance relative to measures that look at the failure of clinical infections.

Despite the important insights gained by extending the classical models of malaria, other factors, such as immunity, heterogeneous biting, and differential patterns of drug use were not fully explored due to the complexity of modeling superinfection. Thus, in the second chapter of Section III an individual-based model of malaria is introduced that enables a more robust analysis of these issues. Structured to be a stochastic analogue to classical Ross-Macdonald type models, the model is nonetheless based on individuals, and thus aspects of within-host competition can be explored. Importantly, because the model is analogous to the classical models, which have been well-described and analyzed, it is possible to determine how small changes in the assumptions of the model affect the dynamics of the disease. This model is specifically used to examine how competition between drug-resistant and drug-sensitive parasites is impacted by heterogeneous biting. An additional aspect of the model that makes it useful is the ability to examine how competition across multiple evolutionary axes impacts the dynamics of the disease. Prior studies have noted that superinfection can greatly impact the evolution of virulence; however, the relationship between virulence and other phenotypes has not been explored. In the last chapter of Section III the coevolution of virulence and drug-resistance is examined. First the issue of virulence evolution in long-lived infections, such as malaria, is examined. We find evidence in archival malariatherapy data to support the notion that the primary tradeoff with virulence is duration of infection rather than host-mortality. In other
words, increased reproduction at the outset of the infection leads to longer infections, but this saturates and then at even higher levels actually decreases the length of infection. We then explore the implications for this type of tradeoff at the individual level on the population level dynamics of infection. In particular, we explore how this may impact, and be impacted by, the evolution of resistance. In theory, because drug-resistant parasites are at an advantage when there is drug treatment, as therapy removes their competitors, virulence may actually increase as resistance spreads through a population. Anecdotal reports have suggested a link between the emergence of resistant parasites and increases in both clinical cases of malaria and the virulence of infections. Despite this possible link, little theoretical evidence exists to confirm this association. We show that, on average, when resistant parasites are rare, and thus competing only against drug-sensitive parasites, they are likely to have a higher virulence level than drug-sensitive parasites. However, as resistance spreads to more individuals, drug-resistant parasites are more likely to be in competition with other drug-resistant parasites and the average virulence level falls.
Chapter 1

A Century of Theory for the Dynamics and Control of *Plasmodium falciparum*

**Summary**
In this chapter, an introduction to the biology of malaria and mathematical theories for control is given. Specific aspects of the life-cycle of malaria are discussed as well as the history of mathematical models and their impact on the control of the disease.
Introduction

Malaria is an ancient disease. References to a disease with paroxysmal fevers that was associated with an enlarged spleen and a tendency to occur epidemically, hallmarks of malaria, were mentioned more than 4,700 years ago in ancient Chinese texts (Carter and Mendis 2002). Similar references on papyri as well as hieroglyphic inscriptions indicate its presence in ancient Egypt in the 16th century BCE (Livingstone 1971), while writings by Hippocrates leave little doubt about the presence of malaria in ancient Greece (~400 BCE) (Livingstone 1971; Carter and Mendis 2002).

Currently malaria is estimated to cause approximately 500 million clinical cases every year (Snow, Guerra et al. 2005), and more than one million deaths (Murray, Rosenfeld et al. 2012).

Historically, malaria has likely caused the death of more individuals than any other disease (Livingstone 1971). The burden of malaria in certain parts of the world, particularly Africa and parts of southern Europe, has been so high that genetic disorders, such as sickle-cell anemia and thalassemia, have been selected for because they provide some protective effect against malaria when individuals have one copy of the gene (heterozygous), even though they may be lethal when homozygous (Haldane 1949; Allison 1954; Allen, O'Donnell et al. 1997; Sachs and Malaney 2002). Malaria may also have contributed to the decline of ancient Greece, the collapse of the Roman Empire (Jones 1907), and the failure of the crusades (Livingstone 1971).

Despite its historical importance, it was not until the later part of the 19th Century that it was determined that mosquitoes transmitted malaria parasites (Smith, Battle et al. 2012a). Prior to that it was believed to have been cause by ‘bad air’, or malarìa in Italian, from which it got its
name (Ross 1911). And it was not until the early part of the 20th Century that Sir Ronald Ross first described the full life-cycle of the parasite (Ross 1911). Since then, numerous technological advancements have allowed for a significantly enhanced understanding of the disease, including the ability to clone and cultivate strains in the lab (Trager and Jenson 1978), which has expanded our understanding of the molecular aspects of the parasite.

Mathematical models of transmission though, have played a significant role in the history of malaria control. In addition to being the first to describe the parasite life-cycle, Sir Ronald Ross also wrote the first model of malaria (Ross 1911). Ross devised these equations, in part, to argue for new control methods (Smith, Battle et al. 2012a). Later, revised and updated by George MacDonald to include superinfection (MacDonald 1950a) and a quantitative theory of control (MacDonald 1957), malaria models provided the intellectual justification for the creation of the Global Malaria Eradiation Programme (Smith, Battle et al. 2012a), established in 1955 (GMEP). While the collapse of the GMEP in 1969 resulted in a tremendous setback for malaria control and prevention, and set the stage for a global resurgence in malaria, its collapse was due more to the structure of the campaign than any major flaws in the underlying theory (Smith, Battle et al. 2012a).

Though a number of individuals contributed, the comprehensive theory of malaria transmission which has largely influenced the way not only malaria epidemiology is understood and modeled, but all mosquito-borne pathogens, is generally regarded as the “Ross-MacDonald” model. In the following chapter, I discuss the development and formulation of the key aspects of the theory,
the basis of which serves as a jumping off point for later chapters. I also detail the life-cycle of malaria, so that the concepts and terminology used throughout will be fully understood.

**Life-Cycle of the Malaria Parasite**

Malaria is caused by a protozoan parasite of the genus *Plasmodium*, which attacks the red blood cells. A number of species besides humans can acquire malaria, including monkeys, mice, birds and lizards, but each is impacted by distinct *Plasmodium* specie(s). There have been more than 200 species of *Plasmodium* described (Perkins and Austin 2009), although there are only five species of malaria that commonly infect humans, *P. falciparum*, *P. vivax*, *P. ovale*, *P. malariae* and *P. knowlesi*. Until recently it was assumed that only four species commonly infected humans, but recent technological advances have found that *P. knowlesi* can be transmitted to humans (Jongwutiwes, Putaporntip et al. 2004) in the wild and this happens quite frequently across a large range, though it is commonly misdiagnosed as *P. malariae* (Singh, Sung et al. 2004; Cox-Singh, Davis et al. 2008). A number of other primarily primate malarias have also been shown (Contacos, Coatney et al. 1970) or are thought to be (Coatney and Roudabush 1936) capable of infecting humans, but are not commonly found, and are not considered to be a significant threat to human health. Discussions of malaria, whether it is in relation to morbidity and mortality, or eradication, largely refer to *P. falciparum*, which is the primary causative agent in the majority of malaria deaths worldwide. The use of the term ‘malaria’ in the ensuing dissertation will thus refer to *P. falciparum*, unless otherwise indicated.

Transmission of malaria occurs through a vector, the mosquito. The *Anopheles* genus of the family Culicidae is responsible for transmission of the *Plasmodium* species that cause disease in
humans. There are more than 70 species of *Anopheles* that can transmit human malaria, though only about 40 of them do so commonly (Service and Townson 2002). Only the female *Anopheles* is involved in transmission, as the blood is needed for production of eggs. The first phase of transmission occurs when an uninfected mosquito bites an infected human and ingests gametocytes—the sexual form of the parasite—along with its blood-meal. Gametocytes are both male and female, and they mate within the gut of the mosquito about 30 minutes after being ingested (Sinden 1998), producing a diploid zygote. The zygote then undergoes two meiotic divisions, during which reassortment and recombination of the two parental genomes occurs, producing four haploid copies within an ookinete (Sinden 1998). The ookinete then migrates through the midgut wall of the mosquito and forms an oocyst (Sinden and Billingsley 2001). The four haploid cells then undergo mitotic division producing anywhere from 1,000 to 10,000 sporozoites over an 8-15 day period depending on the species (Pringle 1965; Rosenberg and Rungsiwongse 1991; Beier and Vanderberg 1998). The sporozoites are then released and migrate to the salivary glands of the mosquito where they are injected into a human during the next blood-meal(s).

As the eggs are fertilized within the mosquito, the human is merely an intermediate host in which the haploid parasite replicates asexually. Within a human host, the parasite has two stages: an exoerythrocytic and an erythrocytic phase. In the first stage, the injected sporozoites rapidly

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1 Because of this time-constraint it is generally assumed that the majority of infections within a mosquito are generated by gametes from a single host. Rates of selfing and outcrossing are thus generally related to the number of genetically distinct clones in an individual, which is related to the rate of transmission. Higher transmission rates lead to higher rates of outcrossing.

2 While in general, transmission occurs through the *Anopheles* vector, other transmission routes are possible, including blood transfusions, congenital transmission from mother to fetus, and through needle sharing. For example, direct transmission of the asexual forms of *P. falciparum* between ‘drug addicts’ was endemic in New York and other large cities in the 1930s and 1940s (Most 1940).
make their way to the liver and migrate across the liver wall in a manner that is very similar to the mechanisms used by the ookinete to cross the midgut wall in the mosquito (Sinden 1998) and infect hepatocytes. Within a single hepatocyte the parasite multiplies asexually for a period of 6-15 days depending on the species, producing approximately 30,000 merozoites within a schizont, which then burst out and infect erythrocytes, red blood cells.  

Within the red blood cells, the parasite replicates over a period of approximately 48 hours (Reilly, Wang et al. 2007). Each replication produces approximately 16 new merozoites on average (Reilly, Wang et al. 2007), which infect other red blood cells when the cell ruptures. The characteristic fever cycle that embodies the clinical manifestations of the disease are a result of this cyclical process. With each replication, some of the merozoites, instead of producing new merozoites, develop into gametocytes, which can then infect susceptible mosquitoes, bringing the transmission cycle full circle. A diagram of the life-cycle can be found in Chapter 2.

**The Ross–MacDonald Model**

**Sir Ronald Ross**

The first person to fully describe and demonstrate the life-cycle of the malaria parasite was Sir Ronald Ross (Ross 1911). In addition to being a biologist, Ross was also an amateur mathematician, and sketched out the first mathematical models of malaria transmission based on the life-cycle of the parasite (Ross 1908; Ross 1911). His major advance was to sketch out

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3 In *P. falciparum* and *P. malariae*, the liver infection clears fully after development of the blood-stage merozoites, but in *P. vivax* and *P. ovale*, there may be a period of quiescence during the liver stage leading to delayed clinical manifestations or relapses months or even years later.

4 Other species have different replication periods, which range from 1-3 days depending on the species.
mathematically the principal factors involved in transmission so as to calculate an estimate for
the number of new infections,

(1.1) Number of new infections = \( fisbaimp \)

where \( p \) is the average population, \( m \) is the proportion of the population infected, \( i \) is the
proportion of infected individuals that are infectious, \( a \) is the average number of adult female
anopheline mosquitoes per person, \( b \) is the proportion of uninfected mosquitoes that bite an
individual, \( s \) is the fraction of infected mosquitoes that survive to become infectious, and \( f \) is the
proportion of infectious mosquitoes that bite an individual (Ross 1908). Using this formula, and
adding in a recovery rate, \( r \), Ross calculated the number of infected individuals in an area based
on the assumption that \( 1 - m \) individuals would be susceptible at a given time period, and in an
endemic area recoveries (\( rmp \)) needed to balance new infections,

(1.2) \( m = 1 - \frac{r}{iab^2s} \)

assuming that \( f = b \). By plugging in calculated values for \( r, i, b \) and \( s \), Ross was able to estimate
the relationship between \( a \) – the number of mosquitoes per person – and \( m \), the number of
infected individuals. He used this to demonstrate mathematically that to eliminate malaria one
only needed to reduce the number of anopheline mosquitoes below a critical threshold, not
eliminate all mosquitoes (Ross 1908). Future efforts of the GMEP, which involved spraying insecticides, primarily DDT, relied in part upon this idea.

In an addendum to his work, Ross then developed a system of finite difference equations that allowed one to determine the incidence and prevalence over time of a specific ‘happening’ (Ross 1911). The equations were of the form:

\[ a_{t+1} = (1-h)v a_t + HV z_t \]

\[ z_{t+1} = h v a_t + (1-H) V z_t \]

where \( a \) is the fraction of the population unaffected, \( z \) is the fraction of the population affected, \( v \) and \( V \) is the variation in unit time due to births, deaths, immigration and emigration, \( H \) is the recovery rate and \( h \) is the happenings rate, or the rate the unaffected become affected. Then taking the unit of time to be infinitesimal, Ross calculated continuous differential equations

\[ \frac{dA}{dt} = (v-h) A + (N + r) Z \]

\[ \frac{dZ}{dt} = h A + (V - N - r) Z \]

---

5 This was a controversial finding at the time and was regarded as nonsense by the nascent public health community (Fine 1975b), who were generally resistant to early modeling efforts in this and other diseases (Kingsland 1995).

6 In Ross’s “Theory of Happenings,” his calculation of a happenings rate endeavored to explain not just malaria, or even infectious diseases in general, but questions posed by “statistics, demography, public health, the theory of evolution, and even commerce, politics and statesmanship” (Ross 1916).
where the terms are as in equation 1.3 and \( N \) is the birth rate of the affected individuals (assuming that individuals are not born affected). In the case where \( v = V \), these equations can be simplified to calculate solely the proportion affected among the total population

\[
\frac{dx}{dt} = b(1-x) - (N+r)x
\]  

where \( x = Z / P \) and \( P \) is the total population, and the other variables are as before.

Solving this equation, assuming \( b, r \) and \( N \) are constants, we get

\[
x = \frac{b}{b+r+N} - \left( \frac{b}{b+r+N} - x_0 \right) \exp(-t(b+r+N))
\]

where \( x_0 \) is the initial infected population at time \( t = 0 \). These equations assumed that \( b \) was a constant, which as Ross acknowledges is true at the stable equilibrium, but is not the general solution to the equation. For the general solution, one must keep track of the number of infected mosquitoes. So the general solution to the problem is a set of non-linear equations of the form,

\[
\frac{dz}{dt} = \frac{b'fz'z'}{p}(p-z) - rz
\]

\[
\frac{dz'}{dt} = \frac{b'f'z}{p}(p' - z') - N'z'
\]
where the primed variables refer to the mosquito population. In this case, the number of infected mosquitos, \( z' \), is a function of the number of bites \( b' \) per unit time on the fraction of infectious, \( f \), affected, \( z \), individuals. Similarly, the number of infected individuals depends on the number of bites by affected and infectious mosquitoes. With a constant population of mosquitoes, the mortality rate can be replaced by the birth rate, \( N' \). The equations also ignore the natural mortality of humans as it is such a slow process compared to the dynamics of disease.

The link between the two different versions of equations is the happenings rate, \( h \), which is related to, but not exactly the same as the functions that describe the biting patterns from equation (1.1). An analysis by Lotka (Lotka 1923b), showed that under a certain set of assumptions, the results from equations (1.2) and (1.7) would be very similar, and in particular the case where a small focus of malaria develops into an endemic stable solution (Lotka 1923b).

**Reexamination of Ross’s Initial Assumptions**

Despite an exhaustive examination of these equations by Lotka (Lotka 1923a; Lotka 1923b; Lotka 1923c; Lotka 1923d; Lotka 1923e), they were not significantly re-evaluated until the 1950s. One of the serious flaws of these equations was the fact that while Ross acknowledged that happenings occur to both affected and unaffected individuals, he assumed that additional happenings were wasted, i.e. they did not alter the dynamics of an infection or the rate at which an individual would clear an infection.\(^7\) This assumption was re-evaluated by MacDonald in

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\(^7\) In the original formulation, \( z \) is the sum of \( b, c, d \), etc, where \( b \) is the proportion of individuals to which the happening has occurred only once, \( c \) is the proportion of individuals to which the happening has occurred only twice, and so on. Ross, however, assumed that re-affecting, or additional happenings, have no impact on the recovery rate, and thus this is merely a bookkeeping function with no impact on the dynamics.
1950 (MacDonald 1950a; MacDonald 1950b),\(^8\) who in fitting Ross’s equations to data found that recovery rates of 0.001 and often below 0.0005 were needed to fit the equations to data. This corresponds to infections lasting from three to more than five years, which did not accord with established understanding of the biology of the disease. In an attempt to rectify this problem, MacDonald made the following assumption: “The existence of infection is no barrier to superinfection, so that two or more broods of organisms may flourish side by side, the duration of infection due to one being unaltered by others” (MacDonald 1950a).\(^9\) In addition to superinfection, MacDonald included assumptions about mosquito mortality during sporogony (MacDonald 1952) and first calculated the basic reproductive number \((R_0)\) as the average number of secondary cases produced by an index case in a totally susceptible population (MacDonald 1957). The resulting equations, which have been at the heart of most models of malaria since, are generally termed the Ross-MacDonald equations. These equations were re-derived by Smith and MacKenzie (Smith and McKenzie 2004) with particular focus on the entomologic parameters. They first derive vectorial capacity \((V)\), or the potential number of humans infected, per infected human, per day, assuming perfect transmission as\(^{10}\)

\[
V = \frac{ma^2 e^{-gq}}{g}
\]

\(^8\) Walton (Walton 1947b) actually examined multiplicity of infection in malaria 1947. He used a Poisson distribution to estimate the proportion of infected individuals as \(1 - e^{-m}\), where \(m\) is the number of infective bites per person per year, and \(q\) is the proportion of the year when parasitemia was present. However, this equation does not accord with the data (MacDonald 1950a), and seems to have had no lasting impact on the theory.

\(^9\) Unfortunately, though the resulting equations gave a better fit to the data with realistic parameters, they did not accurately depict the assumptions (Bailey 1982; Dietz 1988a).

\(^{10}\) Alternatively, this is referred to as the number of infectious bites by a mosquito over its lifetime.
where $m$ denotes the number of mosquitoes per human and $a$ the human feeding rate (the number of bites on humans per mosquito per day). The instantaneous death rate is $g$ ($e^{-g}$ is the probability of a mosquito surviving one day), and $n$ is the number of days required for sporogony. The entomological inoculation rate (EIR), the number of infectious bites per human per day, is then derived as the product of the human biting rate ($ma$) and the sporozoite rate, the proportion of mosquitoes that are infectious, $(aPe^{-gn}/(g + aP))$. Thus,

$$EIR = \frac{ma^2cPe^{-gn}}{g + acP}$$

(1.9)

where $P$ is the fraction of humans infected and $c$ is the transmission efficiency from humans to mosquitoes, or the fraction of bites on infected humans that result in infections in mosquitoes. Lastly, the force of infection, or Ross’s happenings rate, can be derived as the number of infectious bites per human per day that result in infection,

$$b = bEIR$$

(1.10)

where $b$ is the transmission efficiency, or the proportion of infectious bites that produce an infection in a human.

The static entomological parameters can then be substituted into dynamic equations of the population to give a single equation for the proportion of the human population that is infectious,
A New Direction

The next wave of innovation in malaria modeling concerned the role of immunity. Assumptions concerning the development of blood-stage immunity and transmission-blocking immunity in the human host were incorporated into a model of control and tested in the Garki region of Nigeria in the early 1970s (Dietz, Molineaux et al. 1974; Molineaux and Gramiccia 1980). The model assumed that individuals were infectious for only a short period of time, and they either recovered or developed immunity. The model further assumed that while these immune individuals could become re-infected, they were not infectious. Because the state of immune individuals was not relevant for transmission these compartments were collapsed in later models into familiar SIRS equations (Aron 1988a; Aron 1988b), where immunity was boosted by exposure.

And there the innovation in malaria modeling has generally tended to remain (Reiner, Perkins et al. Submitted). While these now classic models of malaria have generally described the dynamics of malaria, they still ignore a number of important biological phenomena (Smith and McKenzie 2004). Despite this, these formulae have been extensively re-used with little critical evaluation of factors that could impact the dynamics, such as the role of immunity as well as heterogeneity in

\[
\dot{P} = \frac{ma^2 b c e^{-g_n}}{g + aeP} P(1-P) - rP
\]

where \(r\) is the recovery rate.
transmission both on a spatial and temporal scale (Reiner, Perkins et al. Submitted). In fact, in general, there has been little in the way of critical evaluation of the Ross-MacDonald theory in the last several decades (Reiner, Perkins et al. Submitted). This is in part because it is difficult to parameterize more complex models. However, a more thorough understanding of the dynamics of malaria and the impact of control efforts is needed.

This is particularly true with regards to understanding how to control the emergence and spread of drug resistance. The classic formulae in fact completely ignore the role of clinical infections. By grouping all infections as either infected or immune, there is little that can be understood about how treatment, and resistance to treatment, impacts the dynamics of the disease. Thus, in part, this dissertation is intended to examine how factors, such as heterogeneous biting, impact the dynamics of resistance. By pushing beyond the basic assumptions of the Ross-MacDonald formulae, I hope to find a new direction for understanding how individual variation impacts the dynamics of malaria, and thus improve the ability to make more robust, accurate, and useful predictions for addressing the profound public health challenge posed by malaria and in particular antimalarial drug resistance.
Chapter 2

Antimalarial Drug Resistance:
A Review of the Biology and Strategies to Delay Emergence and Spread

Summary
The emergence of resistance to former first-line antimalarial drugs has been an unmitigated disaster. In recent years, artemisinin class drugs have become standard, and they are considered an essential tool for helping to eradicate the disease. However, their ability to reduce morbidity and mortality and slow transmission requires the maintenance of effectiveness. Recently, an artemisinin delayed clearance phenotype was described, threatening local elimination and global eradication plans. Understanding how resistance emerges and spreads is important for developing strategies to contain its spread. Resistance is the result of two processes: (1) the selection for resistance; and (2) the spread of resistance. In this review, we examine the factors that lead to both the selection and spread of resistance, and why resistance is more likely to emerge in low-transmission areas. We then examine strategies for controlling the spread of resistance, pointing out the complexities and deficiencies in predicting how resistance will spread.
Introduction

Malaria is the most important parasite species of humans, affecting more than 2.2 billion people, and causing hundreds of millions of clinical cases of malaria every year (Snow, Guerra et al. 2005). Five species of the malaria parasite cause disease in humans: *Plasmodium falciparum*, *Plasmodium vivax*, *Plasmodium malariae*, *Plasmodium ovale*, and *Plasmodium knowlesi*. Of these species, *P. falciparum*, causes the most severe disease and is the leading cause of death in children under the age of five in Africa (Mathers, Lopez et al. 2006). The discovery in the 1940s that the synthetic drug, chloroquine (CQ), could effectively treat individuals safely and cheaply helped spur malaria eradication efforts in the 1950s. However, the emergence of CQ resistance diminished its therapeutic efficacy and doomed initial efforts to eradicate the disease.

The demise of these initial eradication efforts led to a resurgence in the disease, and a significant change in the ecology, as CQ-resistant parasites spread from South-East Asia to Africa (Payne 1987). In the ensuing years, CQ was replaced as a first-line drug by sulphadoxine-pyramethamine (SP), but resistance soon emerged to SP and spread widely (Nair, Williams et al. 2003; Roper, Pearce et al. 2003). While drugs are only one tool in the eradication effort, they are crucial to the effort, and thus, in this review we examine the mechanisms that lead to the emergence of resistance and the factors that contribute to the spread of resistance. This is of particular importance because in recent years, artemisinin class drugs have become widely available and are being promoted as a significant tool in the renewed fight to eradicate the disease. However, a delayed-clearance phenotype has already been reported in both western Cambodia (Dondorp, Yeung et al. 2010) and Thailand (Phyo, Nkhoma et al. 2012). This delayed-clearance phenotype, while not of clinical significance yet (Phyo, Nkhoma et al. 2012), is
the first indication that resistance to artemisinin is beginning to emerge. This has important implications for global eradication efforts, as it will likely be at least a decade before a new compound is capable of replacing the artemisinins (Olliaro and Wells 2009). Thus, it is important to understand how resistance emerges as well as mechanisms for controlling its spread.

**Selection for Resistance**

Antimalarial drug resistance is mediated by two processes; 1) the rate that de novo mutations conferring resistance are selected; and 2) the spread of those resistant alleles. CQ, SP and more recently the artemisinin class drugs have been widely adopted as first-line drugs because they are highly efficacious in eliminating *P. falciparum* infected erythrocytes and they are well-tolerated by almost all patients (Mehlotra and Zimmerman 2006; Schlitzer 2008). In addition, unlike other drugs, such as atovaquone and pyrimethamine (when not combined with sulfadoxine), the rate that de novo mutations conferring resistance occur is low.

Heritable drug resistance is enabled through a number of mechanisms including reductions in the active or passive uptake of a drug, abrogation of drug activity by conversion of the drug to an altered form, increased expression of the drug target, or a decrease in the ability of an inhibiting agent to bind due to alterations in the enzyme target (Cowman 1998). These heritable phenotypic changes, of which more than one is possible, are the result of mutations, which can be either single point mutations, alterations to multiple loci, or the result of gene duplication (White 2004).
Because resistance-conferring mutations generally result in significant changes in metabolic pathways (Dorsey, Fidock et al. 2001; Plowe 2001), they are likely to be deleterious in the absence of drug-treatment. Evidence for this has been found in in vitro experiments, where significant growth differentials were observed for specific CQ-resistant point mutations (Peters, Chen et al. 2002; Hayward, Saliba et al. 2005). A biological fitness cost also explains the significant reductions in the prevalence of resistant parasites after the removal of CQ as a first-line therapy in Malawi (De-quan, Rui-jun et al. 1995; Laufer, Thesing et al. 2006). However, while new resistance-conferring mutations are likely to have a significant associated biological fitness cost, this cost is likely abrogated over time by the evolution of compensatory mutations (Jiang, Patel et al. 2008). A similar phenomenon is known to occur in resistance evolution in bacteria (Andersson and Levin 1999).

The likelihood that a specific mutation conferring resistance will be present in a population is a function of the mutation rate, the selection coefficient against the mutation, and the probability that the resistant allele is selected from an infection (Hastings 1997). If resistance requires more than one mutation, than the initial frequency of resistance will be less. For example, if the frequencies of two necessary resistance-conferring mutations are both 0.01%, then parasites with both mutations will have an initial frequency in an infection of 0.0001%. This process underlies the recommendation that all malaria infections should be treated with combinations of two or more drugs (White 1998; White 1999b; National Research Council 2004). Assuming that each drug requires a single non-identical mutation in order for the parasite to acquire resistance, then the initial frequency of multi-drug resistant parasites will have a similar calculation as above.
The total parasite load also plays an important role. The malaria parasite life-cycle requires a mosquito in which meiosis occurs, but the vast majority of cell divisions in its life-cycle occur mitotically in the human host and thus is the more likely place where resistant mutations arise (White and Pongtavornpinyo 2003). Though the density at which symptoms occur can vary widely depending on the immune status of the individual, they are generally associated with blooms in parasite biomass. In non-immune individuals, symptoms may occur at densities of about 50 parasites μl^{-1} of blood, or between 10^8 and 10^9 asexual parasites (children who have less total blood volume, have correspondingly lower total parasites). Immune individuals may tolerate higher parasite loads, but parasite loads above 10,000 parasites μl^{-1} or about 10^{11} parasites are typically symptomatic even in immune individuals in highly endemic areas (Smith, Schellenberg et al. 1994).

Clinical attacks are short, lasting on average only a couple days. However, infections can persist for a long time without further symptoms. Estimates suggest infections last on average 200 days (Eyles and Young 1951; Smith, Dushoff et al. 2005), but can last significantly longer. Thus, most individuals harboring parasites at any one time are asymptomatic with low levels of parasitemia. However, because individuals that are symptomatic have such high levels of parasitemia, the majority of malaria parasites in the world at any one time are likely in individuals that are symptomatic (White and Pongtavornpinyo 2003), suggesting that symptomatic individuals are more likely to harbor resistant parasites (White 1999a).
Carriage of resistant mutants is only important for selection if those individuals harboring these mutants use drugs. Increased drug use within a population thus leads inexorably to a greater probability of selection for resistant mutants, a relationship that has been well documented in both models and experimentally (Austin, Kristinsson et al. 1999). However, widespread use of drugs has significant benefits both for the individual—reduced likelihood of morbidity and mortality—and the population—as a treated individual is less likely to transmit an infection. Thus, decisions about the distribution and use of drugs must balance the positive benefits of treatment with the negative externality of faster selection for resistance and reduced future effectiveness of the drug (Klein, Laxminarayan et al. 2007).

Selection for resistant mutants at the individual level depends on the concentration of the drug over time in the blood (pharmacokinetics) and the inhibitory effects on the malaria parasite at those concentrations (pharmacodynamics). Together the pharmacokinetics and pharmacodynamics give the concentration and length of exposure to a drug that parasites will face, however antimalarial drugs differ significantly in the length of time they are maintained in the body. Some drugs, such as chloroquine, have long half-lives (one to two months), while others such as artemisinin have much shorter half-lives (one hour) (White 1999a). As the concentration of a drug falls, its therapeutic efficacy falls as well. If the dosing is incomplete, meaning it fails to effectively eliminate all the parasites, either because of non-compliance or too low a dosage, parasites that may be inhibited at higher concentrations could survive and recrudesce. Alternatively, new infections may be exposed to subtherapeutic levels of drugs used
for treatment with long half-lives (White 1997), or because of prophylactic use (though this is rare except in travelers), or the use of substandard-drugs.\textsuperscript{11}

There have been some cases of high level drug resistance arising in individual infections during therapy (Clyde and Shute 1957; Looareesuwan, Viravan et al. 1996), however, in general, the mechanism by which resistant parasites are selected is believed to be through subtherapeutic drug levels (White 1997). At low drug concentrations, parasites with resistant mutations are able to survive and over time increase their fitness through compensatory mutations. Studies suggest that large proportions of the population in Sub-Saharan Africa may have subtherapeutic concentrations of CQ in their blood (Mockenhaupt, May et al. 2000) and that these are often associated with resistance (Wichmann, Egelte et al. 2007).

The stage of asexual parasite development (Figure 2.1) also impacts the efficacy of a drug (Dieckmann and Jung 1986; ter Kuile, White et al. 1993), with the maximum inhibitory effect of the drug typically occurring in the late ring and early trophozoite stages (ter Kuile, White et al. 1993). The artemisinin compounds are active during a broader time window of development (ter Kuile, White et al. 1993), which is the likely reason they tend to clear parasites at a faster rate than other drugs (White, Nosten et al. 1992; Barnes, Mwenechanya et al. 2004; Karunajeewa, Reeder et al. 2006). However, the artemisinin delayed clearance phenotype reported (Dondorp, Nosten et al. 2009; Dondorp, Yeung et al. 2010; Phyo, Nkhoma et al. 2012) is believed to be the

\textsuperscript{11} Substandard drugs encompass both counterfeit drugs, which have been deliberately manufactured with insufficient active ingredients, as well as products that have degraded. Drugs with no active ingredient, while significantly impacting the individual taking the medication, have no impact on resistance.
result of reduced efficacy of the drug against ring-stage parasites (Saralamba, Pan-Ngum et al. 2011).

Lastly, immunity to malaria, which develops with exposure (Schofield and Mueller 2006) and age (Baird 1995), plays a role in selection for resistance. Immunity to malaria provides protection against clinical manifestations of the disease only, and not against further asymptomatic infections (Schofield and Mueller 2006). Clinically-immune individuals also respond better to treatment (Yorke and Macfie 1924), with shorter treatment courses necessary to generate good therapeutic results even when drug resistance is present (White 1997). Thus, even though individuals that are clinically-immune tend to support higher parasite loads without showing symptoms—which increases the possibility that they may harbor a resistant parasite—the immune system is more likely to eliminate mutant resistant parasites.

**Spread of Resistance**

Once resistance has been selected for through drug treatment in an individual, to become a population problem, these parasites must be transmitted. Transmission of a *de novo* resistant mutant out of the primary host is conceivably the largest hurdle that resistant parasites face. The parasite first must survive long enough to produce infective gametocytes that are transmitted to a mosquito vector. Within the vector, the resistant mutation must not be lost during meiosis, and then the mosquito must survive sporogony and transmit a viable infection to a new individual.

The most important factor impacting the spread of resistance within a population is the total amount of drug use in the population. The higher the frequency of drug use in the population,
the more likely a resistant parasite is to encounter drugs, and the faster it will spread through the population (Wernsdorfer 1994; Mackinnon 1997). However, competition, both within the host (Hastings 1997) and among hosts (Klein, Smith et al. 2008), which changes at different transmission levels can play a significant role in the spread of resistance. In low transmission areas, individuals tend to be infected by fewer genetically distinct parasites, so resistant parasites face lower competition within the host. In addition, increases in the transmission rate increase the probability that multilocus resistant genotypes will be broken up by the action of Mendelian segregation because high transmission areas have higher rates of multiple infections (Dye and Williams 1997).

Between hosts, the transmission rate impacts the rate of spread in two ways. First of all, as transmission intensity increases, the time after a person uses drugs until they are re-infected is reduced. This means that individuals with resistant parasites harbor them without competition for a shorter period of time in high transmission areas. Secondly, because immunity results in a reduction in the frequency and severity of clinical malaria, clinical manifestations of the disease, while higher overall, are lower, per infection. The result is that in lower transmission areas there is a higher frequency of resistant alleles per infection as individuals are less likely to be clinically immune, thus each infection is more likely to result in a higher parasite load. Combined with higher rates of treatment per infection (since individuals are less likely to have immunity, they are more likely to become symptomatic and treat each infection), this results in a higher likelihood of resistant parasites being selected (Hastings 1997). In addition, clinically-immune individuals are less likely to take drugs and thus can act as a reservoir for drug-sensitive parasites (Klein,
Smith et al. 2008). As the proportion of individuals that are immune increases at higher transmission intensities, this abrogates the ability of the resistant parasite to spread. In other words, the fitness cost of resistance must be significantly less in high transmission settings as compared to low/unstable transmission settings, for resistant parasites to spread. However, once resistance is capable of spreading in high transmission areas (due to compensatory mutations that reduce its fitness cost (Jiang, Patel et al. 2008)) it will spread through the population much faster than in a low transmission setting. These theoretical results are consistent with the emergence of CQ-resistance along the Thai-Cambodia border, a low transmission area, in the late 1950s, and its slow spread across the continent which took until the late 1970s. CQ-Resistance wasn’t detected in Africa until the late 1970s, but then spread across the continent within a decade (Wellems and Plowe 2001).

Spread between populations is mediated by the movement of individuals and vectors. While flight distances of some species of *Anopheles* can be significant (Service 1997), in general, they only average around a kilometer from breeding sites (Thomson, Connor et al. 1995). Local-scale movement patterns of mosquitoes are due to searches for hosts and aquatic environments for oviposition (LeMenach, McKenzie et al. 2005), but long-distance dispersal can occur due to natural air movement or by riding along with humans (Service 1997). While there are reports of mosquitoes being swept on long-distance journeys of a couple hundred miles by natural winds or at the fore of a front (see Service 1997 for a review), the inherent brutality of the trip likely results in significant population loss, suggesting that this is not a major source of resistance spread. On the other hand, conveyance of mosquitoes in vehicles between countries and across
oceans, and the subsequent spread of malaria, has been an issue since at least the 1800s (Service 1997). There have also been many reports of malaria occurring in individuals living near airports in developed countries (Isaäcson 1989), which makes this a viable mechanism for the spread of resistance.

The short life-span of the mosquito compared to the average length of infection in the human host (10 days vs. 200 days), suggests however, that human movement plays a more important role in the spread of resistance. For instance, in the 1950s, resistance to pyrimethamine spread rapidly from village to village along well traveled trade routes (Clyde and Shute 1957).

**Resistance Emergence**

The rate at which resistance emerges is generally measured as the time after a drug is introduced into a population until a specific proportion of clinical infections are caused by resistant parasites. This measure implicitly assumes both the selection of resistant mutations and their spread within a population, and is important as this measure is a large determinant of when to switch first-line drugs (Bloland and Ettling 1999; Fèvre and Barnish 1999). The rate that resistance emerges depends in part on how resistance is encoded. Polygenically encoded resistance means that there is a reduced likelihood of selecting a resistant parasite, but it can also impact the emergence rate, depending on the relationship between the genes. If the effects of the mutations are additive, i.e. each subsequent mutation increases the tolerance or competitive ability of the parasite, then resistance will emerge faster than if every mutation is needed for resistance (Cross and Singer 1991; Mackinnon and Hastings 1998; Hastings, Watkins et al. 2002).
Transmission of malaria occurs through a vector, the mosquito, which ingests gametocytes—the sexual form of the parasite—when feeding on an infected human. Gametocytes, which are both male and female, mate within the gut of the mosquito, and undergo meiosis and then migrate through the midgut wall of the mosquito and form an oocyst (Sinden and Billingsley 2001), within which thousands of sporozoites develop (Beier and Vanderberg 1998). These are then injected into a human during the next blood-meal(s), where they rapidly make their way to the liver and infect hepatocytes and begin asexually (mitotically) replicating (Sinden 1998). After a period of ~6-15 days, the liver schizonts rupture releasing thousands of merozoites into the blood where they invade red blood cells. Over the next ~48 hours, the parasite begins replicating mitotically, progressing through a set of stages (ring, trophozoite, and schizont), and produces an average of 16 new daughter merozoites per schizont (Reilly, Wang et al. 2007). The schizonts then burst in near synchrony with other parasites producing the characteristic fever cycle that embodies the clinical manifestations of the disease. With each replication, some of the merozoites, instead of producing new merozoites, develop into gametocytes, which can then infect susceptible mosquitoes, bringing the transmission cycle full circle.
For a number of reasons, low and unstable transmission favors the faster emergence of resistance:
(a) the starting frequency of resistance is likely higher due to higher biomass infections (i.e. a
larger percentage of infections result in parasite blooms) (White and Pongtavornpinyo 2003); (b)
there is more drug treatment per parasite as more infections result in clinical symptoms
(Hastings 1997; Plowe, Kublin et al. 1998; White 1998); (c) immunity is less developed in low
transmission areas, so mutant parasites are more likely to survive the host immune response
(Plowe, Kublin et al. 1998; Gatton, Hogarth et al. 2003); and (d) as noted above, clinically-
immune individuals can serve as a reservoir for drug-sensitive parasites (Klein, Smith et al. 2008).

Genetic and epidemiological evidence supports the role of low and unstable areas in generating
resistance that then spreads to other areas. Chloroquine resistance was first noted around 1960
in western Cambodia (Payne 1987), a low transmission area (Gething, Patil et al. 2011).
Resistance to CQ also arose independently in low transmission areas of South America around
the same time (Payne 1987). Genetic evidence suggests that resistance genes from these founder
events (and from one additional site in Papua New Guinea) then spread to other regions,
including sub-Saharan Africa (Wellems and Plowe 2001; Wootton, Feng et al. 2002; Ariey,
Fandeur et al. 2006).

Sulfadoxine-pyrimethamine resistance (Hurwitz, Johnson et al. 1981; Verdrager 1986) as well as
mefloquine resistance (Boudreau, Webster et al. 1982; Nosten, ter Kuile et al. 1991; Fontanet,
Johnston et al. 1993) also arose in low-transmission areas of South-East Asia in the 1970s and
1990s, respectively. Thus, unsurprisingly, Plasmodium falciparum parasites with reduced in vivo
susceptibility to artemisinin derivatives were first reported in 2008 in western Cambodia (Noedl, Se et al. 2008; Dondorp, Nosten et al. 2009).

Resistance to CQ did not emerge as high-level resistance, but instead was marked by recrudescence following treatment. Accumulation of additional mutations over time led to the ability to survive higher drug concentrations (Payne 1987). A similar step-wise accumulation of mutations has been associated with increased resistance in SP as well (Plowe, Cortese et al. 1997). Emergence of resistance to artemisinin seems to be following a similar pattern, with the primary manifestation currently being described as delayed clearance (Dondorp, Yeung et al. 2010; Stepniewska, Ashley et al. 2010).

More recently, a similar artemisinin resistance phenotype has been described in western Thailand (Carrara, Zwang et al. 2009; Phyo, Nkhoma et al. 2012). This new foci may be due to spread of resistance from Western Cambodia or may be due to a separate de novo mutation. As there have also been reports of elevated artemisinin tolerance due to drug misuse (Shahinhas, Lau et al. 2010) as well as possible artemisinin related mutations (Thomson, Connor et al. 1995) in other areas, this suggest that artemisinin resistance may emerge in multiple locations. Thus, malaria control strategies must continue to target resistance evolution as well as strategies that slow the spread of resistance where it has arisen. Part of this must be through increased surveillance to identify new pockets of resistance and the rate that it is spreading (Escalante, Smith et al. 2009; Hastings 2011).
Controlling the Spread of Resistance

When resistance to CQ spread, countries shifted to SP as their nationally recommended treatment, and as resistance to SP spread, countries again shifted to artemisinin combination therapies (ACTs) – the WHO recommended standard. Resistance to artemisinin class drugs has now been reported (Noedl, Se et al. 2008; Dondorp, Nosten et al. 2009; Noedl, Socheat et al. 2009; Phyo, Nkhoma et al. 2012), and while not clinically relevant as of yet, developing and instituting control strategies to delay its emergence is important for future malaria control efforts, particularly as it will likely be at least a decade before a new compound is capable of replacing the artemisinins (Olliaro and Wells 2009). Mathematical models of malaria have been important for studying the evolution of drug resistance as they provide a means of integrating and synthesizing the results of studies done in many different academic disciplines to better understand how drug resistance emerges and spreads through and between populations. They are also useful for predicting the impact, feasibility, and cost of control strategies.

Modeling of malaria has a long history dating back to the first descriptions of the life-cycle of the disease by Sir Ronald Ross in 1908 (Ross 1908). Ross demonstrated mathematically that to eliminate malaria one only needed to reduce the number of anopheline mosquitoes below a critical threshold, not eliminate all mosquitoes. This simple finding, later confirmed and elaborated by MacDonald (MacDonald 1952), formed the basis for the eradication effort of the 1950s based on widespread application of insecticides, which was hugely successful in many parts of the world and failed due to operational constraints and lack of funding rather than any inherent flaws in the theory (Smith, Battle et al. 2012b).
More recently, mathematical models of antimalarial drug resistance played a significant role in policy adoption of artemisinin combination therapies as the recommended first-line drugs for combating malaria. Though the theoretical benefit of using multiple drugs dates back to the early 20th Century (Ehrlich 1913), mathematical models showed that not only would ACTs delay the evolution of resistance, but that they could also slow the spread of resistance once it had emerged (Laxminarayan 2004; Laxminarayan, Over et al. 2006). These results helped underpin the decision to subsidize the widespread adoption of ACTs through the implementation of the Affordable Medicines Facility–malaria (AMFm).

Despite the useful role played by mathematical models in understanding the emergence and spread of drug resistance, there is still significant progress to be made, particularly in devising strategies to maintain the effectiveness of artemisinins. Though, the recent emergence of artemisinin resistant phenotypes is certainly a cause for considerable concern, high-level resistance to artemisinin has not been reported (Phyo, Nkhoma et al. 2012), meaning containment strategies (Dondorp, Yeung et al. 2010; White 2010) can still be effective, but must be based on a sound understanding of the biology.

Mathematical models of antimalarial drug resistance have elucidated important aspects of resistance evolution, particularly concerning the role of transmission intensity, superinfection (i.e. multiple simultaneous malaria infections (MacDonald 1950a)), and clinical immunity (Hastings 1997; Hastings and D’Alessandro 2000; Hastings 2006; Klein, Smith et al. 2008; Pongtavornpinyo, Yeung et al. 2008; Chiyaka, Garira et al. 2009; Artzy-Randrup, Alonso et al.
2010). However, these models also make some simplifying assumptions that limit their usefulness for predicting how control strategies will work. One of the primary issues is the mechanism by which resistance evolves. As noted above, the step-wise accumulation of resistance genes, or tolerance, is the presumed mechanism by which most antimalarials, including artemisinin, will acquire high-level resistance. Thus, factors such as the frequency of drug use and the number of individuals with subtherapeutic drug concentrations (Hastings, Watkins et al. 2002; Hastings and Watkins 2006) play a crucial role in spread of resistance, and need to be considered in future models to better understand how the current artemisinin tolerant phenotype may progress to high-level resistance, and what strategies would be most appropriate in delaying increasing tolerance as well as spread of resistance.

A second issue is the role of within-host competition due to superinfection. Most epidemiological models of drug resistance have ignored the role of superinfection. The one exception (Koella and Antia 2003) used a formulation that allowed co-existence of drug-resistant and drug-sensitive parasites under mathematical conditions that were not biologically realistic (Hastings 2006; Klein, Smith et al. Submitted). A consequence of not accounting for superinfection is that many models consistently predict that once resistance emerges it will rapidly spread to reach 100% (Curtis and Otoo 1986; Cross and Singer 1991; Hastings 1997; Hastings and D’Alessandro 2000; Aneke 2002; Bacaër and Sokhna 2005), despite empirical evidence showing resistance does not always fix (Hastings 2006). Including complex within-host competition provides one means of explaining this result (Hastings 2006), but a better

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12 Heterogeneous drug use due to clinical immunity can also generate coexistence (Klein, Smith et al. 2008; Chiyaka, Garira et al. 2009). Alternatively, coexistence can occur if the de novo emergence rate of resistance is high and
understanding of how competition within the host impacts resistance is still needed. This is particularly important, because as CQ resistance spread through Africa, there were reports of increased clinical cases of malaria (Bødker, Kisinza et al. 2000; Shanks, Biomndo et al. 2000; Craig, Kleinschmidt et al. 2004) and increased morbidity and mortality risk (Zucker, Lackritz et al. 1996; Trape, Pison et al. 1998b) that was not attributable to treatment failure. In other words, there may be a genetic linkage between drug-resistance and increased morbidity and mortality. Since an increase in clinical disease would be associated with an increase in drug usage, this could generate a significant advantage for drug-resistant parasites in competition with drug-sensitive parasites. A clearer understanding of how superinfection and within-host competition affects the relationship between drug-resistance and virulence is needed to better predict how this relationship may drive the spread of drug resistance.

Lastly, a more complete understanding of how heterogeneity in vector movement and biting patterns impacts the spread of resistance is needed. Studies have shown that mosquito biting patterns are highly heterogeneous due to differences in size (i.e. children receive fewer bites than older individuals) (Port, Boreham et al. 1980), ecology (Kligler 1928; Kligler 1930; Wanji, Tanke et al. 2003), host infection status (Lacroix, Mukabana et al. 2005) as well as innate differential attraction (Mer, Mirnbaum et al. 1947; Lindsay, Adiamah et al. 1993; Knols, de Jong et al. 1995). In addition, movement patterns by both mosquitoes and hosts can localize biting intensity even further (Stoddard, Morrison et al. 2009). Estimates suggest that heterogeneity in biting results in approximately 20% of the individuals receiving 80% of the bites (Woolhouse, resistance is continually introduced into the population (Esteva, Gumel et al. 2009), though this is not a particularly likely scenario for important first-line drugs.
Dye et al. 1997; Smith, Dushoff et al. 2005; Smith, Drakeley et al. 2010). As heterogeneity in biting patterns likely has a significant role in the rate the resistance spreads through a population, a more extensive understanding is urgently need, particularly at the regional spatial scale that informs where resistance is likely to spread.

**Summary**

While the holy grail for malaria eradication is the development of a cheap, effective vaccine, currently none exists, and the most promising candidate to date has, at best, only a moderate impact on the incidence of malaria (Alonso, Sacarlal et al. 2005). Despite this, significant progress against morbidity and mortality is possible with the tools we already have. However, the emergence of an artemisinin resistant phenotype threatens one of the key components of elimination and eradication plans, and new control strategies are urgently needed. While eliminating the parasite from the area where resistance has emerged has been suggested (White 2010), the history of malaria elimination suggests that this is an unlikely scenario. While more surveillance and epidemiological studies are needed to help determine the extent of the problem as well as the effectiveness of interventions (Escalante, Smith et al. 2009; Hastings 2011), a better understanding of how drug resistance emerges and spreads is urgently needed to devise other control strategies. Determining what type of intervention to implement, as well as when and where and how much it will cost, requires a detailed understanding of how drug use, clinical immunity, superinfection, and heterogeneous biting impact the spread of resistance. Addressing these issues will allow for a better, more robust understanding of how to control this urgent challenge.
Chapter 3

Cross-Reactive Immune Responses as Primary Drivers of Malaria Chronicity\textsuperscript{13}

Abstract
The within-host dynamics of an infection with the malaria parasite \textit{Plasmodium falciparum} are the result of a complex interplay between the host immune system and the parasite. Continual variation of antigens displayed on the surface of infected red blood cells enables the parasite to evade the immune system and prolong infection. Despite the importance of antigenic variation in generating the dynamics of infection, our understanding of the mechanisms by which antigenic variation generates long-term chronic infections is still limited. We examine the role of cross-reactivity in generating infection dynamics that are comparable to measured experimental infections. We present a hybrid model, which mixes discrete replication events and continuous interaction with the immune system, and evaluate the dynamics of a single malaria infection over time. We then examine three major mechanisms by which the dynamics of an infection can be structured: cross-reactive immune responses, differential parasite adherence phenotypes and heterogeneous parasite switch rates. Our results demonstrate that cross-reactive immune responses play the primary role in generating the dynamics observed in experimentally untreated infections and in lengthening the period of infection. Importantly, we also find that it is the primary response to the initially expressed VSA that structures the cascading cross-immune dynamics.

\textsuperscript{13} With Andrea L. Graham, Manuel Llinás, Simon Levin
Introduction
Naïve individuals infected with the malaria parasite *Plasmodium falciparum* for the first time invariably develop significant clinical symptoms. Those that survive the initial acute infection generally remain chronically infected with low and diminishing levels of parasitemia for many months. With repeated infections, individuals develop clinical immunity, which is characterized by lower levels of parasitemia and a reduced probability of clinical disease (Schofield and Mueller 2006), although immunity to severe disease generally develops quickly (Gupta, Snow et al. 1999). Severe pathology is thought to be mediated by the expression of variant proteins, particularly *P. falciparum* erythrocyte membrane protein 1 (PfEMP1), on the surface of infected red blood cells (RBCs), which results in the sequestration of parasites in the microvasculature of vital organs (Miller, Baruch et al. 2002). While many aspects of clinical immunity formation remain unknown (Langhorne, Ndungu et al. 2008), the decrease in the frequency and severity of disease episodes over several years despite almost continual infection risk suggests that an immune response directed against the polymorphic PfEMP1 antigens plays a significant role in the acquisition of clinical immunity (Kyes, Horrocks et al. 2001). Continual switching of the expressed immune target allows the parasite to evade the immune system’s response to specific variants and thus prolong the duration of infection (Miller, Good et al. 1994).

PfEMP1 is encoded by the highly variant *var* multigene family that is spread across all 14 chromosomes (Kyes, Horrocks et al. 2001). Each haploid genome contains approximately 50-60 distinct *var* genes (Kyes, Horrocks et al. 2001) that have been grouped into four major groups (A, B, C, and E) and two intermediate groups (B/A and B/C) based on their upstream region, chromosomal organization and similarities in coding and non-coding regions, as well as in the
domain structure of the encoded proteins (Kraemer and Smith 2003; Lavstsen, Salanti et al. 2003).

Severe clinical infections in non-immune individuals are generally dominated by parasites expressing a subset of PfEMP1 variant surface antigens (VSAs) which are more conserved than other groups (Nielsen, Vestergaard et al. 2004) and have been associated with severe malaria (VSASM) (Bull, Kortok et al. 2000). At present, the best evidence suggests that VSASM is primarily associated with up-regulation of group A var genes (Jensen, Magistrado et al. 2004; Rottmann, Lavstsen et al. 2006; Cham, Turner et al. 2009). Repeated re-infection appears to generate a strong immune response against the antigens causing severe malaria and infections in older, chronically infected individuals are generally dominated by VSAs associated with uncomplicated malaria (Bull, Kortok et al. 2000; Nielsen, Staalsoe et al. 2002).

\textit{P. falciparum} parasites express VSAs in a mutually exclusive fashion (Scherf, Hernandez-Rivas et al. 1998). However, while multiple VSAs may be expressed by different parasites within a host at any one time, the dynamics of infections are characterized by sequential waves of parasitemia that are dominated by specific VSAs (Handunnetti, Mendis et al. 1987; Brannan, Turner et al. 1994; Kaestli, Cortes et al. 2004). The decrease in frequency of a particular VSA is associated with the production of antibodies specific to that VSA, though evidence suggests that a particular threshold density must be reached before a long-lasting immune response against a particular VSA is induced (Krause, Gatton et al. 2007). After clearance of parasites expressing a
VSA, antibodies generated in response gradually decrease, though immunological memory remains and is rapidly restored upon re-exposure (Staalsoe, Megnekou et al. 2001).

While there is clear evidence for modulation through transcription regulation, the process of switching is still not well understood. Rates of switching appear to be variable and controlled epigenetically (Chookajorn, Ponsuwanna et al. 2008). Theoretical models and in vitro data suggest that either differential switch rates (Paget-McNicol, Gatton et al. 2002; Horrocks, Pinches et al. 2004), differential growth rates (Molineaux, Diebner et al. 2001), or structured switching (Recker, Buckee et al. 2011) constitute the main mechanism by which the parasite avoids exhausting its entire VSA repertoire early in the infection. However, unlike other organisms that use antigenic variation to evade the immune system, P. falciparum has only a limited number of var genes per haploid genome. Because at the peak of a primary acute infection there are typically between $10^9$ and $10^{13}$ asexual parasites (White 1999a), even low rates of switching would result in the display of all variants to the immune system early in an infection.

In this study we describe a model that combines variant switching with cross-reactive immune responses to suggest how P. falciparum produces chronic asymptomatic infections despite a limited var gene repertoire. While prior models have suggested that cross-reactive immune responses play a significant role in structuring the dynamics and length of malaria infections (Recker, Nee et al. 2004), we demonstrate how cross-reactive immune responses may interact with antigenic switching to produce infection patterns that are qualitatively similar to experimental P. falciparum infections. Linking cross-reactivity patterns with PfEMP1 protein
coding data, and a simplified immune system, our results suggest that cross-reactive immune responses, rather than differences in growth or switch rates, play the most significant role in structuring the long chronic dynamics of a *P. falciparum* infection. Significantly, we demonstrate the importance of the initial immune response in generating the cascade of dominant VSAs and the resulting structure of the dynamics.

**Methods**

The within-host dynamics of malaria or antigenic variation have generally been modeled using ordinary differential equations (Sasaki 1994; Molineaux and Dietz 1999). Since these models have used continuous time to describe the interaction of the parasite with the immune system, they fail to describe the discrete reproduction of the parasite, which occurs in a synchronized fashion approximately every 48 hours (Reilly, Wang et al. 2007). Thus, the existing models neglect the fact that new variants can only switch on after a minimum of 48 hours. In this paper, we use an approach that combines both mechanisms of modeling (Haydon, Matthews et al. 2003; Mideo, Barclay et al. 2008; Miller, Råberg et al. 2010); the interaction of the parasite and the immune system occurs in continuous time punctuated by the replication of the parasite at discrete intervals.

We assume that parasites that survive the 48 hour period of replication without being cleared by the immune system produce 18 (Cheng, Lawrence et al. 1997; Bejon, Andrews et al. 2005; Reilly, Wang et al. 2007) new merozoites that find and infect new RBCs nearly instantaneously (Kyes, Horrocks et al. 2001). We also assume that as parasite density increases, increased immune activity, including fever, as well as possible resource constraints, limit the growth rate of
the parasite. This density dependent mechanism, which has been shown to be important in structuring the dynamics of malaria infections (Kwiatkowski and Nowak 1991), impacts the parasite most significantly during the initial parasitemia, when unrestrained growth leads to significant parasite density. This is consistent with both theoretical (Gravenor, McLean et al. 1995) and murine models which have demonstrated that an innate immune response is necessary for controlling the initial level of parasitemia (Su and Stevenson 2002), but antibody specific mechanisms of the adaptive immune response are necessary for eliminating the parasite thereafter (Stevenson and Riley 2004).

The discrete reproduction events of the parasite are described by the following equation,

\[ P' = \frac{K \lambda P}{K + \lambda P} \]

where \( P' \) is the number of new parasites, \( \lambda \) is the number of new parasites produced per parasite and \( K \) is the carrying capacity.

Between discrete replication events, the parasite interacts with the immune system in a continuous manner. We assume that parasitized cells are removed by the spleen or other mechanisms of the innate immune system at a constant rate \( \mu \), while the adaptive immune effectors (\( A \)) and memory cells (\( M \)) that are specific for that antigen remove parasitized cells in a mass-action manner related to the concentration of both parasite and immune effector (\( y \)).
Undifferentiated adaptive immune effectors are activated by the parasite at a rate that approximates their concentration in the blood ($\kappa$). Once activated, they become specific for the removal of parasites expressing that antigen, and the removal of those parasites stimulates the production of new adaptive immune effectors at rate $\rho$, inducing a positive feedback loop that is characteristic of the immune system (Fearon and Locksley 1996). Adaptive immune effectors are assumed to have a short life-span and wane at rate $\delta$. However, once the immune system is activated, it is assumed to remain active until the infection is contained. Thus, the removal of parasites is assumed to stimulate the production of cytokines ($V$) that keep the adaptive immune effectors from degrading and from generating memory cells. Cytokines have a short life-span and degrade quickly at rate $\theta$. Thus as interactions between parasites expressing a particular VSA and adaptive immune effectors specific for that VSA wane, the immune system will down-regulate.

As the immune system down-regulates, a proportion of the generated effectors ($\varepsilon$) become memory cells ($M$), which are not assumed to wane (Struik and Riley 2004), though they are lost when activated by the parasite. Compared to naïve immune effectors, memory cells have higher sensitivity to antigenic stimulation, and provide significantly more positive stimulatory feedback to the immune system (Sallusto, Geginat et al. 2004). Thus, when a particular VSA is expressed again (in the same or a new infection), memory cells are assumed to rapidly produce new adaptive immune effectors at a significantly higher rate ($\pi$). To ensure that memory cells are only created as the immune system is down-regulating, we instituted a binary function $\varepsilon(A_t=0)$, which regulates the production of memory cells. At the beginning of a 48 hour cycle, if the production of new immune effectors is outpacing their destruction, which only occurs when the immune
response is actively increasing, \( \varepsilon = 0 \), and no memory cells are produced. The dynamics of the interaction between the parasite and the immune system between replication events are described by the following set of ordinary differential equations.

\[
\begin{align*}
\dot{P} &= -\mu P - \gamma PA - \gamma PM \\
\dot{A} &= \kappa P + \gamma PAP + \gamma PM\pi - \delta Ae^{-V} \\
\dot{M} &= \delta Ae^{-V} \varepsilon(A_{r=0}) - \gamma PM \\
\dot{V} &= \gamma P[A + M]\omega - V\theta
\end{align*}
\]

\[\text{(3.2)}\]

**Antigenic Variation**

The primary benefit of the hybrid approach is that we can analyze the major choice that the parasite makes at each replication event: 1) become merozoite clones expressing the same VSA; or 2) switch to express a new VSA.\(^{14}\) An additional implication of the model is that we can simulate the extirpation of particular VSAs. We assume that once a parasite expressing a certain VSA has fallen below a threshold value, and antibodies specific for that VSA exist, it will be cleared by the immune system with no further switches or replications. *P. falciparum* antibodies are capable of reacting with VSAs from heterologous isolates (Bull, Lowe et al. 2002; Ofori, Dodoo et al. 2002), suggesting that antibodies for specific variants are cross reactive with other variants. We thus introduce cross-reactivity: immune effector cells and memory cells specific for a particular VSA are assumed to be capable of eliminating parasites expressing other VSAs, but at a reduced rate depending on the relatedness of each VSA.

\(^{14}\) We ignore the role of gametocytes as they only account for a small fraction of the newly produced merozoites, and the adaptive immune system is not generally targeted at gametocytes (Smalley and Sinden 1977).
While there are other multigene families that have been associated with variant surface protein expression and undergo antigenic variation (Kyes, Horrocks et al. 2001; Cortés, Carret et al. 2007), we limit the VSAs undergoing antigenic variation to those expressed by PfEMP1. The structure of PfEMP1 is similar across groups and plays a significant role in the adhesion and binding properties as well as immune evasion (Smith, Gamain et al. 2001). Divergence in the protein sequence is related to variant proteins that bind alternative receptors linked to cerebral and placental malaria (Smith, Gamain et al. 2001). Thus, we use relatedness of the expressed protein sequences ($\sigma$) as a proxy for calculating the potential cross-reactivity of specific VSAs.

Predicted protein sequences were obtained from PlasmoDB (http://www.plasmodb.org) for 62 var genes in the 3D7 reference strain (Kraemer, Kyes et al. 2007), which has been shown to be representative of typical sequence diversity both across regions and strains (Rask, Hansen et al. 2010). The sequences were then aligned and an identity matrix calculated using clustalX2 (Larkin, Blackshields et al. 2007). The resulting identity matrix is based on protein sequence similarity (Figure 3.1) and organized by var gene group. A similar analysis was done for each of the first three Duffy binding-like and Cysteine-Rich Interdomain Region domains with the similar result that average relatedness was lowest and variation was greatest in group A vars (Table 3.A1 in the appendix). In addition, analysis of the full model using these alternative matrices was similar to the results produced from the full protein sequences. Thus in our analysis, we calculated the cross-reactivity, or ability to bind, of an adaptive immune effector specific for VSA $j$ to a parasite expressing VSA $i$ based on the relatedness of the full protein sequence using the following functional relationship,
where $\beta_{ij}$ is the distance between protein sequences $i$ and $j$, measured as the proportion of homologous amino acids, and $D$ is the e-folding distance, or the exponential rate at which cross-reactivity wanes. Cross-reactivity is assumed to be due to heterogeneity in the affinity of immune effectors for alternative VSAs. Thus, when a parasite expressing VSA $j$ is cleared by an immune effector specific for VSA $i$, the assumption is that this is due to a subdominant epitope that is more specific for VSA $j$. Due to the positive feedback inherent in the immune system (Fearon and Locksley 1996), we assume that this results in the production of more immune effectors specific for VSA $j$. This approach is used to account for the role of subdominant epitopes that were originally stimulated by an alternative VSA, but may be more efficient at binding to a different VSA. Memory cells are assumed to function similarly. The full model of parasite interaction with the immune system between replication events can thus be described by the following set of ordinary differential equations,

\begin{align*}
\dot{P}_i &= -\mu P_i - \gamma P_i \left[ \sum_j A_{ij} \sigma_{ij} + \sum_j M_{ij} \sigma_{ij} \right] \\
\dot{A}_i &= \kappa P_i + \gamma P_i A_i \rho + \gamma P_i \left[ \sum_j A_{ij} \sigma_{ij} + \pi \sum_j M_{ij} \sigma_{ij} \right] - \delta A_i e^{-\nu_i} \\
\dot{M}_i &= \delta A_i e^{-\nu_i} \epsilon(A_{i=0}) - \gamma M_i \sum_j P_j \sigma_{ij} \\
\dot{V}_i &= \gamma P_i [A_i + M_i] \omega + \gamma P_i \left[ \sum_j A_{ij} \sigma_{ij} + \sum_j M_{ij} \sigma_{ij} \right] - V_i \theta
\end{align*}

(3.4)
where \( i \) refers to each specific VSA. Variation in the expressed antigen is assumed to occur in a structured manner where the order of switching is determined randomly at the onset of infection and then the parasite loops through that pattern until the infection is cleared. The discrete reproduction equations for the full model are,

\[
(3.5) \quad P'_i = \left( \frac{K \lambda P_i}{K + \lambda \sum P_j} \right)
\]

for which each VSA is calculated separately, but density dependence is measured based on total parasite density; thus \( j \) refers to all active VSAs including \( i \).

Each simulation is assumed to be an infection of a naïve individual (except where we examine re-infection) by a sporozoite that produces on the order of 50,000 genetically identical merozoites that all burst from the liver on day 0 expressing the same \( \textit{var} \) gene, though we relax this assumption later. The initial parasitemia corresponds to a parasite density of 0.01/μl in an adult with a 5.0 l blood volume, which is approximately 2,000 times less than the microscopic detection level of 20/μl. At each replication event a proportion of the replicating merozoites, 2% in the base simulation, switch to express an alternative VSA. Once an immune response has been initiated to a particular VSA it will be eliminated when the density is less than 0.0002/μl, though it can reappear later once the immune effectors have waned and other VSAs switch expression to this particular VSA. An infection ends when the immune system has completely eliminated all parasites, though we also determine the last day that parasites are visible at or above the
microscopic detection level. Parameter specifications for the full model can be found in Table 3.1.

Lastly, we ran each simulation until the parasite had been eliminated and all adaptive immune effectors had waned leaving only memory cells. We then initiated a second infection, but assumed an alternative switching order. Thus, the parasite expressed a different VSA at the outset of the infection than in the initial infection, and had a different switching order. This allowed us to determine if the parasite was able to re-infect individuals, as well as their capability in generating a second clinical infection.

<p>| Table 3.1: Parameter Values used in the Analysis |</p>
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\lambda$</td>
<td>18</td>
<td>Number of merozoites per merozoite (i.e. growth rate)</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>$10^{-4}$</td>
<td>The rate of removal of infected red blood cells per innate immune effector per day</td>
</tr>
<tr>
<td>$\delta$</td>
<td>1/10 days$^{-1}$</td>
<td>Death rate of immune effector</td>
</tr>
<tr>
<td>$\kappa$</td>
<td>$10^{-3}$</td>
<td>Rate of interaction between parasite and undifferentiated immune effector</td>
</tr>
<tr>
<td>$\mu$</td>
<td>0.01</td>
<td>Death rate of parasite in the spleen</td>
</tr>
<tr>
<td>$\epsilon$</td>
<td>0.05</td>
<td>Percent of immune cells that become memory cells</td>
</tr>
<tr>
<td>$\rho$</td>
<td>4</td>
<td>Immune cells produced per effector-parasite interaction</td>
</tr>
<tr>
<td>$\theta$</td>
<td>1/2 days$^{-1}$</td>
<td>Cytokine rate of decline</td>
</tr>
<tr>
<td>$\pi$</td>
<td>10000</td>
<td>Immune cells produced per memory cell-parasite interaction</td>
</tr>
<tr>
<td>$\omega$</td>
<td>10000</td>
<td>Up regulation of immune system per immune cell-parasite interaction</td>
</tr>
<tr>
<td>$D$</td>
<td>21</td>
<td>The e-folding distance – the exponential rate at which cross-protection wanes</td>
</tr>
<tr>
<td>bloodVol</td>
<td>5 Liters</td>
<td>Blood Volume</td>
</tr>
<tr>
<td>$K$</td>
<td>800,000 parasites/µl</td>
<td>Parasite carrying capacity</td>
</tr>
</tbody>
</table>
Figure 3.1: PfEMP1 Protein Similarity
Predicted protein sequences were obtained from PlasmoDB (http://www.plasmodb.org) for all 62 var genes in the 3D7 reference strain. The sequences were then aligned and an identity matrix calculated using clustalX (Gap Opening 10, Gap Extension 0.2). The resulting matrix is organized by var gene group, A, B/A, B, B/C, C and var2CSA. Group A var genes are the least similar to each other, and the most varied, which is consistent with their predicted extra-cellular domains. var2csa is also significantly dissimilar to other proteins as would be expected by its alternative binding patterns. Group B and C are more similar to each other than the other groups, though are slightly more similar within each group.
Results
The model was first calibrated with a parasite that was unable to vary its antigens to ensure that the dynamics mimic a typical immune response. After an initial exponential increase in the density of the parasite, the immune system controls the infection, eliminates the pathogen, and dispatches subsequent challenges relatively quickly (Figure 3.2). The model was also calibrated to ensure that the initial peak parasite load was consistent with experimental P. falciparum infections (Eyles and Young 1951).

We first demonstrate the basic mechanism by which cross-reactivity generates infection dynamics by examining a model with only three var genes. We simulated the model 60,000 times, assuming differing levels of cross-reactivity between each VSA. In the majority of simulations the overall infection length was nearly indistinguishable from a system with no cross-reactivity. However, we found that certain combinations of cross-reactivity generated infections that were more than four times longer than infections with no cross-reactivity (Figure 3.3). This result occurs when the binding between the immune effectors is mismatched so that the immune effectors for one VSA strongly bind only one of the other VSAs. For example, immune effectors for VSA1 and VSA2 are strongly cross-reactive and so are immune effectors for VSA2 and VSA3, but, immune effectors for VSA1 and VSA3 are not. Thus, when VSA1 is expressed by parasites at the outset of an infection, immune effectors can reduce parasites expressing VSA2, so a strong immune response against VSA2 isn’t generated, which in turn means that there is only a small impact of cross-reactivity on parasites expressing VSA3. However, as parasites switch to expressing VSA3 after VSA1 and VSA2 there are only a limited number of parasites expressing this VSA. This means that, while weak, the slight cross-reactivity keeps the parasite level low
enough that the immune system doesn’t generate a strong response against VSA3 allowing it to recrudesce and persist at low levels.

Expanding to the full repertoire of 62 VSAs, an infection with cross-reactive responses can structure the qualitative dynamics of the infection to be similar to the dynamics from experimental chronic infections (Eyles and Young 1951) (Figure 3.4 and Figure 3.A1 in the appendix). Without cross-reactivity, the infection is shorter and the density of the parasite stays nearly constant at a level near the initial max parasitemia throughout the course of the infection. Conversely, with cross-reactivity, the infection peaks and then drops significantly and stabilizes around the microscopic detection level before dying out, a result qualitatively similar to the dynamics observed in natural infections (Eyles and Young 1951). In addition, similar to the sequential wave-like pattern exhibited in natural infections (Handunnetti, Mendis et al. 1987; Brannan, Turner et al. 1994; Kaestli, Cortes et al. 2004) the peaks of the infection with cross-reactive responses are dominated by different VSAs (Figure 3.5).

Figure 3.2: Calibration of immune system dynamics for a non-varying pathogen.
To calibrate the immune response, a generic pathogen was introduced that had similar infection dynamics to malaria but did not possess the ability to vary its displayed antigens. After an initial inoculation, the immune system is able to control the parasite by ~day 14 and generates immunological memory. A second infection was introduced at day 400 to ensure that the immune system can control future infections. The parasite was introduced with the same initial density as the original infection, but the rapid proliferation of immune effectors (which proliferate from the memory cells remaining) kept the parasite from exceeding the limit of microscopic detection (which is approximately 20 parasites per μl), and rapidly eliminated the parasite.
The dynamics of an infection with only three antigenic variants generally lasted a very short period of time. However, in a small minority of cases antigenic variation increased the length of the infection significantly (A). The insets B & C show a single infection lasting approximately 120 days. In this example, VSA1 and VSA2 are closely related, thus the generation of a strong immune response to the initial VSA1, is also capable of mostly clearing VSA2. However, the presence of a third VSA that is only slightly cross-reactive with VSA1 but strongly cross-reactive with VSA2. This inhibits the immune system from mounting a memory response that is capable of eliminating VSA3 initially, resulting in an incomplete immune response. As the immune response begins to down-regulate with the collapse in parasite levels, the parasites expressing VSA3 are able to increase, with the result being a recrudescent infection.
Figure 3.4: Example parasite invasion
The dynamics of infection differ significantly between a parasite with no cross-reactivity (A) and with cross-reactivity between VSAs (B). Without cross-reactivity, the infection lasts ~200 days, but does not conform qualitatively with the expected dynamics of a long chronic malaria infection. On the other hand, cross-reactivity of VSAs generates an infection that is qualitatively similar in both the dynamics and duration to long-term chronic infections of malaria (Eyles and Young 1951).

Figure 3.5: The sequential dominance of different variant surface antigens
The densities of each VSA in the infection shown in the figure are color-coded and each peak is labeled with the dominant VSA. Each number corresponds to a particular var gene.
The specific dynamics produced by cross-reactive immune responses in the model depend on the order and mechanism of switching. To account for the stochasticity inherent in this process, we simulated 5,000 infections with randomly generated switch orders. The average infection length, measured as the last day the infection was above 20 parasites/μl, or the detectable limit of microscopic evaluation, which is how infections were analyzed in experimental infections of individuals (Eyles and Young 1951), was 282 days (SD: 71 days). While this was slightly longer than the approximate duration of 222 days (SD: 154) of infection measured in experimental infections of individuals (Eyles and Young 1951), we assumed, unlike the original experiments, that none of the infections were modified by drug treatment. Infections in the model were capable of persisting for a much longer period below detectable limits. The longest infection was approximately 5 years. Although atypical, reports of infections lasting for several years after leaving an endemic zone have occasionally been reported in the literature (Szmitko, Kohn et al. 2009).

To establish the primacy of cross-reactive responses in structuring the dynamics of a *P. falciparum* infection, we examined a number of different alternatives. First, we examined whether or not differential switch rates can structure similar infections by assuming that the parasite had variable switch rates and no cross-reactive responses. Switch rates were assumed to vary from 0.2% to as high as 16%, and were randomly generated. Over thousands of simulations, we found that while the infection dynamics fluctuated more than in the case with a constant switch rate, they still approximated the same dynamics as with no cross-reactivity (Figure 3.6a). In addition, we tried numerous variations, such as high switch rates for *var* A genes and low for *var* B and C
genes as well as vice versa, with the same general results in all cases. Second, as VSAs can have different adherence phenotypes and thus different rates of clearance, we examined if differences in clearance rate, rather than cross-reactivity, can structure an infection. The resulting dynamics could produce long infections, but not until after a prolonged period of high parasitemia that would likely be lethal (Figure 3.6b).

Lastly, we examined the mechanism of switching. In our primary model we assumed that each VSA switched to a single new VSA in an ordered pattern determined at the onset of the infection. We therefore varied this in two ways. First we assumed that each VSA switched to a single random VSA in each generation. In the absence of cross-reactivity the resulting infection was typically short and was quickly extinguished. Including cross-reactivity resulted in long infections similar to our primary model; however, the rate that parasitemia levels dropped was more precipitous compared to our initial model and the model tended to oscillate in a more regular manner (Figure 3.6c). Recent evidence suggests that rather than switching randomly, parasite antigens are switched in a structured manner from a group of dominant VSAs through intermediaries, finally resulting in a new dominant VSA (Recker, Buckee et al. 2011). Under these assumptions we found similar dynamics: without cross-reactivity the exposure of multiple VSAs quickly led to the parasite being eliminated, while cross-reactivity increased the longevity of the infection significantly. Notably, in both cases, the number of activated VSAs rose at a faster rate than in the primary model, with the majority of VSAs being turned on by day 10 and all of them turned on by day 14.
These results suggest that increasing the number of VSAs expressed at the onset of infection may determine how rapidly the parasite enters the chronic stage. To test this, we examined how the dynamics of an infection were structured when more than one VSA was expressed at a time, assuming that each expressed VSA started at the same initial concentration. We found that an increase in the number of expressed VSAs at the onset of infection significantly reduced the probability that an infection would be cut short due to a switch to an antigen that was too similar antigenically. While expressing a few different VSAs had no significant impact on the dynamics, as more VSAs were expressed the qualitative dynamics became less and less similar to experimental infections as the initial crash in the population became more and more significant and reduced the probability the infection would persist. The dynamics reached a threshold at approximately five VSAs expressed at infection onset, after which the infection never persisted. We also examined the dynamics when all VSAs were expressed initially at differing levels, and again the infection never persists in our model. This suggests that the initial VSA or a limited group of VSAs initially expressed by the parasite plays a key role in initiating a cascade of cross-immunity which structures the response of the immune system.

A secondary benefit for the parasite from an incomplete immune response is the ability to re-infect the same individual causing a new long chronic infection (Figure 3.6d). For each simulation, we allowed all adaptive immune effectors to wane leaving only memory cells, and then reinfected individuals with the same parasite, but with an alternative switching order. In this case we found that the parasite could re-infect individuals, but while infections could persist for a significant time period below microscopic detection levels, they never were capable of
generating a clinical infection. Thus, in line with field (Bruce, Donnelly et al. 2000) and experimental results (Collins and Jeffery 1999a), the immunity conferred from a primary infection does not prevent future infection, but rather reduces the probability of clinical symptoms, and shortens the length of new infections. This ability to re-invade suggests that incomplete immune responses due to cross-reactivity may allow specific var genes to persist in the population for long periods of time, a result consistent with other studies examining cross-immunity between genotypes (McKenzie and Bossert 2005).

Discussion
A major question in the biology of malaria infections is how the limited repertoire of VSAs (~60) in a single malaria parasite is capable of maintaining a chronic infection. We present a hybrid model that mixes continuous interaction between the parasite and the immune system with discrete replication events. The inclusion of both long and short-term immunity as well as antigenic switching and heterogeneous clearance rates provides a more robust framework than prior models of cross-reactive immune responses (Recker, Nee et al. 2004), for examining how the malaria parasite prolongs infection. From our results, we suggest that not only are cross-reactive immune responses central to prolonging infection, and that the resulting dynamics are qualitatively similar to experimental infections (Eyles and Young 1951), but that it is the initial immune response that is important for structuring the long chronic phase of the infection.

The generation of a strong immune response is a key element for eliminating a pathogen and for developing a memory of the pathogen. However, P. falciparum appears to take advantage of this strong immune response. High initial parasite levels, which result from replication in the liver,
induce the immune system to produce specific immune effectors against one or a few VSAs that are initially expressed. Our model suggests that this strategy is central to generating the cascade of sequential antigens. Because the initial response of the immune system is significant, as the parasite switches to express other VSAs, even weak cross-reactivity limits the growth of other VSAs. However, this response only controls but does not eliminate other VSAs, resulting in an incomplete immune response to these new VSAs. The infection is then able to recrudesce as the response to the first VSA wanes. Thus, while a strong, specific primary response may cause significant harm to the host, it may also provide a mechanism by which the parasite can persist chronically.

This result is not inconsistent with experimental infection data showing that most if not all VSAs are expressed by day 10-12 in an infection (Wang, Hermsen et al. 2009), as even low rates of switching per generation would expose all VSAs by about day 10-12. Our results suggest that if more than approximately five VSAs are expressed at once, the parasite is unable to take advantage of cross-immunity and the immune system is able to eliminate the infection after a short period. On the other hand, if only a single VSA, or at most a handful, are initially expressed, the immune system's response against the initial VSA(s) weakens the response against other cross-reactive VSAs. As the first subset of VSAs wane, other VSAs are then able to proliferate. This suggests that the parasite should maximize the long-shadow of cross-reactivity by switching to VSAs that are not too similar antigenically. In vitro data suggests that the parasite evades the host by always switching the location of expressed var genes either to var
genes on different chromosomes or to var genes located in central versus telomeric clusters on the same chromosome (Recker, Buckee et al. 2011).

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**Figure 3.6: Alternative methods of structuring P. falciparum infections**

A) Differential switch rates: While a mixture of slow and fast switch rates increases the fluctuations of the parasite levels, without cross-reactivity the dynamics are still not consistent with experimental infections. B) Differential clearance rates: Variance in patterns of adherence can result in differential clearance rates of parasites expressing different VSAs, however, the limited impact on the dynamics suggests that this is not a central mechanism in structuring the dynamics. C) Random switch patterns: Instead of an ordered progression, parasites may switch randomly in each generation to different VSAs. The dynamics in this case, with cross-reactivity, seem consistent with experimental infections, and similar to ordered switching. Thus, the model cannot discriminate between the best switching method. D) Reinfection: An infection with the same strain but with an alternative switching order can produce a chronic infection that only occasionally rises to the level of microscopic detection.
Other mechanisms have been proposed for how var genes generate chronicity. However, in our analysis we found that neither variation in the rate that different var genes switch on and off (Horrocks, Pinches et al. 2004) nor differences in the growth rate of different VSAs (Molineaux, Diebner et al. 2001) is likely to detract from the central role that cross-immune responses play in generating a chronic infection. This is not to suggest that differential adherence properties play no role in structuring the dynamics of an infection, as certain VSAs are clearly only successful in pregnant women (var2csa), while others have been associated with severe malaria (Jensen, Magistrado et al. 2004; Rottmann, Lavstsen et al. 2006), however, we found that without some cross-reactivity, the resulting dynamics do not conform to observed in vivo infection dynamics.

In addition to structuring the dynamics of the infection, we also found that cross-reactive responses may influence the rate that immunity to particular var genes is acquired over multiple infections. Because the proteins encoded by the Group A var genes tend to be less similar and have greater variability in how similar they are to each other than other groups, cross-reactivity does not dampen their growth as significantly. Thus, during the long chronic stage of the infection they tend to generate a larger number of parasites, which means a larger number of specific immune effectors. While in a single infection the initial VSA generates the largest response, in repeated infections, the higher parasitemia generated by the Group A var genes would generate stronger immune responses after fewer infections, a result consistent with field data (Cham, Turner et al. 2009). Though higher parasitemias would likely contribute to the perceived link between virulence and certain VSAs, other factors, such as differences in adherence, will likely play a larger role.
Lastly, the structured dynamics that result from cross-reactive responses suggest some factors about the ecology and epidemiology of the disease. Faster growth rates, which increase the probability of clinical symptoms, would generate a more robust immune response, and a longer cross-immune shadow that retards the formation of an immune response to other VSAs and allows the parasite to maintain a chronic infection longer. In addition, faster growth rates allow the parasite to switch expressed VSAs at a faster rate, which is useful in individuals already partially immune. Our model also suggests a reason why long chronic infections have been shown to be greatly lengthened by the presence of multiple infections (Nassir, Abdel-Muhsin et al. 2005): An increasing number of infections would result in a greater number of different VSAs being expressed at any particular time. Because antibodies are cross-reactive, they reduce the density of other VSAs, which reduces the rate that undifferentiated immune effectors encounter VSAs and slows the positive feedback loop of antibody creation. Thus, the generation of an immune response capable of eliminating each VSA takes longer to develop, and the overall length of the infection is increased.

While the results from our model are robust to a wide range of parameters, the model is only an abstraction of the complicated interaction between the parasite and the immune system. In particular, we assumed that the parasite replicated in synchrony every 48 hours and that each invasion produced an equal number of merozoites with each replication. Evidence suggests that synchronous replication is generally maintained until late in the infection (Bruce, Donnelly et al. 2000), and we feel that small deviations near the end of an infection would not significantly impact the described dynamics as by that point the infection is primarily regulated by VSA-
specific immune effectors, and mechanisms that slow growth (which mimic the role of the innate immune response) would, at most, play a very minor role. Thus, asynchrony would only impact the timing of the late peaks, but would not alter the fundamental interaction between the immune system and the parasite. In addition, our model is only an approximation of the human immune system, and we made a number of simplifications, chief among them that immune effectors are generated immediately after interacting with the parasite rather than after a delayed period. However, there is evidence that this type of model better approximates the exponential increase of immune effectors as opposed to a strict delay model, because the amount of interaction an undifferentiated immune effector has with a pathogen is important in determining the effector growth rate (Antía, Bergstrom et al. 2003).

We also make the simplifying assumption that cross-immunity is specifically related to similarities in protein sequences, despite the fact that protein sequence similarity is no guarantee that the proteins are structurally similar or that the immune response is likely to cross-react with these structures. However, we feel this is a good approximation as protein structure has played a significant part in how \textit{var} genes have been classified (Kraemer and Smith 2003; Lavstsen, Salanti et al. 2003; Kraemer, Kyes et al. 2007), and some functional differences have been observed between groups (Smith, Gamain et al. 2001; Bull, Berriman et al. 2005), suggesting that variant proteins are more similar within groups than across groups, and may be targeted similarly by the immune system. In addition, our analysis of sequence similarity generated patterns that would be expected based on the extracellular domain structure and the results were similar when we looked only at a single domain. While the model assumes that cross-reactive
responses are due to subdominant epitopes that were originally stimulated by an alternative VSA, but are more efficient at binding to a different VSA, the implementation of the model groups all VSA-specific immune effectors together. Thus, to account for the fact that these subdominant epitopes are binding more effectively, we assume that when they are stimulated, the positive feedback of the immune system makes them more effective at the new VSA and thus we group them there. The result is that a proportion of VSA-specific immune effectors are created through these cross-immune responses (Figure 3.A2 in the appendix). While this loses some of the complexity of subdominant epitopes, we feel that it best represents the way that cross-immunity functions in this simplified system. Lastly, our results were qualitatively robust to large variations in merozoite replication number, switching mechanisms and rates, e-folding distance (see methods), and immune system parameters, suggesting that the magnitude of cross-reactivity is not as important in generating chronicity, but rather that the system possesses cross-reactivity. This robustness would be important in effectively surviving and replicating within heterogeneous host environments.

Understanding how immunity to *P. falciparum* develops is crucial for progress in the quest for an effective vaccine. Models of the within-host dynamics of malaria infections have generally been unable to capture the complex dynamics of the interaction between host and parasite without relying on complicated assumptions about the biology of the parasite (Molineaux, Diebner et al. 2001; Paget-McNicol, Gatton et al. 2002). We believe our model, on the other hand, relies primarily upon the demonstrated role of cross-reactivity and is robust to large variations in the biological assumptions. The model also provides a good mathematical framework for
understanding how the malaria parasite generates long-term chronic infections with a limited antigenic repertoire. Significantly, we suggest that it is the expression of only one or a very limited number of antigens at the onset of an infection that drives the cascade of cross-immune responses required to establish a lasting infection. Finally, we find that the high initial parasite density, which can have severe consequences for the host, and is the main source of clinical symptoms, is an important modulator of the immune response that ultimately allows the parasite to maintain a productive infection.
Appendix

Table 3.A1

<table>
<thead>
<tr>
<th>%ID</th>
<th>Std Dev</th>
<th>Min</th>
<th>Max</th>
</tr>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Group A</td>
<td>29.3</td>
<td>23</td>
<td>6</td>
</tr>
<tr>
<td>Group B/A</td>
<td>49.3</td>
<td>3.7</td>
<td>6</td>
</tr>
<tr>
<td>Group B</td>
<td>38.8</td>
<td>5.1</td>
<td>6</td>
</tr>
<tr>
<td>Group B/C</td>
<td>32.9</td>
<td>11.2</td>
<td>6</td>
</tr>
<tr>
<td>Group C</td>
<td>37.4</td>
<td>8.4</td>
<td>6</td>
</tr>
<tr>
<td><strong>DBL/CIDR Domain 2</strong></td>
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<tr>
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<td>8</td>
</tr>
<tr>
<td>Group B/A</td>
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<td>6</td>
<td>8</td>
</tr>
<tr>
<td>Group B</td>
<td>35.4</td>
<td>8.5</td>
<td>8</td>
</tr>
<tr>
<td>Group B/C</td>
<td>37.8</td>
<td>8.3</td>
<td>8</td>
</tr>
<tr>
<td>Group C</td>
<td>45.2</td>
<td>9.5</td>
<td>8</td>
</tr>
<tr>
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<td></td>
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<td>Group B/A</td>
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<tr>
<td>Group B</td>
<td>38.8</td>
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<td>Group B/C</td>
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<tr>
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<td>16</td>
</tr>
<tr>
<td>Group C</td>
<td>44.9</td>
<td>4.8</td>
<td>16</td>
</tr>
</tbody>
</table>

Predicted protein sequences were obtained from PlasmoDB (http://www.plasmodb.org) for all 62 var genes in the 3D7 reference strain. To calculate the similarity of different domains, we obtained domain predictions from the VarDom 1.0 Server (Rask, Hansen et al. 2010). Predicted protein sequences of each domain were then aligned and an identity matrix calculated using clustalX2 (Larkin, Blackshields et al. 2007). We then calculated the average identity and standard deviation of each group.
Figure 3.A1: Comparison of representative infection dynamics from model with malariatherapy data

Archival data of malariatherapy—the purposeful infection of humans with \textit{P. falciparum} to treat neurosyphilis (Collins and Jeffery 1999b)—were averaged over each day to create a mean parasite density for each day (A). This represents the average dynamics of an infection (the grey area is the confidence interval around the average). Figures B–F are representative runs of the model. Source: \textit{Plasmodium falciparum} malaria therapy data of neurosyphilis patients in Georgia and South Carolina (as described in Collins and Jeffery 1999b). William E. Collins gave us permission to use the data and Klaus Dietz sent us the data.
Figure 3.A2: Generation of VSA-specific Immune Effectors

Immune effectors are generated from parasite interactions with undifferentiated as well as primed immune effectors. Cross-immunity is assumed to be due to subdominant epitopes that have a higher binding affinity for other VSAs than for the VSA for which they were initially activated. Through the feedback mechanisms of the immune system, we assume that this results in the generation of new immune effectors that are specific for the new VSA. This chart represents the immune effectors created through VSA-specific interactions (dark) and through cross-immune interactions (light).
Chapter 4

Superinfection and the Evolution of Resistance to Antimalarial Drugs

Abstract
A major issue in the control of malaria is the evolution of drug resistance. Ecological theory has demonstrated that pathogen superinfection and the resulting within-host competition influences the evolution of specific traits. Individuals infected with Plasmodium falciparum are consistently infected by multiple parasites, however, while this likely alters the dynamics of resistance evolution, there are few robust mathematical models examining this issue. We developed a general theory for modeling the evolution of resistance with host superinfection and examine: (1) the effect of transmission intensity on the rate of resistance evolution; (2) the importance of different biological costs of resistance; and (3) the best measure of the frequency of resistance. We find that within-host competition retards the ability and slows the rate at which drug-resistant parasites invade, particularly as the transmission rate increases. We also find that biological costs of resistance that reduce transmission are less important than reductions in the duration of drug-resistant infections. Lastly, we find that random sampling of the population for resistant parasites is likely to significantly underestimate the frequency of resistance. Considering superinfection in mathematical models of antimalarial drug resistance may thus be important for generating accurate predictions of interventions to contain resistance.

15 With David L. Smith, Ramanan Laxminarayan, Simon Levin
Introduction

More than a century ago, the Nobel prize-winning German scientist Paul Ehrlich predicted that pathogens subjected to drugs would evolve resistance (Ehrlich 1913). The conjecture has been proven true so often and in so many contexts that it can be regarded as Ehrlich’s Law. There is, nevertheless, uncertainty about virtually every other question of academic or public health interest—in particular, the role of superinfection, defined as the simultaneous infection with multiple strains of the same pathogen, and its influence on the evolution of drug resistance. Even though superinfection has been shown to alter the evolution of specific traits, particularly virulence (Levin and Pimentel 1981; Nowak and May 1994; van Baalen and Sabelis 1995), a robust theory of the effect of superinfection on the evolution of drug resistance is still lacking.

This is important in the study of malaria, since superinfection, and the concomitant increase in within-host competition, is likely to change the dynamics of resistance evolution and have ramifications for control efforts. Here, we extend some of the classic models in malaria to consider the consequences of within-host competition among drug-sensitive and drug-resistant Plasmodium falciparum malaria parasites, the species of malaria associated with the highest levels of morbidity and mortality.

Early studies of P. falciparum malaria suggested that high rates of exposure to malaria could result in simultaneous infection with multiple parasites, termed superinfection (MacDonald 1950a). This was not conclusively confirmed until the 1970s (McKenzie, Smith et al. 2008), but it is now known that P. falciparum malaria infections are typically composed of several genetically distinct lineages, called the multiplicity of infection (MOI), even in areas of low transmission (Paul, Hackford et al. 1998). Because of the complexity of the parasite’s life cycle, in which parasite
meiosis and recombination occurs in the mosquito, genetic and phenotypic variation between clonal \textit{P. falciparum} populations can be significant, which can impact their competitive ability.

Antimalarial drug resistance is encoded by mutations in, or changes to, the copy number of genes relating to the drug’s target or influx-efflux pumps that affect intraparasitic concentrations of the drug (White 2004). These changes, which are beneficial in the presence of drugs, also have a biological fitness cost to the parasite, lowering its competitiveness (Felger and Beck 2008; Babiker, Hastings et al. 2009). In the presence of therapy, the drug-resistant parasites have an advantage. Removal of the drug exposes the parasites to increased competition and can lead to a decline in the frequency of resistance-conferring mutations, as occurred in Malawi after the country stopped using chloroquine (Laufer, Thesing et al. 2006). However, in areas with lower transmission (and lower MOI and lower rates of competition), the frequency of resistance has declined at a slower rate (De-quan, Rui-jun et al. 1995). Thus, understanding how superinfection affected historical examples of resistance to the former first-line antimalarial drugs chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) is of great interest for understanding the factors that are likely to be important for the evolution of resistance to artemisinin-class drugs and to the artemisinin combination therapies (ACTs) that are now the first-line treatment for \textit{falciparum} malaria in much of the world.

The existing mathematical theory describing the evolution of resistance is poorly developed with respect to superinfection and within-host competition. Models of superinfection in other organisms (Levin and Pimentel 1981; Nowak and May 1994) either have assumed that a dominant strain could displace another pathogen and take over the host, but not vice versa, or
have not allowed for transmission from coinfected states (Levin 1983a; Levin 1983b). Neither of these approaches is biologically relevant in malaria, where superinfection is the rule rather than the exception, and evidence from the field (Daubersies, Sallenave-Sales et al. 1996; Arnot 1998) and murine models (Snounou, Jarra et al. 1989; Taylor, Walliker et al. 1997; De Roode, Read et al. 2003) has demonstrated the impact of within-host competition on the survival and transmissibility of genetically distinct malaria clones.

In malaria, the mathematical theory of superinfection was developed by MacDonald, Irwin, Dietz, and Bailey (Walton 1947a; MacDonald 1950a; Dietz, Molineaux et al. 1974; Fine 1975a; Bailey 1982; Dietz 1988b). Their models allow for superinfection, meaning that multiple genetically distinct clones coinfect a host, and were developed to aid in matching assumptions of the transmission rate with clearance rates. Although the models generally fit the data on prevalence better than the assumption that additional infections have no effect on the clearance rate (Dietz 1988b), epidemiological models of this type have not been applied to problems of within-host competition and drug resistance, where it is often assumed that individuals are infected by either drug-resistant or drug-sensitive parasites only (Aneke 2002; Klein, Smith et al. 2008; Esteva, Gumel et al. 2009).

In their model of antimalarial resistance, Koella and Antia (2003) assume that individuals can be superinfected by both drug-resistant and drug-sensitive infections (heterotypic) but not by two drug-resistant or drug-sensitive types (homotypic). They assume that individuals with heterotypic infections transmit both parasites at the same rate regardless of the composition of
drug-resistant and drug-sensitive parasites in the population. This significantly biases transmission, particularly when resistance is first emerging and drug-resistant parasites are rare. As has been shown in species coexistence models (Cohen 1970; Slatkin 1974), the only way that one species can exclude another is if there is significant competition in superinfected hosts, and in malaria, there is strong evidence that drug-resistant and drug-sensitive parasites compete in a host, and removing one will increase the fitness advantage of the other (Wargo, Huijben et al. 2007). Because the full costs of competition are not embedded in the model (i.e., individuals cannot be multiply infected by the same type of parasite), when the fitness cost of resistance is low (but not zero), the model predicts coexistence even when there is no treatment in the population (Koella and Antia 2003), a result that is not biologically plausible (See Appendix I).

In this paper, using a general formulation of malaria superinfection, we present a general theory for the evolution of resistance when superinfection occurs. Building on population genetics models for malaria (Hastings 1997; Hastings 2006; O’Meara, Smith et al. 2006) as well as past epidemiological models (Klein, Smith et al. 2008), the approach presented here allows for an examination of some of the basic questions involved in how competition affects the spread of drug resistance in different environments. Our model provides a new way of thinking about modeling antimalarial drug resistance, incorporating many of the concepts that are in common use in malaria epidemiology today (Smith, Battle et al. 2012b), and suggests ways to better understand the important control points and identify new directions for future research.
Methods

Superinfection model

To model the evolution of resistance with superinfection, we modified a Markov-Chain model for superinfection and clearance to include clinical malaria and antimalarial drugs; the original model was developed for malaria (Bailey 1982). Multiplicity of infection, the number of pathogens per host individual, increases as new infections occur, but decreases as they clear. The state variables in the model represent the fraction of the population that has a given MOI: $X_i$ denotes the fraction of hosts with an MOI of $i$. We have extended the model to track the MOI of sensitive and resistant types (Figure 4.1). Let $X_{i,j}$ denote the fraction of the population with $i$ sensitive and $j$ resistant strains. The equations are formulated so that $\sum_{i,j} X_{i,j} = 1$, and hence the sum of the time derivatives $\sum_{i,j} \dot{X}_{i,j} = 0$. Thus, the values of the state variables describe the joint distribution of resistant and sensitive phenotypes in a population.

Entomology

The dynamics of infection in the model follow similar notation to single-infection models by Macdonald (MacDonald 1957), as modified by Smith and McKenzie (Smith and McKenzie 2004). Vectorial capacity ($V$), the number of infectious bites by a mosquito over its lifetime, is given by the formula $V = ma^2e^{st}/g$, where $m$ denotes the number of mosquitoes per human and $a$ is the number of bites on humans per mosquito per day. The instantaneous death rate is $g$ ($e^{st}$ is the probability of a mosquito surviving one day), and $n$ is the number of days required for sporogony.
The daily entomological inoculation rate (EIR), the number of infectious bites per person per day, is calculated as the product of vectorial capacity and the fraction of mosquitoes that are infectious, \((P / (1 + aP / g))\), where \(P\) is the proportion of the bites on the infected human population that infect mosquitoes, assuming transmission efficiency \(c\) from humans to mosquitoes \((P = cX_{i,j}, i \neq 0 \text{ or } j \neq 0)\). The force of infection, or happenings rate \((b)\), is \(bEIR\), where \(b\), the infectivity rate, is the fraction of bites on humans that produce a patent infection.

**Competition and a biological cost of resistance**

The model ignores fluctuations in the abundance of parasites within a host, but competition is naturally incorporated into the model as a transmission bottleneck at the mosquito. In the absence of any biases, the probability of a mosquito transmitting either a resistant or a sensitive parasite is a function of the proportion of each type ingested (assuming there is no preference for selfing). We assume that resistant genotypes in each gamete in the mosquito are lower than the proportion of the resistant genotypes in the human infection; thus, the parameter \(\lambda\) weighs all the factors that could produce a biological cost of resistance in the dynamics of parasites from hepatocyte to gametocyte.

The number of genotypes that are transmitted by each infectious bite is limited both by the number of ookinetes that have contributed sporozoites, and by the number of liver-stage schizonts that arise from each infectious bite. The proportion of sporozoites in a mosquito’s salivary glands injected during a feeding event is typically small (Shute 1945; Rosenberg, Wirtz et al. 1990; Medica and Sinnis 2005), though the number of genetically distinct sporozoites injected is unclear (Druilhe, Daubersies et al. 1998). Because the bottlenecks inherent within the
system likely make the infection of an individual with a large number of genetically diverse sporozoites an uncommon event (Sinden and Billingsley 2001), we assume that each mosquito transmits the offspring of only one gamete, regardless of the MOI of the host that infected it.

Figure 4.1: The MOI model with sensitive and resistant types
The state variables describe the proportion of the population with a given MOI for both drug-sensitive and drug-resistant parasites, and the solid lines show changes that are the result of transmission and clearance. Antibiotic use (dashed lines) reduces the MOI of drug-sensitive parasites, but not drug-resistant ones. Biological costs of resistance are introduced as an increased propensity to clear or a reduced propensity to transmit drug-resistant types.
Because competition for transmission occurs only when a mosquito bites an individual with a mixed infection, the overall probability that an individual sporozoite in the next generation is sensitive ($\tilde{X}_w$) or resistant ($\tilde{X}_r$) can be represented as

\[
4.1a) \quad \tilde{X}_w = \sum_i \sum_j \frac{i^2 + \lambda ij}{(i + \lambda j)^2} X_{i,j}
\]

\[
4.1b) \quad \tilde{X}_r = \sum_i \sum_j \frac{(\lambda j)^2 + \lambda ij}{(i + \lambda j)^2} X_{i,j}
\]

where $\lambda < 1$ (see Appendix II for derivation). Thus, the frequency of resistance in new infections is $\tilde{X}_r / (\tilde{X}_w + \tilde{X}_r)$, and the force of infection for sensitive ($b_w$) and resistant ($b_r$) parasites is defined as

\[
4.2a) \quad b_w = b \left( \frac{\tilde{X}_w}{\tilde{X}_r + \tilde{X}_w} \right)
\]

\[
4.2b) \quad b_r = b \left( \frac{\tilde{X}_r}{\tilde{X}_r + \tilde{X}_w} \right)
\]

We also assume that resistant parasites face an additional cost in clearance. This is denoted by $q$, where $q_{ij} = [(i + j) / j]^\alpha$, and $\alpha > 0$. Thus, $\alpha$ describes the bias in the proportion of cleared parasites that are drug resistant, relative to the frequency of drug-resistant types among all types.
in a host. While numerous reports have presented evidence of fitness costs of resistance in malaria (De-quan, Rui-jun et al. 1995; Laufer, Thesing et al. 2006; Babiker, Hastings et al. 2009), there is no clear understanding of how these costs are paid (Babiker, Hastings et al. 2009). However, as the resistance alleles are seemingly lost at a much higher rate in high transmission areas relative to low transmission areas (De-quan, Rui-jun et al. 1995; Laufer, Thesing et al. 2006), we assume that within-host competition is the most important driver and thus drug-resistant parasites are assumed to have a biased clearance rate only when in competition with drug-sensitive parasites.

**Clinical infections and drug use**

Drug use is assumed to be associated with clinical symptoms (primarily fever), which develop at rate $\psi$. The rate that clinical symptoms arise is independent of MOI. A fraction, $\rho$, of symptomatic patients are assumed to use drugs and successfully clear all sensitive parasites. The drug usage rate is assumed to be constant over each simulation, but is varied among simulations as noted. Treatment of resistant parasites is assumed to be ineffective.

**Equations**

The dynamics of the state variables, $X_{i,j}$, which denotes the fraction of the population with $i$ sensitive and $j$ resistant strains, are thus described by the following set of coupled ordinary differential equations.

The equation describing the change in the proportion of uninfected hosts ($i = 0, j = 0$) is
4.3a) \[ \dot{X}_{0,0} = -bX_{0,0} + r_{1,0}X_{1,0} + r_{0,1}X_{0,1} + \sum_{i=1}^{N} \rho_{i}X_{i,0} \]

where \( r \) is the recovery rate of an infection consisting of \( i \) sensitive and \( j \) resistant strains, \( b \) is the force of infection, \( \psi \) is the rate that clinical symptoms develop and \( \rho \) is the fraction of those patients that are treated and clear the infection successfully.

For individuals infected only with drug-sensitive clones \((i > 0, j = 0)\),

4.3b) \[ \dot{X}_{i,0} = -bX_{i,0} - r_{i,0}X_{i,0} + r_{i+1,0}X_{i+1,0} + \rho_{i}X_{i-1,0} + \rho_{i}X_{i,0} - \rho_{i}X_{i,0} \]

where \( q \) is the reduction in the duration of infection due to the biological cost of resistance, and \( b_{w} \) is the force of infection for drug-sensitive parasites.

For individuals infected only with drug-resistant clones \((i = 0, j > 0)\),

4.3c) \[ \dot{X}_{0,j} = -bX_{0,j} - r_{0,j}X_{0,j} + r_{0,j+1}X_{0,j+1} + \rho_{j}X_{0,j-1} + \rho_{j}X_{0,j} + \sum_{i=1}^{N} \rho_{i}X_{i,j} \]

where \( h_{r} \) is the force of infection for drug-resistant parasites.

For individuals infected with both drug-sensitive and drug-resistant clones \((i > 0, j > 0)\),
The equations are constrained such that the model has a “triangular” formulation (as in Figure 4.1).

**Triangularity and the neutrality condition**

The model presupposes coexistence in the parasite population of a large number of distinct genotypes, but it considers competition solely between drug-sensitive and drug-resistant parasites, which differ both genetically and phenotypically. To describe the effect of competition on the evolution of resistance, it was necessary to establish that the model satisfied general principles of ecological and population genetic neutrality as described by Lipsitch, Colijn et al. (2009). These principles note that models of competition between genotypically different, but phenotypically similar strains, should meet two criteria: (1) the relative fraction of infected and uninfected hosts should not depend on the frequency of either strain; and (2) the relative frequency of both strains should remain stable for all time greater than zero. In other words, the structure of the model should not, in and of itself, generate coexistence of indistinguishable strains, but that mechanisms that could induce coexistence should be introduced explicitly. These principles suggest that the prior superinfection model published by Koella and Antia (2003) needs modification because, as formulated, the model structure itself promotes coexistence. In Appendix I we describe a model with similar properties to the Koella and Antia superinfection model, in which homotypic superinfection was not allowed, and rigorously demonstrate that coexistence is always an outcome of the model, even when the resistant strain has a fitness cost.
To ensure that coexistence in our model of superinfection, described above, is not an artifact of the model formulation, we analyzed a special case of the general form of the model, in which the maximum MOI was two. In this case, the model allows for both heterotypic superinfection as well as homotypic superinfection. Numerical simulations confirm that in the absence of drug treatment, drug-resistant parasites can neither invade nor persist when they have a cost of resistance. We also found that in the absence of either drug pressure or a biological cost of resistance, the model is neutrally stable, provided the transmission rate and the recovery rate of each type in a heterotypic superinfection is exactly equal to each type in a homotypic superinfection. In other words, the rate that individuals become doubly infected with either the same type or a different type must be equal, and the transmission rate of heterotypic and homotypic infections must be the same regardless of strain composition, with an equal rate of transmission of each type from heterotypic infections. Thus, the model in this form does not predict coexistence when there is no specific mechanism promoting its spread.

Both conditions remain true as MOI increases, provided that the model structure maintains a triangular formulation in which every possible combination of the maximum MOI is included (i.e., in a model with a maximum MOI of three, all possible states where the MOI is equal to three must be possible: \(X_{3,0}\) and \(X_{2,1}\) and \(X_{1,2}\) and \(X_{0,3}\)). Otherwise, the model structurally creates a niche that allows for coexistence. All our numerical simulations were done using a general form of the model with the triangularity condition in place and in which the maximum MOI was 30. Parameters used in simulations were consistent with malaria epidemiological literature (Garrett-Jones 1964; Smith and McKenzie 2004; Smith, Battle et al. 2012b), and are listed in Table 4.1.
**Frequency of resistance**

We employ two measures of the frequency of resistance in complex infections: (1) the fraction of the population that is infected by at least one resistant strain \( 1 - \sum_{i} X_{i,0} \); and (2) the fraction of the parasite load that is resistant \( \sum_{i,j} j / (i + j) X_{i,j} \). The former is important because it tracks nearly exactly with the frequency that a treated clinical infection is resistant, which is the measure by which resistance is often tracked in a population (Bloland, Kazembe et al. 1998; Hastings, Nsanzabana et al. 2010). On the other hand, the latter is particularly useful when discussing the role that asymptomatic infections play in the spread of resistance.

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<th>Table 4.1: Parameters used in the model</th>
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Results
The model is based on the ideas of malaria superinfection as first laid out by Walton (Walton 1947a) and Macdonald (MacDonald 1950a) and written down in equation form by Bailey (1982). However, unlike previous models of drug resistance in malaria (Koella and Antia 2003; Klein, Smith et al. 2008) we explicitly model the effect of within-host competition between drug-resistant and drug-sensitive parasites. This allows for an examination of the importance of different types of fitness costs as well as how competition affects the rate at which resistance spreads across differential transmission rates.

To evaluate the model, we ran the system to equilibrium without resistance and then introduced resistance at low frequency ($10^{-7}$). At low transmission rates (an annual EIR of approximately one or less), the model predicts that drug-resistant parasites will invade and spread and eventually dominate the drug-sensitive parasites. However, as the transmission rate increases, the ability of drug-resistant parasites to competitively exclude drug-sensitive parasites decreases (Figure 4.2). This is because the mean MOI increases with the intensity of transmission, measured in terms of either vectorial capacity or EIR, and so competition increases. Thus, at low transmission rates, most individuals are infected with approximately one infection only, and the influence of within-host competition is limited. As the mean MOI increases, it becomes more difficult for resistant parasites to invade because competition within the host increases, overcoming the ability of resistant parasites to spread when they are rare. This is consistent with historical suggestions that resistance to the former first-line malaria drugs, CQ and SP,
Increases in transmission intensity reduce the competitive advantage of the resistant parasite because the mean MOI increases and resistant parasites must compete more within the host than between hosts. Increasing the drug treatment rate increases the competitive advantage of the drug-resistant parasite. All cases assumed that both the fitness cost of transmission and the fitness cost of clearance were equal to 20%.

both emerged from areas of low or unstable transmission (White 1998; White and Pongtavornpinyo 2003). Changing the drug treatment rate for any particular transmission level increases the competitive ability of resistant parasites allowing them to invade even when within-host competition increases at higher transmission levels.

The model also predicts that superinfection modulates coexistence. This result depends on the biological fitness costs as well as the treatment rate. At high treatment rates (80% of clinical infections treated), the drug-resistant parasite competitively excludes the drug-sensitive parasite at all fitness costs at low transmission. As the transmission rate increases (annual EIR ~7), coexistence can occur over a large range of fitness costs—even where the fitness cost of clearance
is zero (Figure 4.3). At higher transmission rates (annual EIR ~25), the range over which coexistence is possible contracts. Lowering the treatment rate (20% of clinical cases treated) shifts the results and allows for coexistence at lower transmission rates, and shrinks the parameter range over which coexistence can occur at higher transmission rates (Figure 4.4).

**Figure 4.3: Fitness costs and coexistence**

The cost of resistance can occur through either transmission or clearance. The transmission cost is measured as the relative difference in the contribution of resistant and sensitive parasites to transmission when in equal abundance. The fitness cost of clearance is measured as the reduction in the rate of clearance of a resistant parasite relative to a sensitive parasite when in competition. Thus, when there is little or no cost of clearance but a significant cost of transmission, the result is coexistence. As the transmission rate is increased (B), the parameter space over which coexistence can occur is abrogated. In addition, there are significant differences in the range of each parameter over which coexistence can occur. Areas of coexistence are marked by low to moderate fitness costs of clearance and high costs of transmission. In fact, in low-transmission areas, resistant parasites can coexist with sensitive parasites even when there is no fitness cost of clearance. Moderate transmission is defined as an annual EIR ~7 and high transmission is an annual EIR ~25. The treatment rate was assumed to be 80%.
Figure 4.4: Fitness costs and coexistence at low treatment rates
Parameter range where coexistence can occur is affected by the treatment rate. Whereas drug-resistant parasites completely exclude drug-sensitive parasites at low transmission rates when the treatment rate is high (Figure 4.3), decreasing the treatment significantly (in this case to 20%) allows for coexistence at low transmission rates, but the parameter range over which coexistence can occur shrinks significantly at higher transmission rates.

The fitness of drug-resistant parasites is proportional to both the average duration of an infection and the ability to transmit. The former is a function only of within-host competition, whereas the latter is a function of both within-host competition and among-host competition, since transmission potential is a function of both transmissibility to a susceptible vector (a function of competition between clones within the host) and the propensity for infectious vectors to infect a new host (the relative fraction of each type). Results from the model suggest that resistant parasites can invade even with significant fitness costs of transmission, but not when the fitness cost of clearance increases significantly. This suggests that the ability to remain competitively infectious within hosts is a stronger determinant of the invasion capacity of drug-resistant parasites than their probability of transmission at any single event.
Competition also affects the rate that resistance spreads in a population. At low transmission rates, increasing the transmission intensity increases the rate that resistance spreads in a population because, as noted above, most infected individuals have an MOI of one and there is no competition. Thus, the ability of drug-resistant parasites to competitively exclude drug-sensitive parasites increases as transmission increases. However, as the average MOI increases and competition begins to inhibit the ability of the drug-resistant parasite to exclude drug-sensitive parasites, the rate at which resistance spreads in a population decreases—or stated another way, the waiting time for resistance to reach a certain threshold value grows longer (Figure 4.5). This change is rapid at first, but as the transmission rate continues to increase, the rate of increase slows.

Superinfection also affects how the frequency of resistance is calculated. Although the prevalence of resistance (the fraction of infected people that harbor at least one resistant parasite) increases concomitantly with the fraction of the population that harbors at least one resistant parasite, the resistance load (the proportion of types that are resistant) changes at a different rate (Figure 4.6). As the transmission intensity increases, the resistance load increases at a rate that is similar to the other measures. However, the rate of increase slows after a point and then falls, while the prevalence of resistance continues to increase. At high transmission levels this spread can be more than five percentage points different when the fraction of clinical infections reaches 10%.
Figure 4.5: Rate of resistance spread as a function of transmission

Each line measures the waiting time until the percentage of clinical infections that contain drug-resistant parasites (and thus fail treatment) reach a predefined threshold level (1%, 5%, 10%, 20%, and 50%). As vectorial capacity increases, so does the mean MOI and the degree of competition, which slows the rate at which drug-resistant parasites spread and delays the time until each threshold is reached. Discontinuities are due to the fact that the model is continuous, but we are calculating the time in discrete intervals.

Figure 4.6: Measuring the frequency of resistance

There are multiple ways to calculate the frequency of resistance in a population: (1) the prevalence of resistance (the fraction of individuals infected with a resistant parasite); (2) the fraction of clinical infections that fail treatment; and (3) the resistance load (the proportion of the parasite population that is resistant). Depending on the measurement, the frequency of resistance could be wildly different. To measure these differences, we calculated the resistance load at the point in time when the percentage of clinical infections that fail drug treatment reached 10%. Although both measures increase nearly concurrently at low transmission rates, as transmission increases (and thus so does competition), the fraction of the parasite population that is resistant soon peaks and then begins to fall, even as the proportion of clinical infections harboring resistant parasites increases.
Discussion

A prior model of superinfection in malaria with drug-resistant and drug-sensitive parasites assumes that heterotypic superinfection is possible but homotypic superinfection is not (Koella and Antia 2003). However, this assumption means that coexistence will always occur (see Appendix I). The structure of this prior model (Koella and Antia 2003) is also similar to earlier models of species coexistence (Cohen 1970; Slatkin 1974), which always predict coexistence unless there is some type of competition in individuals heterotypically superinfected. Thus, coexistence is an artifact of the mathematical model, not a generic property of the underlying biological process.

In this paper we replace those assumptions with a model that implements a more robust competition framework that allows for a thorough examination of the effect of competition on the spread of drug-resistant malaria parasites in an epidemiological context. We found general conditions for the evolution of resistance as the outcome of within- and among-host competition between two classes of parasite types with differing fitness; the conditions depend on competition, drug pressure, and the biological cost of resistance.

Results from a general model demonstrate that the estimate of disease prevalence as a function of the transmission rate was qualitatively similar to that found in other models (MacDonald 1950a) and similar to estimates of the same relationship in the field (Smith, Dushoff et al. 2005; Sama, Owusu-Agyei et al. 2006; Smith and Hay 2009). Our results also support earlier studies showing a strong relationship between the fitness cost of resistance and transmission (Hastings 1997; Klein, Smith et al. 2008). When the fitness cost of resistance is low, drug-resistant parasites are
able to invade and spread across all transmission levels. However, as the fitness cost of resistance increases, the ability of drug-resistant parasites to invade and spread is reduced. One possible reason previously suggested for this relationship is that the parasite is exposed to a higher level of drug pressure per infection in low-transmission areas because a higher fraction of infections in these areas result in clinical symptoms (Hastings 1997). Our results suggest that the low force of infection, when recolonization of infected individuals is rare, plays a significant role in low-transmission areas as well. After drug use eliminates drug-sensitive parasites, individuals harboring resistant parasites are less likely to be recolonized by drug-sensitive parasites, reducing within-host competition.

Although a biological cost of resistance has been measured in in vitro experiments (Peters, Chen et al. 2002; Hayward, Saliba et al. 2005) and estimated from the field (De-quan, Rui-jun et al. 1995; Laufer, Thesing et al. 2006), reductions in the competitive ability of the parasite are not expected to be equal across different axes of competition. This has significant biological and epidemiological importance. Based on in vitro experiments in which CQ resistant parasites had an estimated 25% loss of fitness per generation (Hayward, Saliba et al. 2005) as well as murine models demonstrating slower growth of drug-resistant parasites in the mouse (Chawira, Warhurst et al. 1986; Walliker, Hunt et al. 2005), it has been suggested that mutations conferring resistance decrease the reproductive efficiency of the parasite and slow growth (Babiker, Hastings et al. 2009). The end result of the lower growth rate is assumed to be a decrease in the probability of a parasite being transmitted when a mosquito feeds on blood because of the lower relative numbers of gametocytes. A similar mechanism is also presumed to
affect the duration of infection; however, evidence on the relative cost of resistance on clearance is lacking, particular in relation to transmissibility. We found that although the reductions in transmission probability were important, the ability to persist in an infection may be a more important barometer of the resistant parasite’s competitive ability, particularly in lower-transmission settings. These results can be partly explained by the vast time-scale differences between these processes. Because the time from infection of a susceptible mosquito to infection of a human is an order of magnitude faster than the duration of infection, the benefit of increasing the duration of infection is significantly greater than the benefit of increasing infectiousness within an infection. The result is that resistant parasites can invade even with significant fitness costs of transmission, but not when the fitness cost of clearance increases significantly.

Because the parasite has evolved an extremely long duration of infection to maximize transmission opportunities and can transmit efficiently at very low densities (Barnes and White 2005), it is not surprising that the effect of changes in the clearance rate is more pronounced on the viability of drug-resistant parasites than on reductions in transmission. However, the effect on attempts to control the spread of resistance is important. Recent evidence has shown the emergence of a delayed clearance phenotype to the drug artemisinin in western Cambodia (Dondorp, Yeung et al. 2010), which has led to calls for implementing a resistance containment strategy. Our results suggest that interventions that can shorten the duration of infection for resistant parasites, such as mass drug administration or mass screening and treating, may be more
beneficial than has been recognized by earlier models that ignored superinfection (Pongtavornpinyo, Yeung et al. 2008).

Within-host competition can also produce coexistence of the two different parasite phenotypes at the population level. Coexistence can occur when the relative advantage, measured as transmission potential over recovery rate, of the sensitive parasite is greater in mixed infections. However, the parameter range over which coexistence can occur is altered by the transmission rate. At low transmission rates, competition is lessened both between different phenotypes as well as with the same phenotypes. Individuals with drug-resistant parasites can then be easily reinfected, because they have only a few parasites. Thus, if drug-sensitive parasites have an advantage in mixed infections, the result for a fixed treatment rate will be a greater tendency to coexist. On the other hand, as the transmission rate increases, individuals who are treated with drugs are likely to harbor multiple drug-resistant parasite clones. In this case, the invasion capability of drug-sensitive parasites is reduced, making the range over which coexistence can occur smaller.

In terms of designing control strategies, the time until resistance reaches a critical level, which is critically influenced by the transmission and treatment rates, may be a more important measure than the final equilibrium level of resistance. Currently, there is no standardized methodology for assessing the frequency of resistance, though it is generally measured as the proportion of a type specimen isolated from a clinical infection that did not adequately respond to treatment (Bloland, Kazembe et al. 1998). However, screening surveys of the human population using in
vitro tests to determine resistance of a type specimen has also been suggested as a means to ascertain the frequency of resistance in the population (Picot, Olliaro et al. 2009). These sampling mechanisms are not identical when individuals have complex infections, and it is important to understand how these sampling differences may end up measuring radically different quantities. In high-transmission areas, when the frequency of resistance is low, complex infections will primarily consist of drug-sensitive parasites. Because sampling is imperfect, the probability of false negatives (i.e., at least one resistant parasite is present but was not detected) is high (Hastings, Nsanzabana et al. 2010), which suggests that random screening surveys as a measure of resistance in higher-transmission areas may result in biased results. This is likely also true when withdrawing a drug. For instance, even though the detection of chloroquine-resistant mutations has dropped to undetectable levels in Malawi (Laufer, Thesing et al. 2006; Laufer, Takala-Harrison et al. 2010), the complexity of malaria infections suggests that drug-resistant parasites may still be present at low frequencies and that the reintroduction of chloroquine may be followed by a rapid resurgence in resistant infections.

In this model of malaria superinfection, which was extended to incorporate competition between drug-sensitive and drug-resistant parasites, our results are limited to examining only the axis of competition between drug-resistant and drug-sensitive phenotypes. The evolution of antimalarial drug resistance is obviously a complicated process involving superinfection and many interacting processes, including immunity (Klein, Smith et al. 2008), the patterns of drug use, and heterogeneous biting (Smith, Dushoff et al. 2005). The results of this simple model clearly demonstrate that within-host competition is a significant component in the emergence and
spread of drug resistance even at lower transmission rates. Models that examine the best ways to control or contain the emergence of resistance must take account of superinfection and a variable degree of within-host competition across the spectrum of transmission. In particular, the assumption of a blanket fitness cost of resistance is less reasonable when the duration of infection dominates the probability that resistance will spread. Future studies in this area will include how heterogeneous biting by the mosquito vector changes the dynamics of competition, particularly in low-transmission areas, and how host immunity interacts with virulence to change the dynamics of the emergence of drug-resistance.
Appendix I

Superinfection and competition in an SIS model

Models of superinfection based on SIR models in the tradition of Kermack and McKendrick (1927) have generally assumed that superinfection meant one strain could infect and take over the host of another, but not vice versa (Levin and Pimentel 1981; Nowak and May 1994). However, not all infections are characterized by one phenotype’s immediately “taking over” a host already infected with an alternative strain, and this is especially true in malaria, where individuals are often coinfected by multiple genetically distinct clones at any one time. Although models have been developed to look at how multiple infections affect prevalence (MacDonald 1950a; Bailey 1982; Dietz 1988b), they have not been used to look at how phenotypic competition affects the population structure. The lone exception is a model by Koella and Antia (2003), who modified a simple SIR model for malaria to include different phenotypes (resistant to drugs and sensitive to drugs) and superinfection in a mixed class. In their model for superinfection, they find the surprising result that resistant parasites can persist in the absence of drug treatment even if they have a biological cost of resistance. This, as has been pointed out, makes little biological sense in a population that is well mixed (as is the case in an SIR model).

To understand why this type of model is inherently problematical, we consider a simple SIS model of superinfection, where $S$ is the population of susceptible individuals, $I_w$ and $I_r$ are the populations of infected individuals with either the wild type or the resistant strain, respectively, and $I_m$ is the population infected with both (Figure 4.A1).

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In this generalized model, like Koella and Antia (2003) we assume that there are only two strains, and the strains differ in their phenotypic response to drug treatment: the resistant strain is unaffected by drug treatment, but the sensitive strain is eliminated by drug treatment. It is also further assumed that the resistant strain suffers a fitness cost of resistance that can alter its ability to transmit or the length of time an individual is infected, or both. Because the dynamics of the mosquito population are encapsulated in a single term describing the force of infection as proportional to the infected population, we simplified this to make the results more tractable and defined the transmission parameter as $\beta$, which determines the rate that contact between different individuals results in infection. To incorporate the differential fitness on transmission, we assume that $\beta_r < \beta_w$, where the subscript $r$ stands for resistance and $w$ for wild type or sensitive. We further assume that individuals clear an infection at rate $\gamma$, and that $\gamma_r < \gamma_w$; that is, individuals clear resistant infections faster than they clear sensitive infections. Lastly, like Koella and Antia (2003), we also assume that individuals who have mixed infections can transmit both phenotypes.
In this formulation there are four states: susceptible (S); infected with the wild-type parasite only ($I_w$); infected with a resistant strain of the parasite ($I_r$); and infected with both strains ($I_m$).

Because we are interested in drug resistance, we assume that infected individuals develop clinical symptoms at rate $\psi$, and a proportion $\rho$ of those clinical infections are treated with drugs. Drug use is assumed not to have any effect on individuals infected with drug-resistant infections. The equations for the model are

4.A1a) $\dot{S} = -S \left( \beta_w (I_w + I_m) + \beta_r (I_r + I_m) \right) + I_w (\gamma_w + \psi \rho) + I_r \gamma_r$

4.A1b) $I_w = \beta_w S (I_w + I_m) - I_w \left( \beta_r (I_r + I_m) + \gamma_w + \psi \rho \right) + I_m \gamma_r$

4.A1c) $I_r = \beta_r S (I_r + I_m) - I_r \left( \beta_w (I_w + I_m) + \gamma_r \right) + I_m (\gamma_w + \psi \rho)$

4.A1d) $I_m = I_w \beta_w (I_r + I_m) + I_r \beta_w (I_w + I_m) - I_m (\gamma_w + \gamma_r + \psi \rho)$
For this model there are potentially four equilibria: (1) the disease-free equilibrium; (2) an endemic equilibrium with only wild-type infections; (3) an endemic equilibrium with only resistant infections; and (4) a coexistence equilibrium. Because we are interested in the invasion ability of the resistant parasite in an environment dominated by sensitive parasites, we focus solely on the endemic equilibrium with only wild-type infections (E$_2$), for which it is shown easily that $S^* = \frac{\gamma_w}{\beta_w}$, $I_w^* = 1 - S^*$, $I_r^* = 0$ and $I_m^* = 0$. To most clearly demonstrate the inherent problems with this model, we examined the model when there is no drug treatment ($\rho = 0$), i.e. where there is no factor to promote the invasion of resistant parasites. The stability of E$_2$ can be determined by calculating the eigenvalues of the Jacobian matrix of this system. The linearization matrix at E$_2$ is of the form

$$J = \begin{pmatrix} J_1 & J_3 \\ 0 & J_2 \end{pmatrix}$$

where the eigenvalues of $J_1$ have a negative real part by the assumption that the sensitive equilibrium is stable in the absence of the resistant, and where $J_2$ is a diagonal matrix with eigenvalues ($\beta_r - \gamma_r$) and ($-\gamma_r - \beta_w$). Thus, as long as the reproductive number of the resistant parasite is greater than one (i.e., $\beta_r > \gamma_r$), the equilibrium point E$_1$ is not stable, even if the resistant parasite faces a fitness cost of resistance ($\beta_w > \beta_r > 0$ and $\gamma_w > \gamma_r > 0$). In other words, no matter what the fitness cost of the resistant parasite, it is able to invade even without an explicit mechanism (i.e., drug treatment) promoting its spread.
Thus, both this model and previous models of superinfection (2003) are incapable of generating a biologically correct result. The main problem is related to how the parasites interact in the mixed class. As has been shown in species coexistence models (Cohen 1970; Slatkin 1974), the only way that one species can exclude another in this type of model is if there is significant competition in the mixed class. In the case of the above system, if an individual is infected with a sensitive parasite, the sensitive parasite can neither prevent the individual from becoming infected, nor does it change the clearance rate of the resistant parasite. As was shown in the multiple species model, increasing these effects can result in the exclusion of one or the other. In malaria, there is strong evidence that parasites compete in a host, and removing one will increase the fitness advantage of the other (Wargo, Huijben et al. 2007).

The results of the above model can be extended to malaria models, and a similar formulation can be written down. Because in most malaria models that follow the Ross-MacDonald format, the dynamics of the mosquitoes are encapsulated in a single term describing the force of infection as proportional to the infected population, the results (not shown) are identical under similar assumptions.

**Fixing the problem**
The problem described above can be fixed by including all the possible combinations of states with an MOI of two. In other words, compared with the model described in Figure 4.A1, there are two additional states that describe homotypic superinfection (Figure 4.A2). This is, in fact, a special case of the general model described in the text with an MOI of two. Numerical simulations confirm that in the absence of drug treatment, the parasite is incapable of invading
or persisting when it has a fitness cost. In addition, numerical simulations also show that similar to other models of multistrain pathogens (Lipsitch, Colijn et al. 2009), neutral stability in this model is possible in the absence of drug treatment if the rules describing net clearance and net transmission from heterotypic superinfection (i.e., \( I_{r,w} \)) are equal to the homotypic superinfections (i.e., \( I_{w,w} \) or \( I_{r,r} \)) and symmetric (i.e., the rates for the resistant and the wild types are equal).

Figure 4.A2: Fixing the simple SIS model of superinfection with drug resistance
This formulation is similar to the model in Figure 4.A1 but allows for homotypic infection in addition to heterotypic infection. Thus, in addition to the four states as in the prior formulation, there are two new states: infected with two wild-type strains (\( I_{w,w} \)); and infected with two resistant strains (\( I_{r,r} \)). The inclusion of the two homotypic states allows for “neutral stability” of two indistinguishable strains and ensures that resistance cannot spread without a mechanism promoting it. This formulation is a special case of the more general model described in the text.
Appendix II

Biological cost of resistance

When a mosquito bites a human, it picks up gametocytes, the sexual stage of the parasite. In the mosquito these gametocytes differentiate into gametes and then unite to form a zygote, meiosis occurs, and the next generation of the haploid parasite, the sporozoite, is formed through mitosis from the four haploid daughter cells. If $i$ is the proportion of sensitive gametes and $j$ the proportion of resistant gametes in an infection, then the probability that a zygote will be sensitive, resistant, or mixed is just a function of the proportion of each that a mosquito ingests (assuming no hybridization or propensity for selfing):

Proportion sensitive: $\left( \frac{i}{i+j} \right)^2$

Proportion resistant: $\left( \frac{j}{i+j} \right)^2$

Proportion mixed: $\left( \frac{2ij}{i+j} \right)$

If we assume that the resistant parasites suffer from some biological cost of resistance ($\lambda$), then the proportions become

Proportion sensitive: $\left( \frac{i}{i+\lambda j} \right)^2$

Proportion resistant: $\left( \frac{\lambda j}{i+\lambda j} \right)^2$
Proportion mixed: \( \frac{2i\lambda j}{i + \lambda j} \)

Thus, the probability that any single sporozoite from an infection is either resistant or sensitive is

Proportion sensitive: \( \frac{i^2 + \lambda ij}{(i + \lambda j)^2} \)

Proportion resistant: \( \frac{(\lambda j)^2 + \lambda ij}{(i + \lambda j)^2} \)

To expand to the population, we multiply the proportion of each type of infection by the probability that each is either resistant or sensitive and then sum over all possible infection levels. This analysis assumes that the only factor affecting the transmission of resistant and sensitive parasites is defined by the parameter \( \lambda \). Other factors, such as selfing rates or heterogeneous biting, may skew the proportion of resistance from one generation to the next, but overall we assume the population remains at Hardy-Weinberg equilibrium except for factors assumed in \( \lambda \).
Chapter 5

Individual-Based Model of Malaria Transmission

Abstract
Despite the important insights gained by extending the classical models of malaria, other factors, such as immunity, heterogeneous biting, and differential patterns of drug use have not been fully explored due to the complexity of modeling superinfection. In this chapter an individual based model of malaria is introduced that enables a more robust analysis of these issues. Structured to be a stochastic analogue to classical Ross-Macdonald type models, the model is nonetheless based on individuals, and thus aspects of within-host competition can be explored. Importantly, because the model is analogous to the classical models, which have been well-described and analyzed, it is possible to determine how small changes in the assumptions of the model affect the dynamics of the disease. This model is specifically used to examine how competition between drug-resistant and drug-sensitive parasites is impacted by heterogeneous biting.
**Introduction**

Antimicrobial chemotherapies have substantially reduced the burden of numerous infectious diseases and changed the face of medicine since they first became widely available. However, the evolution of drug resistance threatens the efficacy of these drugs and by extension portends a situation in which individuals suffer severe morbidity and mortality from common infections now easily treated. Malaria therapy, for instance, has undergone this type of transformation twice when the first-line drugs chloroquine (Payne 1987) and then sulphadoxine–pyrimethamine (Nair, Williams et al. 2003; Roper, Pearce et al. 2003) failed, which significantly undermined the ability to control the disease (Wongsrichanalai, Pickard et al. 2002) and increased the morbidity and mortality of the disease (Trape, Pison et al. 1998a; Snow, Trape et al. 2001).

While the evolution of drug resistance is the result of a two-step process of mutation and selection, it is also the start of competition between drug-sensitive and drug-resistant parasites. This competition occurs both between hosts (Klein, Smith et al. 2008) and within hosts (Hastings 1997). Most theoretical studies of drug resistance evolution focus primarily on between-host competition and ignore the role of within-host competition. While this is done primarily for pragmatic reasons, i.e. it is complicated to develop and analyze a model in which individuals can be infected by multiple pathogens, within-host competition can dramatically impact the dynamics of the disease and can have impacts on the evolution of resistance (Alizon and van Baalen 2005; Klein, Smith et al. Submitted).

Theoretical analysis of the evolutionary implications of multiply-infected hosts has generally been confined to implications for virulence evolution in single pathogen multi-strain models.
Cointfection with two different pathogens may also impact the dynamics of a disease (Abu-Raddad, Patnaik et al. 2006; Graham 2008; Williams, Granich et al. 2010), but, with respect to the evolution of resistance in malaria, we are primarily concerned with multi-strain models of a single pathogen. A key aspect of multiple-strain infection dynamics is that the population dynamics of the pathogen are intertwined with the evolution of strategies to deal with within-host conflict. Thus, the force of infection, which is mediated in part by the frequency of multiple infections, also influences the likelihood that a host will be multiply infected (Day, Alizon et al. 2011). This means that in order to understand the dynamics of the pathogen, it is necessary to understand how competition impacts and is impacted by the dynamics of transmission. For instance, the mechanism of competition with respect to the evolution of virulence, whether it is scramble or contest, results in different population compositions. Scramble competition, in which the probability of transmission is similar among all infecting strains in a host, results in a monomorphic level of virulence. On the other hand, contest competition, in which the most virulent pathogen outcompetes less virulent pathogens for all transmission events from a host, can lead to polymorphisms in the virulence levels of the population (van Baalen and Sabelis 1995). Similar methods of competition may impact the evolution of resistance.

Mathematical models have been an important mechanism for studying the evolution of drug resistance, particularly in malaria, as they provide a means of integrating and synthesizing the results of studies done in many different academic disciplines. However, despite the demonstrated importance of within-host competition, and the importance of this problem facing
public health today, few models of infectious diseases, particularly vector-borne diseases, have examined the impact of multiple infections on the emergence of drug resistance. As noted above, this is in part due to the complexity of the models, which need to take account of multiple axes of competition. For instance, the vast majority of mathematical models of malaria have been based on the “Ross-Macdonald” framework (Reiner, Perkins et al. Submitted), which, while simplifying the dynamics, makes it difficult to embed within-host competition. On the other hand, individual based models of malaria in which within-host competition can be embedded fairly easily, can be difficult to analyze, requiring vast computational power and numerous parameters to fit (Smith, Killeen et al. 2006; Smith, Maire et al. 2008). To bridge this difference, we have developed an individual-based model that is a direct analogue of a mathematical model of mosquito-borne pathogen transmission based on the assumptions of the Ross-MacDonald model.

The model builds on prior epidemiological models of vector-borne transmission (MacDonald 1950a; Dietz, Molineaux et al. 1974; Bailey 1982), and takes a stepwise approach to incorporating both competition and drug resistance. This makes the analysis tractable and allows for a careful examination of how deviations from the orthodoxy of the Ross-Macdonald framework impact the dynamics of infection and the evolution of resistance. We apply the model to malaria, the most important parasitic species in humans, and a disease with both significant within-host competition and significant problems with drug resistance. In this chapter we present the model and demonstrate that the model in its base formulation is a direct analogue to a well-characterized malaria model. Then we extend the model to examine how the introduction
of drug-resistance impacts the dynamics of the disease, examining the impact of different transmission rates and different biological costs of drug-resistance. Lastly, we use the individual-based aspect of the model to further extend the analysis and examine how heterogeneous biting by mosquitoes impacts the evolution of resistance.

**Methods**

**Deterministic Superinfection Model**

Malaria is a vector-borne disease transmitted by the bite of an anopheles mosquito. Individuals living in malarious regions of the world are bitten regularly by infected mosquitoes. This continuous re-exposure results in simultaneous infection, or superinfection (MacDonald 1950a), with multiple parasites. The number of genetically distinct parasite genotypes, called the multiplicity of infection (MOI), generally increases on average in the population with transmission (Bendixen, Msangeni et al. 2001; Sama, Owusu-Agyei et al. 2006), but varies based on mosquito biting patterns (Smith, Dushoff et al. 2005) and the age of the individual (Bendixen, Msangeni et al. 2001; Sama, Owusu-Agyei et al. 2006), which likely has to do with the level of acquired immunity (see Chapter 2).

Our deterministic model of malaria is based on a Markov-Chain model for superinfection and clearance developed by Bailey (Bailey 1982) based on work by both MacDonald, Irwin, Fine and Walton (Walton 1947a; MacDonald 1950a; Dietz, Molineaux et al. 1974; Fine 1975a). The premise of the model is based on the mathematical description of superinfection by Macdonald that assumed “[t]he existence of infection is no barrier to superinfection, so that two or more broods of organisms may flourish side by side, the duration of infection due to one being unaltered by others” (MacDonald 1950a).
The dynamics of the model assume that MOI increases with new infections and it decreases as they clear. The state variables in the model represent the fraction of the population that has a given MOI: \( I_i \) denotes the fraction of hosts with an MOI of \( i \). To ensure that our formulation will be analogous to an individual based version, we explicitly model the mosquito population’s contribution to transmission. Mosquitoes can either be susceptible to infection (\( M_S \)), latently infected (\( M_L \)), or infected and infectious (\( M_I \)). Figure 5.1 provides a schematic of the model.

Figure 5.1: Schematic of deterministic Model

\( S \) refers to susceptible individuals, \( I_i \) is individuals infected with \( i \) genetically distinct clones, and \( M_S, M_L, M_I \) refer to susceptible, latent and infectious mosquitoes, respectively.

**Entomology**

Susceptible adult female mosquitoes, the only ones capable of transmitting the disease, emerge at a constant rate per individual (\( m \)) and are assumed to die at a constant rate (\( g \)). The rate that mosquitoes become infected is dependent on the density of mosquitoes, the biting rate (\( a \)) and the efficiency of transmission from human to mosquito (\( c \)). Mosquitoes that become infected
must then survive sporogony \((n)\) before becoming infectious. The dynamics of the mosquito population are described by the following set of ordinary differential equations:

\[
\begin{align*}
\dot{M}_s &= mN_H - gM_s - \frac{acI_H}{N_H}M_s \\
\dot{M}_L &= \frac{acI_H}{N_H}M_s - (g + n)M_L \\
\dot{M}_i &= nM_L - gM_i
\end{align*}
\]

where \(N_H\) is the total number of humans and \(I_H\) is the total number of infected humans. The number of genotypes that are transmitted by each infectious bite are limited both by the number of ookinete\(\text{s} that have contributed sporozoites, and by the number of liver-stage schizonts that arise from each infectious bite. The proportion of sporozoites in a mosquito’s salivary glands injected during a feeding event is typically small (Shute 1945; Rosenberg, Wirtz et al. 1990; Medica and Sinnis 2005), though the number of genetically distinct sporozoites injected is unclear (Druilhe, Daubersies et al. 1998). Because the bottlenecks inherent within the system likely make the infection of an individual with a large number of genetically diverse sporozoites an uncommon event (Sinden and Billingsley 2001), we assume that each mosquito transmits the offspring of only one gamete, regardless of the MOI of the host that infected it. We also assume that the mosquitoes do not recover from infection.

**Human Dynamics**

The human dynamics are formulated so that \(\sum_i I_i = 1\) and \(\sum_i \dot{I}_i = 0\), where \(I_0\) are susceptible individuals. Thus, the values of the state variables describe the distribution of sensitive phenotypes in a population over time. Humans are infected by infectious mosquitoes at a rate dependent upon the mosquito density, the biting rate, and the infectivity rate \((b)\), or the fraction
of bites on humans that produce a patent infection. The equation describing the dynamics of the population of susceptible humans is,

\[
\dot{S} = I_0 = B - \left( \frac{abM_l}{N_H} + \mu \right) I_0 + \gamma I_1 + \psi \rho \sum_{j=1}^{n} I_j
\]

and the equations describing infected humans is,

\[
\dot{I}_j = \frac{abM_l}{N_H} I_{j-1} - \left( \frac{abM_l}{N_H} + \mu + \gamma j + \psi \rho \delta \right) I_j + \gamma (j + 1) I_{j+1}, \quad j \geq 1
\]

where \(\mu\) is the background mortality rate, \(\gamma\) is the rate infections are cleared, and \(\delta\) is the disease induced death rate. We also assume that deaths are balanced by births \((B)\) so the population size remains constant (though this can be relaxed). Lastly, drug use is assumed to be associated with clinical symptoms (primarily fever), which develops at rate \(\psi\). The rate that clinical symptoms arise is assumed to be independent of MOI. A fraction of symptomatic patients, \(\rho\), are assumed to use drugs and successfully clear all sensitive parasites immediately upon treatment. Treatment of resistant parasites is assumed to be ineffective. For numerical simulations we assume that the max MOI is 100, thus to avoid a population leak, the equation for the maximum MOI differs slightly,

\[
\dot{I}_{j_{\text{max}}} = \frac{abM_l}{N_H} I_{j_{\text{max}} - 1} \left( \mu + \gamma j_{\text{max}} + \psi \rho + \delta \right) I_{j_{\text{max}}}
\]

though this has negligible impact because of the high assumed MOI.

**Individual-Based Model**

**Gillespie Algorithm**

Our individual model of malaria was developed to be, in its base form, a stochastic analogue of the deterministic model described above. Thus, the model is composed of individual humans
that can be infected through infectious bites from mosquitoes and individual mosquitoes that can be infected by biting infected humans.\textsuperscript{17} Simulation of the model is based on Gillespie’s Direct Method, an algorithm that can generate statistically rigorous solutions to systems of stochastic equations (Gillespie 1976; Gillespie 1977). Originally developed for simulation of chemical reactions, it has been used extensively in examining biological questions. The basic premise of the algorithm is that the transition of a homogenous mixture of species from one state to another can be described by probabilistic methods.

In the Direct Method of the algorithm, the system is started in an initial state and then the next reaction and the time to that reaction are selected by sampling from probability distributions that describe the temporal behavior of the system (Gibson and Bruck 2000). Thus, to implement the Direct Method all that is needed to find the state of a system given its current state is to calculate what and when is the next event. The answer to these two questions can be found by specifying the “reaction probability density function”, which specifies the probability that given the current state of the system, the next event, $x$, occurs at time $\tau$ (Gillespie 1977). The reaction probability density function is defined as:

\begin{equation}
(5.5) \quad P(\tau, x)d\tau = a_x \exp\left(-\tau \sum_{\nu=1}^{M} a_{\nu}\right) d\tau
\end{equation}

where $a_x d\tau$ is the probability of event $x$ occurring in an infinitesimal time ($d\tau$) and $\nu = \{1, 2, \ldots, M\}$ is the list of all possible events.

\textsuperscript{17} We keep track of individual mosquitoes in order to keep track of individual parasites and to maintain the developmental lag between infection and infectiousness. Future simulations may also allow for multiply infected mosquitoes.
Integrating this equation over all values of $\tau$ from 0 to $\infty$ gives the probability distribution for each event:

$$P(x) = \frac{a_x}{\sum_{1}^{M} a_v}$$

(5.6)

And the probability distribution of time until the next event can be calculated by summing $P(\tau, x)$ over all $x$ such that:

$$P(\tau) d\tau = \left(\sum_{1}^{M} a_v\right) \exp\left(-\tau \sum_{1}^{M} a_v\right) d\tau$$

(5.7)

These distributions can then be used to find the next event and the time until that event based on the Direct Method algorithm.

1. Initialize the system by defining the initial population values and the setting the time to zero.
2. Calculate the probability distribution for each event.
3. Determine the next event based on the probability of each event occurring.
4. Determine the time until the next event by sampling from exponential probability distribution of time until the next event.
5. Execute the chosen event.
6. Update the time so that $t = t + \tau$.
7. Go to step 2.

Sampling is done by random number draw.
Despite the ability of the Direct Method to calculate statistically rigorous solutions, it requires a significant amount of computational power when the system is complex or the population is large.

**Computationally Efficient Translation**

Translation of the Gillespie Algorithm to individual agents, where the rate at which events occur are specific for each agent, creates another scaling problem as the algorithm requires every rate for every individual to be calculated after every event. Though technically possible, as the population increases linearly, the computational efficiency increases exponentially (Allen and Dytham 2009). A computationally efficient alternative was developed by Allen and Dytham (2009) specifically directed at modeling biological populations. While exactly equivalent to the Direct Method, this alternative is significantly more computationally efficient. The difference lies in the fact that the computational cost per event in the alternative algorithm is independent of population size (Allen and Dytham 2009). As with the Direct Method, time steps are sampled from an exponential distribution of probabilities that are analogous to rates. However, in this case the rates refer to the maximum rate ($c_x$) possible for each event ($x$). At each time step a potential event is identified by selecting an event based on its maximum rate and then determining whether this event is executed by selecting an individual to experience the event. Thus, at each time step there is no guarantee an event will occur. The probability of the event occurring is the probability of the event being selected ($c_x / c$, where $c = \sum c_x$), the probability of the $i$th individual being selected, and the probability that the selected event is executed ($x_i / c_x$).

The following outlines the general steps of the algorithm (Allen and Dytham 2009):
1. Initialize the population and set time to zero.

2. Choose maximum rate constants for each rate as small as possible so that \( c_x \geq \max(x; \forall i) \).

3. Sample the length of this time step from an exponential distribution \( cN \), where \( N \) is the current population, and \( c \) is the sum of all maximum rates.

4. Randomly select an event type \((x)\).

5. Randomly select an individual \((i)\).

6. Execute the chosen event with probability \( x_i / c_x \).

7. If the event is executed, update the population, update the time so that \( t = t + \tau \) and go to Step 2. If no event is executed, update the time so that \( t = t + \tau \) and go to Step 3. [Note that updating means only update the individual and any population level parameters, such as population size if it was a death].

**Malaria Individual-Based Model**

For the human dynamics we calculate the maximum possible probabilities that an individual dies \((\mu)\), becomes infected \((abM_t / N_h)\), recovers from an infection \((\gamma)\) (i.e. loses a parasite clone), becomes clinically ill \((\psi)\) and treats \((\rho)\), and dies from the disease \((\delta)\). We then select the time until the next possible event and the event that may occur. We then select a random individual and calculate if the event occurs based on their individual probability for the selected event. If the event is executed, the population is updated.

To increase the computational efficiency of the model, we decoupled the human and mosquito equations and updated the mosquito dynamics after each time step, \( \tau \). Because the lifespan of the human population is significantly longer than the mosquito, we further simplified the model and
updated births, deaths and sporogony of the mosquito population by an alternative algorithm.

This process takes advantage of the fact that over the interval $t + \tau$, the mosquito population size does not change significantly enough to impact the human dynamics, which operate on a much slower time-scale. Thus, we can sacrifice the accuracy of knowing exactly when a mosquito was born, died or became infectious, for computational efficiency by calculating the expected number of events that occur over that time period and executing them all at one time.

The $\tau$-leap method was developed by Gillespie to deal with larger populations (Gillespie 2001) and can significantly decrease the computational time needed to simulate the dynamics of a population, depending on the length of each leap. The key requirement of the $\tau$-leap method is to ensure that $\tau$ be of short enough duration that the change in the state of the system during the interval $[t, t + \tau]$ will cause no appreciable change in the probability distributions of each variable. The number of times $K_x$ that an event will occur over the interval $[t, t + \tau]$ given the state of the system $X$ at time $t$ is a poisson random variable:

(5.8) \[ K_x(\tau; X, t) = P(a_x(X), \tau) \]

For the infection dynamics of mosquitoes, we use a similar process as for the humans: (1) calculate the time until the next possible mosquito infection; (2) select a random individual from whom a susceptible mosquito acquires the infection; (3) update the mosquito population; (4) update the time. We initiate these dynamics at time $t$ and continue until the next event would be past $t + \tau$. 

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An additional simplification that we make is to assume that susceptible mosquitoes are all equal, i.e. we don’t keep track of individual susceptible mosquitoes. Thus, at every defined interval, we calculate the number of new mosquitoes that emerge using a poisson random variable, $P(m_{NH})$, and we also calculate the number of susceptible mosquitoes that died over this period, $P(g_{MS})$. The total number of susceptible mosquitoes is then updated. We keep track of the individual latently infected mosquitoes and the parasite that they are infected with. For each interval, the number of latent mosquitoes that survive sporogony $P(n_{ML})$ have their status updated accordingly, while those that die, $P(g_{ML})$ are eliminated from the population. Lastly, the number of infectious mosquitoes that died over the interval, $P(g_{MI})$ are eliminated from the population. The model was written in C++ and simulated using the parameter values detailed in Table 5.1.

**Biological Cost of Drug-Resistance**

The key aspect that the current formulation adds to the question of superinfection is the ability to allow for within-host competition transmission events. While the number of genotypes that are transmitted from a human to a mosquito can be numerous, the ultimate number of genotypes transmitted by infectious bites of mosquitoes on humans are limited due to a winnowing process within the mosquito (Sinden and Billingsley 2001). Thus, we assume that each mosquito ultimately transmits the offspring of only one of the parasites that infects a human host, regardless of the host’s MOI, and the ‘winning’ parasite is determined at the time a mosquito becomes infected. This allows us to examine the outcome of within-host competition between parasites with different phenotypes. The key phenotype that we examine in this chapter is the biological cost of drug-resistance.
The acquisition of drug tolerance by parasites is encoded by single mutations, multiple mutations, or changes to the copy number of genes relating to the drug’s target or influx-efflux pumps that affect intraparasitic concentrations of the drug (White 2004). These genetic changes, while providing a benefit in the presence of drugs, can also reduce the parasites competitiveness in the absence of drug therapy (Felger and Beck 2008; Babiker, Hastings et al. 2009). This biological cost of resistance, reduces the reproductive efficiency of the parasite and slows its growth rate (Babiker, Hastings et al. 2009), though this likely has differential impacts on transmission and clearance.

We assume that drug-resistant parasites have both “hard” and “soft” costs of resistance (Babiker, Hastings et al. 2009). Hard selection is where the fitness cost of maintaining resistance genes is
incurred irrespective of the presence or absence of drug-sensitive parasites. Thus, we assume that drug-resistant parasites have a greater probability of clearance at all times, whether they are competing with drug-sensitive parasites or not. Soft selection, on the other hand, is where fitness costs are only apparent in the presence of competition. In this case, we assume that drug-resistant parasites have a lower probability of transmission when they are competing with drug-sensitive parasites in a host. We assume a soft fitness cost with respect to transmission because (a) the correlation between asexual parasite density and gametocyte density is limited (Taylor and Read 1997), (b) transmission probability is both saturating and efficient at low densities (Barnes and White 2005), and (c) there is ample evidence of within-host competition between clones for transmission events (Talisuna, Langi et al. 2003; Wargo, Huijben et al. 2007), suggesting that it is likely that small differences in probability will result in differential transmission. For clearance, a hard cost was assumed because, as noted above, the acquisition of resistance mutations decreases the reproductive rate of the parasite (Babiker, Hastings et al. 2009), which is likely to reduce the duration of infection for drug-resistant parasites regardless of competition with drug-sensitive clones.

The different phenotypes are thus assumed to have different probabilities of transmission and clearance. At each clearance and transmission event, a parasite is selected based on their weighted probability of transmission or clearance. Thus, if we assumed the biological cost of transmission was 20%, then if there were two parasites in an individual, a drug-sensitive one and a drug-resistant one, the drug-resistant one would be 20% less likely to be selected. A similar process was used for the clearance cost, however, whereas we assumed that clearance was a “hard”
cost, and thus drug-resistant parasites always cleared at a faster rate, in the transmission case, the
cost of resistance did not impact the probability of a transmission event occurring overall. Thus,
this cost was only a “soft” cost paid when drug-resistant parasites were in the same host with
drug-sensitive parasites.

**Heterogeneous Biting**

Within-host competition can only occur when an infection is composed of multiple clones, and
there is ample empirical evidence that average MOI increases as the transmission rate increases
(Smith, Dushoff et al. 2005; Sama, Owusu-Agyei et al. 2006; Smith and Hay 2009), suggesting
that at higher transmission rates competition should be greater. However, there is also ample
evidence that biting by mosquitoes is not uniform. At the individual level, it has been shown that
mosquitoes are differentially attracted to some individuals (Mer, Mirnbaum et al. 1947; Lindsay,
Adiamah et al. 1993; Knols, de Jong et al. 1995). Individuals and mosquitoes are also distributed
non-randomly across the landscape (Smith, Dushoff et al. 2004) which generates spatially
heterogeneous biting patterns, which can be further localized by movement patterns of both
mosquitoes and humans (Stoddard, Morrison et al. 2009). Thus, it is likely that MOI is highly
variable across a spatial landscape, even at the scale of a village, which would result in differential
levels of competition depending upon the rate that a host is bitten, which may significantly
impact the evolution of resistance.

Because our model tracks individuals, mosquitoes and parasites, we had the ability to explore in a
tractable manner, the impact of heterogeneous biting on the evolution of resistance. To
incorporate heterogeneous biting, we assumed that every individual has a different biting weight,
We assumed that biting weights were gamma distributed so that, as suggested, ~20% of the individuals receive ~80% of the bites (Woolhouse, Dye et al. 1997; Smith, Dushoff et al. 2005; Smith, Drakeley et al. 2010). To maintain comparability to simulations assuming uniform biting, we used an overdispersed gamma function that generates a mean biting weight that is the same as in the prior simulations, \( \Gamma(k = 1, \theta = 0.3) \).

**Results**

To demonstrate the ability of the model to approximate the dynamics of the deterministic superinfection model, we simulated the system 10,000 times with a population size of 10,000. To avoid the disease stochastically dying out before an epidemic begins, we started with five infected individuals (0.05% of the population). Initially we assumed that individuals could not be multiply infected. The frequency of individuals in each state for both the human and mosquito populations was recorded on each day and compared to the dynamics of infection in the classical model that the stochastic IBM is based upon. The results are depicted in Figure 5.2, and demonstrate that the stochastic IBM can generate statistically rigorous solutions to the system of equations. The simulation was run over numerous transmission intensities and time lengths with the same results (data not shown).

We then allowed individuals to become multiply infected, though we assume as in the deterministic superinfection model that all parasite clones are phenotypically the same (MacDonald 1950a; Bailey 1982). Thus, the probability of any single parasite being selected when a mosquito bites a human is equal. Clearance is also similar to the deterministic superinfection model in that an individual human’s probability of clearing a parasite clone on any day is related to the number of clones they harbor. Thus, if they harbor \( x \) clones, the individual
probability of clearance is equal to $x\gamma$, where $\gamma$ is the clearance rate defined above. All parasites are assumed to have the same clearance probability regardless of infection date. Similar to the results of single infections, the individual stochastic model produces statistically rigorous results (Figure 5.3). In addition, at equilibrium, defined in the stochastic case as the distribution of infections after 5,000 days, the expected distribution of multiply infected individuals in the stochastic case is statistically indistinguishable from the deterministic model (Figure 5.4).

Figure 5.2: Comparison of individual based stochastic model with classical formulation of single infection malaria model
The dynamics of the classical model are described by the solid black lines, while one standard deviation of the mean of 10,000 simulations of the individual based model is overlaid.
Figure 5.3: Comparison of individual based stochastic model with classical formulation of multiple infection malaria model
The dynamics of the classical model are described by the solid black lines, while one standard deviation of the mean of 10,000 simulations of the individual based model is overlaid.

Figure 5.4: Multiplicity of Infection (MOI) Comparison
The frequency of the population with a specific MOI was estimated at the end of the simulation. The distribution of MOIs that result from the classical model is described by the grey bars, and one standard deviation of the mean of 10,000 simulations of the individual based model is overlaid.
Incorporating Resistance

Because the model has competition between parasite clones inherently built in, it can easily incorporate within-host competition between drug-resistant and drug-sensitive parasites. We assumed that with some probability an infected individual would develop resistant parasites *de novo*, regardless of drug pressure. Thus, an individual has the same probabilities as in the prior simulations (death, infection, recovery, clinical symptoms and treatment, and disease induced death), but now, if infected, with some probability one of their parasites may become resistant. We assume that when resistant parasites arise they are fully resistant, and thus treatment is assumed to be ineffective. We further assume that they are immutable, and so their phenotype (including the biological cost of resistance) does not change over the course of the simulation.

To assess the spread of resistance within a population we let the system come to equilibrium in the absence of resistance, and then we assume that resistance could appear in infected individuals with a very low probability. This ensures that resistance will keep appearing at a low rate, so that we can determine if and how it will spread through the population, in other words we assume the development (or importation) of resistance is inevitable and focus on the spread within the population. We found that once resistance emerged it would spread quickly and every infected individual would soon harbor drug-resistant parasites. However, the percentage of the parasite population that was composed of drug-resistant parasites increased at a slower rate, though this was dependent upon the transmission rate. In our base set of parameters, we assumed an annual EIR of ~25. In this case drug-resistance spread quickly but drug-resistant parasites did not exclude drug-sensitive parasites despite a high rate of drug-treatment (80%) (Figure 5.5). However, at lower transmission levels (Figure 5.6), drug-resistant parasites were able to exclude
the drug-sensitive parasites for the same combination of parameters. Thus, in line with the prior results examining the impact of superinfection on drug-resistance (Klein, Smith et al. Submitted), coexistence between drug-sensitive and drug-resistant parasites is a likely outcome, and this effect increases the higher the transmission rate or the greater the fitness cost.

Figure 5.5: Drug-resistance evolution

As drug-resistance spreads through the population, the impact of treatment in reducing the burden of infection is negated (difference between dotted red line and dotted grey line). However, despite the percentage of the population infected with at least one resistant parasite quickly reaching 100%, the percentage of parasites in the population that are resistant (blue line) increases at a slower rate, and in fact never goes to 100%, despite the continual introduction of resistance \textit{de novo} (red dots). Annual EIR was ~25 and drug-resistant parasites were assumed to have fitness costs of 32% and 17% for transmission and clearance, respectively.
Figure 5.6: Impact of Transmission of drug-resistance evolution

The transmission rate has a significant impact on the spread of drug resistance in a population. At low annual EIRs (A), drug-resistance goes to fixation at approximately the same time that the percentage of individuals infected with resistant parasites reaches 100% of infected individuals. This is because the average MOI is ~1 in this case, so there is little impact of within-host competition. On the other hand, as the transmission rate is increased (B), the result is coexistence between drug-resistant parasites and drug-sensitive parasites. Drug-resistant parasites were assumed to have fitness costs for transmission and clearance of 32% and 17%, respectively.

Also in line with prior results (Klein, Smith et al. Submitted), we find that the long-term viability of drug-resistant parasites is more dependent upon maximizing its duration of infection rather than maximizing the possibility of transmission at any single event (Figure 5.7). Thus, fitness costs that shorten the duration of infection are more likely to be detrimental than fitness costs that reduce the probability of transmission. These results are consistent across transmission rates, however, as the transmission rate increases, the parameter space over which coexistence is possible increases. This suggests that in high-transmission areas, the probability of coexistence between drug-sensitive and drug-resistant parasites is greater. In addition, despite the larger areas of coexistence, it would be easier for resistance to emerge in lower transmission areas, when the cost of clearance is not too great, as resistant parasites with significant fitness costs can still invade, which is in accord with previous studies (Klein, Smith et al. 2008).
To ensure that our results were not due to assumptions about the way fitness costs were implemented, we examined whether a “soft” cost of clearance impacts the results. Thus, we re-ran the analysis assuming that increased clearance probabilities for drug-resistant parasites only occurred when drug-resistant parasites were in the same host as drug-sensitive parasites. The results from this analysis were in concordance with the prior results (Figure 5.8), however, there was some fitness benefit for the drug-resistant parasites at the margins when the fitness cost of clearance was high.

**Heterogeneous Biting**

Individual variation in the rate at which individuals are bitten by mosquitoes, means some individuals will receive far more infectious bites than others. This variation is due to individual level differences in attraction to mosquitoes (Mer, Mirnbaum et al. 1947; Lindsay, Adiamah et al. 1993; Knols, de Jong et al. 1995), as well as geographical variation (Smith, Dushoff et al. 2004) and differential movement patterns (Stoddard, Morrison et al. 2009). To account for this heterogeneity, we randomly assigned biting weights so that ~20% of the individuals receive ~80% of the bites (Woolhouse, Dye et al. 1997; Smith, Dushoff et al. 2005; Smith, Drakeley et al. 2010). We then examined how heterogeneous biting impacted the dynamics of the disease in the absence of drug-resistance. Because we assumed a highly skewed distribution, where a small fraction of individuals receive the vast majority of the bites, the average number of infected individuals is significantly less then without heterogeneity in biting (Figure 5.9). More importantly, the distribution of MOIs is now skewed (Figure 5.10), with the majority of infected individuals having an MOI close to one at any one time and a small number of individuals with high MOIs.
Because within-host competition will be reduced in the individuals with lower MOI, we assumed this would impact the evolution of resistance. However, we found that once resistance became established, it generally spread rapidly through the population and reached similar equilibrium levels as in the uniform case (data not shown). This result belied the rate at which resistance became established though. Because we assume that the introduction of resistance is stochastic, any single simulation may produce a different establishment time. However, on average, the time for resistance to become established in the population was longer when heterogeneous biting was included (Figure 5.11). This is due to the high degree of heterogeneity in biting weights which means that when resistance is introduced, resistant parasites will either reside in individuals with a high MOI and face a high-level of within-host competition, reducing the likelihood of onward transmission, or they will be in individuals that have low biting weights and are thus less likely to be bitten and transmit the resistant parasites. However, the establishment and spread of resistance are separate (see chapter 2 and Smith, Klein et al. 2010), and thus, we also examined the rate at which resistance spread once it was established. Despite slowing the rate at which resistance becomes established, once it is established, resistance will spread faster in a population with heterogeneous biting than in a population with homogenous biting. This is because once resistance becomes established, individuals with low biting weights are less likely to get re-infected with sensitive parasites when they are infected and individuals with higher transmission rates will quickly spread resistance to those individuals. These results are qualitatively the same at lower transmission rates, though the difference in waiting time for resistance to establish between the heterogeneous and homogenous biting cases is greater than in the higher transmission case.
Mutations that confer drug resistance can result in fitness costs that impact the duration of infection as well as the probability of transmission. These costs can impact the duration of infection or the ability of the parasite to transmit. The tradeoff between the two suggests two important aspects: (1) there is a large parameter space where drug-resistant parasites and drug-sensitive parasites can coexist in the population; and (2) the drug-resistant parasites long-term viability is more dependent upon maximizing its duration of infection relative to the drug-sensitive parasite than maximizing its transmission ability. These results are true even at low transmission rates (A), but at higher transmission rates (B and C) the parameter range over which coexistence occurs is significantly increased.

**Figure 5.7: Resistance evolution across different levels of fitness costs**

**Figure 5.8: Impact of Soft fitness costs of clearance**

Mutations that confer drug resistance can result in either “hard” or “soft” fitness costs. Hard fitness costs are incurred irrespective of the presence or absence of drug-sensitive parasites, while soft selection is the case where fitness costs are only apparent in the presence of competition. We examined the impact of assuming that the fitness costs of clearance were hard, by re-running the simulations assuming that costs were soft. Though at the margins there were minor differences, the results were qualitatively the same as under the hard assumption.
Figure 5.9: Transmission Impact of Heterogeneous Biting

Heterogeneous biting has a significant impact on the transmission dynamics of malaria. Because the rate of biting on a large proportion of the population is significantly lower, the level of infection in the community is lower. The gray lines are the dynamics of the population in the absence of heterogeneity in biting (dashed is infected individuals and solid is susceptible individuals). The black lines represent the mean of 500 simulations of the individual based model, and one standard deviation of the mean is overlaid.

Figure 5.10: Impact of Heterogeneous Biting on the Multiplicity of Infection

Heterogenous biting significantly impacts the number of individuals that are multiply infected. Because we assumed that only 20% of individuals got 80% of the bites, only a small proportion of the population have high levels of multiple infections, while the majority of the population has only a small number of infection. The black lines are the mean of 500 and 10,000 simulations of the individual based model, and one standard deviation of the mean is overlaid.

Discussion

We developed an individual-based model that is a stochastic analogue to a set of ordinary differential equations. The benefit of this approach is the ability to understand how deviations from the assumptions inherent within class-based models impact the transmission dynamics. We applied this approach to malaria, the most important parasite species affecting humans. Using a differential equations model based on the Ross-MacDonald framework we examined how two important differences between the models can impact the dynamics of transmission: (1) the role of within-host competition between different parasite phenotypes, in this case drug-resistant and
drug-sensitive phenotypes; and (2) differences between individuals, in this case the rate that individuals are exposed to disease.

The emergence of drug-resistance in malaria has been a significant tragedy, resulting in increases in morbidity and mortality (Snow, Trape et al. 2001). However, despite the importance of within-host competition to understanding the epidemiology of this important problem in public health, few models have included this in a manner representative of the dynamics of malaria. In part this is difficult to do in a tractable manner. Using our individual based model, we examined how within-host competition can impact the emergence of drug resistance in a population.

While the results do not upend any classical notions of malaria dynamics, they point to the considerable impact of within-host competition on the spread of resistance. For instance, they indicate that as the transmission rate increases, the range over which drug-resistant parasites can exclude drug-sensitive parasites contracts significantly, while the range over which coexistence can occur increases. In particular, while prior analyses (Klein, Smith et al. Submitted) found the parameter range over which the system transitions from all sensitive to all resistance to be fairly sharp, our new model finds that this is actually a fairly broad range, particularly at higher transmission levels. This suggests that a population of drug-resistant parasites could encompass parasites with a range of fitness costs, making it easier for parasites to either acquire step-wise resistance mutations (Hastings and Watkins 2006) or compensatory mutations (Jiang, Patel et al. 2008).
Figure 5.11: Impact of Heterogeneous Biting on the Establishment and Spread of Resistance
Because resistance was introduced stochastically, we simulated the model 2,000 times and measured the time until resistance reached 0.01%, 0.05%, 0.1%, 0.5%, 1%, 5%, 10% and 25%, with and without heterogeneous biting. In both cases the annual EIR was the same (in A EIR ~25 and in B EIR ~6), and the fitness costs of transmission and clearance were 32% and 17%, respectively. Because a small group of individuals receive the vast majority of bites, resistant parasites that are introduced either face higher levels of within-host competition or are in individuals that don't get bitten much. Thus, on average, the time until resistance became established in the population (calculated as the time to establish at 0.01% frequency) is slower than the homogenous biting case (blue bars). However, once resistance becomes established, it will spread through a population with heterogenous biting faster, as the majority of individuals have significantly less within-host competition. So green bars measure the time for resistance to increase from 0.01% to each resistance frequency. Resistance takes a longer time to establish in lower transmission areas (B), and the difference between heterogeneous biting and homogenous biting is larger than in the higher transmission area. Error bars are 95% confidence intervals, and black lines are the median values.

Emergence of antimalarial drug-resistance has historically occurred in low transmission areas (White 1998; White and Pongtavornpinyo 2003). There are a number of theoretical suggestions as to why that has been the case: (1) within-host competition in higher transmission areas reduces the frequency of resistant alleles (Hastings 1997); (2) immunity, which reduces the rate that parasites see drug treatment (Hastings 1997; Plowe, Kublin et al. 1998; White 1998) and can create a refuge for drug-sensitive parasites (Klein, Smith et al. 2008), and also reduces the
probability of survival for resistant parasites (Plowe, Kublin et al. 1998; Gatton, Hogarth et al. 2003); and (3) higher selfing rates in low transmission areas reduces the likelihood that resistance encoded by mutations at multiple loci will be broken up (Paul, Packer et al. 1995; Dye and Williams 1997; Hastings 1997). However, most of these theories deal with the reasons that resistance is more likely to appear de novo in low transmission areas, and not with the role of establishment. Establishment of resistance in a population is important, because once it becomes established it becomes more difficult to use control methods, such as mass drug administration, to eliminate resistance, as the resistant parasites are more widespread. Our results found that establishment occurred faster, on average, in high transmission settings (see Figure 5.11). In low transmission settings it took an average of ~7 years for resistance to become established under homogenous biting versus only ~5.7 years in a higher transmission setting. This was exacerbated by the role of heterogeneous biting, which increased the difference between low and high transmission areas more than 2.5 fold. This result is counter somewhat to the expectation that multiple infections, and the resulting within-host competition, will slow the establishment of resistance. It suggests in fact that the increased transmission rate can help overcome the within-host costs of competition to establish resistance in a population. As the assumption in our model was that there was a constant low probability that any single individual could be infected with a resistant parasite, this is more akin to the role of resistance spreading into a population from outside. Our results suggest that once resistance has arisen anywhere in the world, its rate of establishment into new areas will be governed, in part, by the rate of transmission, and that contrary to other models (White 1999a; White 1999b), we suggest that this may actually be faster in high-transmission areas.
Once established, the resistant parasites will spread at a rate that is governed by the biological fitness costs of resistance, drug treatment rates, as well as the transmission rate, which impacts individual MOI and within-host competition (White 1999a). We found that holding drug treatment rates constant, the rate that resistance increased from its establishment level is actually faster in the low transmission areas. This was true in both the heterogeneous and homogenous biting cases, however, while heterogeneous biting tended to slow the establishment of resistance relative to homogenous biting, spread was actually faster in the heterogeneous case (regardless of transmission rate). These results demonstrate that heterogeneity, particularly in low transmission areas which saw greater increases in the rate of spread, can significantly impact the spread of resistance. This point is distinct from other models discussing the role of transmission in the initial emergence of resistance which have generally suggested that resistance is more likely to emerge in low transmission areas. Instead it is a reflection of the role that large numbers of individuals with low MOIs combined with a small core of individuals responsible for the majority of transmission play in speeding resistance through an area. Thus, in areas with highly variable transmission patterns, reducing transmission in the “core” group responsible for the majority of transmission is essential to slowing the spread of resistance.

In this chapter we have introduced a computationally efficient individual based model for examining infectious diseases. The model was applied to malaria and the impact of within-host competition and heterogeneous biting on the emergence and spread of drug-resistance. The model results have shown themselves to be extremely computationally efficient and provided statistically rigorous results that conformed with the underlying deterministic model. This
approach allowed us to examine how assumptions of the deterministic equations were impacted by individual differences. The results clearly showed that within-host competition is important for understanding the emergence and spread of drug-resistance, and provided more robust predictions as to how this interacts with transmission intensity. Future extensions to this point will consider the role of immunity, which has been shown to be an important component in the evolution of antimalarial drug resistance (Klein, Smith et al. 2008).

While in this chapter we only examined the role of within-host competition and heterogeneous biting in malaria, the framework allows for robust examinations of other diseases and other factors in the future, such as more realistic distributions for infection and clearance. Similar explorations of this process have shown that this can destabilize epidemic models (Lloyd 2001), but our model allows for more robust examinations of the effect of this process, including things such as the role of multiple infections and immunity. Additionally, this model allows for an examination of multiple facets of evolution concurrently. In the next chapter, we will examine how virulence impacts and is impacted by the dynamics of transmission in malaria and what sort of impact this has on the emergence and spread of antimalarial drug resistance.
Chapter 6
Coevolution of Antimalarial Drug Resistance and Virulence

Abstract
Ecological theory suggests that parasite virulence is the result of a tradeoff between transmission and reproduction. Increased reproduction within the host increases the probability of transmission, but also increases host exploitation and the thus the likelihood of morbidity and mortality. In this chapter we critically analyze this proposition with respect to long-lived infections such as Plasmodium falciparum, the causative agent in malaria. Using data from purposeful infections of individuals with malaria, we find a suggestion that the primary tradeoff with virulence in malaria is the duration of infection. We then examine the consequences of this type of tradeoff within an individual for the dynamics of infection at the population level using the individual based model introduced in Chapter 5. We find that contrary to the established theory, multiple infections can actually decrease the virulence of the parasite. We then examine how the evolution of drug resistance impacts the virulence of the parasite. Here we find that when drug-resistant parasites are rare, on average, they will have a higher virulence level than drug-sensitive parasites. This advantage is due to the benefit of causing clinical symptoms while in competition with drug-sensitive parasites. However, this is short-lived. Once drug-resistant parasites increase in frequency average virulence levels decrease as most individuals will harbor drug-resistant parasites negating the advantage of being virulent.
Introduction

The evolution of parasite virulence (i.e. host morbidity and mortality) is generally considered to be the result of a tradeoff between reproduction, which increases host exploitation and the likelihood of morbidity and mortality, and transmission. While parasites by definition are exploiting the host, and thus causing some level of harm, increasing the level of exploitation results in increased transmission but reduces the host’s life expectancy, reducing the probability of between-host transmission (Anderson and May 1982). Evolutionary theory has predicted that parasites should evolve toward an evolutionary stable host exploitation strategy by balancing the costs and benefits of virulence (Levin and Pimentel 1981; Anderson and May 1982; Ewald 1983; Levin 1983a; Sasaki and Iwasa 1991; van Baalen and Sabelis 1995). These models, which form the cornerstone of the virulence-transmission tradeoff, predicted that rather than evolving towards avirulence (as was the conventional wisdom at the time (Alizon, Hurford et al. 2008)), parasites would evolve towards an intermediate level of virulence.

Despite the clear relationship that theoretically exists between transmission and virulence, experimental evidence has been mixed (Bull 1994; Read and Taylor 2001; Ebert and Bull 2003; Alizon, Hurford et al. 2008). One reason for these equivocal results is the role of multiple infections, which introduce an additional variable: within-host competition (Gandon, Jansen et al. 2001). Models of parasite virulence including within-host competition have found that multiple infections can both increase and decrease the expected virulence level of parasites. On the one hand, because the parasite is forced to compete within the host as the number of concurrent infections increases, transmission becomes more important relative to host preservation (Sasaki and Iwasa 1991) raising the average level of virulence (Levin and Pimentel
1981; Nowak and May 1994; May and Nowak 1995; van Baalen and Sabelis 1995; Gandon 1998). On the other hand, the multiplicity of infection (i.e. the number of concurrent infections), depends on the force of infection, or the rate individuals are infected or superinfected. A lower force of infection means that individuals are less likely to be multiply infected, and/or the number of concurrent infections will be less, which in turn lessens the competition a parasite faces within the host. Thus, factors that decrease the force of infection, such as a high parasite mediated mortality rate, can actually select for reduced parasite virulence (van Baalen and Sabelis 1995; Ebert and Mangin 1997; Gandon, Jansen et al. 2001). The relatedness of infecting parasites is also predicted to impact the virulence of a pathogen. Increasing relatedness of parasites is predicted to lead to lower levels of virulence, while greater distance is predicted to lead to increased virulence (Frank 1996; Brown, Hochberg et al. 2002).

An additional reason that experimental and observational evidence has likely been lacking for the virulence-transmission tradeoff is that most models have assumed that virulence is measured as host-mortality. While the death of a host will obviously shorten the period of parasite infectiousness, host-exploitation can also impact the host response in other ways that limit transmission. For instance, significant morbidity may result in changes in host behavior that decreases the probability of transmission (Klein, Laxminarayan et al. 2007). Of likely greater consequence, though, is the role of host immunity. Several theoretical studies have suggested that virulence may depend on host-immunity (Van Baalen 1998; Brown and Grenfell 2001; Day

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18 This ignores the many microparasites that must kill their hosts in order to successfully transmit (Ebert and Weisser 1997), however, these are primarily relegated to invertebrate infections, and we ignore them in the current analysis.

19 Examples include: (1) self-limiting behavior (such as staying home when you have the flu); (2) outward signs of contagion (such as pustules or a rash) that result in avoidance by other hosts; and (3) increased awareness of disease that changes how people behave (i.e. prevalence-dependent behavior (Klein, Laxminarayan et al. 2007)).
and Burns 2003; Alizon and van Baalen 2005), however the direction of evolution (whether
towards increased or decreased virulence) is mediated by the relationship between parasite
exploitation and the host immune response. This relationship is complex, in part, because often
the most severe effects of pathogen invasion are due to immunopathology (Graham, Allen et al.
2005). Thus, while immunity has been observed to increase parasite virulence (Jäkel,
Scharpfenecker et al. 2001; Mackinnon and Read 2004a), immunity may also modulate virulence
if resident strains suppress the response of the immune system (Graham, Allen et al. 2005;
Alizon and Van Baalen 2008) or reduce the likelihood of superinfection (Brown and Grenfell
2001).

While the relationship between immunity and pathogen virulence is complex, it may also play a
major role in the virulence-transmission tradeoff. The key insight of the virulence-transmission
tradeoff is that for a parasite to increase its transmission potential it must pay a cost (Anderson
and May 1982; Ewald 1983). Transmission potential is both a function of the duration of
infection and the probability of transmission. While the relationship between increased
replication and transmission probability is fairly straightforward, the relationship between
virulence and infection duration is less obvious. It has generally been assumed that the limiting
factor for duration, and thus the main axis along which transmission is balanced, is host-
mortality. Yet, some highly virulent parasites can also generate very long infections by evading or
suppressing the immune system. This is particularly true for many macroparasites (e.g.
helminths), but is also true for microparasites as well (e.g. malaria). While mechanisms differ,
one prime mechanism for immune evasion is antigenic variation, or continual switching by the
parasite of the expressed immune target (Antia, Nowak et al. 1996). This is, for instance, the presumed mechanism by which the malaria parasite *Plasmodium falciparum* prolongs the duration of infection (Marsh and Howard 1986; Biggs, Anders et al. 1992; Miller, Good et al. 1994).

*P. falciparum* is a virulent parasite. It has been estimated to cause ~500 million clinical cases (Snow, Guerra et al. 2005), and kill approximately one million individuals (Murray, Rosenfeld et al. 2012) every year, making it the leading cause of death in children under the age of five in Africa (Mathers, Lopez et al. 2006). However, while it is virulent, only a fraction of clinical infections result in death. In addition, the estimated duration of infection is approximately 200 days per infection (Eyles and Young 1951; Smith, Dushoff et al. 2005), suggesting that, while important, mortality is not the main constraint on the duration of infection, and that immunity may play a more important role in regulating the virulence of the pathogen.

In this chapter, we examine the role of virulence in generating long-term parasite infections in malaria. We examine whether immune evasion may be the target of increased virulence and how this would evolve within a population. Within this framework, clinical symptoms (and occasionally mortality) result from increased parasite virulence, but this is traded off with longer infection duration. An additional factor we consider is drug usage, which is associated with clinical symptoms, and can also impact virulence evolution. In particular, we examine the role of drug usage and the emergence of drug resistance on the evolution of virulence.
Assessing the Transmission–Virulence Tradeoff in Malaria

To understand the virulence transmission tradeoff in malaria we start by examining the dynamics of transmission as defined by Macdonald (MacDonald 1957), and modified by Smith and McKenzie (Smith and McKenzie 2004). For successful human-to-human transmission to occur: (1) the parasite must infect a mosquito during a bloodmeal; (2) the mosquito must then survive until the parasite becomes infectious (a period called sporogony that lasts about ten days, but is temperature dependent (Paaijmans, Read et al. 2009)); and (3) then must infect a human during a second bloodmeal. The transmission potential of the system is controlled by the ecology and environment, and can be described by vectorial capacity (V), which is the number of infectious bites by a mosquito over its lifetime, and is given by the following formula:

\[
V = \frac{ma^2e^{-gn}}{g}
\]

(6.1)

where \(m\) is the number of mosquitoes per human and \(a\) is the number of bites on humans per mosquito per day. The mosquito mortality rate is \(g\), and \(e^{gn}\) is the probability of a mosquito surviving the number of days required for sporogony (\(n\)). Including the human dynamics, gives the basic reproductive number for malaria,

\[
R_0 = \frac{bcV}{r + \mu + \delta}
\]

(6.2)
where $c$ is the transmission efficiency, the fraction of bites that produce a patent infection, from human-to-mosquito, $b$ is the transmission efficiency from mosquito-to-human, $r$ is the recovery rate, and $\mu$ and $\delta$ are the natural and disease induced mortality rates, respectively. A schematic of the basic reproductive number is shown in Figure 6.1.

![Figure 6.1: Basic Reproductive Number in Malaria](image)

The basic reproductive number in malaria can be found by following the circle of transmission. There are $m$ mosquitoes per human and they bite humans at rate $a$. A percentage $c$ of bites on infected humans results in an infection in a mosquito, and a percentage of them survive sporogony ($n$) to become infectious. Infectious mosquitoes can then infect individuals for the duration of their lifespan, though only a percentage, $b$, of bites actually cause infection. Humans that are infected remain infected and infectious for $r$ days.

We then simulated the population dynamics of malaria using a model for multiple infections developed by Bailey (Bailey 1982). The model is deterministic and assumes that infections clear independently (MacDonald 1950a), thus MOI increases with new infections and decreases as they clear. The state variables in the model represent the fraction of the population that has a
given MOI: $I_i$ denotes the fraction of hosts with an MOI of $i$. The force of infection, $h$, in the model is a product of the entomological inoculation rate (EIR), which is the number of infectious bites per person per day, and the transmission efficiency from mosquito-to-human ($bEIR$). EIR is calculated as the product of vectorial capacity and the fraction of mosquitoes that are infectious, $(P / (1 + acP / g))$, where $P$ is the proportion of the bites on the infected human population that infect mosquitoes ($P = cI_i, i \neq 0$). The equation describing the dynamics of the population of susceptible humans is,

\[
(6.3) \quad \dot{S} = \dot{I}_0 = B - (b + \mu)I_0 + \gamma I_1
\]

where $B$ is the birth rate, and the equation describing infected humans is,

\[
(6.4) \quad \dot{I}_j = hI_{j-1} - (b + \mu + \gamma j + \delta)I_j + \gamma(j + 1)I_{j+1}, \quad j \geq 1
\]

We assume that the population birthrate is greater than the natural mortality rate such that the population would be growing in the absence of disease. Thus as long as $B > \mu + \delta$, and $R_0 > 1$ the infection should persist and the population should grow. Results from a simulation of this model are shown in Figure 6.2.
The model assumes that individuals can be multiply infected and that the disease induced death rate is constant over the infectious period. The parameters in the model were $m = 3$, $a = 0.3$, $g = 0.1$, $n = 10$, $b = 0.5$, $c = 0.5$, $r = 1/200$, $\mu = 45 \text{ years}^{-1}$, $B = 0.03 \text{ year}^{-1}$, and $\delta = 0.0002$.

In this case there is no tradeoff with transmission success, but as has been demonstrated by others (Nowak and May 1994; May and Nowak 1995; Alizon and Van Baalen 2008), it is possible to find an optimal trade-off between disease induced death and transmission in this type of model. However, the problem with this model is that it assumes that the probability of disease induced death is constant across the duration of infection. Though the pathophysiology of malaria is complex (Phillips and Warrell 1986; Marsh, Forster et al. 1995) the probability of mortality is greatest in the initial period of time after infection before the acquired immune system can control parasite density. In other words, there is a probability of dying when initially infected, but if an individual survives the initial infection period they generally remain asymptomatically infected for a long period of time. This suggests that a more realistic model for analyzing this tradeoff may be described by the following equation for infected individuals,
\( \dot{I}_j = b(1-\varepsilon)I_{j-1} - (b + \mu + \gamma j)I_j + \gamma(j+1)I_{j+1}, \ j \geq 1 \)

where \( \varepsilon \) is the fraction of individuals that die upon becoming infected.\(^{20}\) This model assumes that each new infection carries a risk of death, regardless of prior exposure or number of infections an individual already has. To compare the results from this model with the prior formulation, we need to set \( \varepsilon \) so that the impact of disease induced death is the same. We do this by setting the \( R_0 \)'s of both models equal. The \( R'_0 \) of the second model is,

\[ R'_0 = \frac{bcV}{r + \mu}(1 - \varepsilon) \]

Setting \( R_0 = R'_0 \), we find that,

\[ \varepsilon = \frac{\delta}{r + \mu + \delta} \]

Substituting this value into the model and using the same parameters as before produces the surprising result that rather than growing, the population crashes (Figure 6.3).

\(^{20}\) We assume that the death rate is basically instantaneous as transmission from clinically ill individuals is negligible. More complex assumptions are unlikely to change the basic results.
Figure 6.3 Simulation of S-I Malaria Model with Multiple Infections

The model assumes that individuals can be multiply infected, but that the disease induced death rate is a function of the transmission rate. The parameters in the model were the same as in figure 6.2.

This result is of interest because the main parameter across which transmission is theoretically balanced is the disease induced death rate. In short infections this result makes sense as the time difference between individuals that recover and individuals that die is negligible. However, as the recovery period increases, this effectively increases $R_0$, meaning individuals are getting more inoculations and thus increasing the probability that they will die (Figure 6.4). This point is important, because it suggests that immunity plays a significant role in protecting individuals from dying. Thus, we could add an immune class that has basically no chance of dying and recover our population, but this begs the question as to what the parasite is trading off increased virulence against if there is little virulence. The seemingly obvious answer is duration, which is also a function of immunity (Marsh and Howard 1986; Biggs, Anders et al. 1992). As transmission potential is a function both of transmission probability as well as duration, there
would be a significant benefit to increased duration. The question then is how duration is related to virulence. In the next part of this chapter, we explore the role of virulence in malaria and its impact on transmission potential.

**Figure 6.4 Differences in Infection Duration**
The infection duration significantly impacts how differences in model structure pertaining to disease induced death impact the population dynamics. In figures A, C, and E, disease induced death is incorporated as a constant probability, while in figures B, D, and F, it is incorporated as the fraction of new infections that result in mortality. As the duration of infection falls from 100 days in A and B, to 20 days in C and D and finally 5 days in E and F, the differences between the model results dissipate. All the parameters in the model were the same as in figure 6.2 and 6.3 except for the recovery rate ($r$).

**Virulence in Malaria**
We first start by examining the evidence for the role of virulence in increasing the probability of transmission from mosquitoes to humans. To examine this, we used archival data of malariatherapy—the purposeful infection of humans with *P. falciparum* to treat neurosyphilis
(Collins and Jeffery 1999b). The data comprise daily records of asexual and gametocyte density in patients between 1940 and 1963. Occasionally mosquitoes were fed on patients to determine the infectiousness of the parasite. As gametocyte counts were not done every day, and because it is difficult to accurately detect gametocytes under the microscope when at low density, we compared the average gametocyte count over the seven days prior to a feed with the percentage of mosquitoes in a batch infected to determine the infectiousness. We found that while on average only ~60% of feeds resulted in transmission to mosquitoes, as the density of gametocytes increased, the proportion of mosquitoes infected increased (Figure 6.5).21 We used locally-weighted regression (LOWESS) (Cleveland, Cleveland et al. 1990) to provide a smoothed fit to the data. This increasing function was then found to be significant by ANOVA (P < .001).22

While these results are in accord with other studies showing a relationship between gametocyte density and transmission success (Jeffery and Eyles 1955; Graves, Burkot et al. 1988; Taylor and Read 1997; Barnes and White 2005; Schneider, Bousema et al. 2007), gametocytes typically represent only a fraction of the asexual parasite density in infected individuals (Bruce-Chwatt 1963; Molineaux and Gramiccia 1980). Thus, they are unlikely to contribute significantly to the virulence (morbidity and mortality) associated with malaria.

Clinical malaria (i.e. fever) is generally associated with higher parasitemia (Greenwood 1987; Gatton and Cheng 2002). As the initial period of infection in malaria is associated with the highest levels of parasitemia, the greatest likelihood of fever, and the highest temperatures

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21 Gametocyte density on the day of feed produced an equivalent result.  
22 Malariatherapy was administered either through sporozoite inoculation (which mimics natural transmission) or a blood transfusion. We found as expected that while this did impact the transmissibility of the parasite, it did not qualitatively change the relationship between gametocyte density and transmission success.
(Gatton and Cheng 2002), we hypothesized that higher initial densities, within the first 14 days of the infection, were a marker of the virulence of a parasite. Two other factors led us to select this as the best marker of virulence: (1) the maximum initial parasite density generally occurs early in the infection before the acquired immune system would be fully active and controlling density; and (2) gametocytes, the transmissible stage of the parasite, are not generally present early in the infection, suggesting there is something important about the initial interaction with the immune system. We examined how the density of asexual parasites impacted the density of the gametocytes, by calculating the average measured gametocyte density over the first 200 days of each infection and comparing it to the maximum measured initial asexual density. The results show that as the max asexual density increases, the average gametocyte density also increases (Figure 6.6). The data was fit using LOWESS (Cleveland, Cleveland et al. 1990), and was found to be significant by ANOVA at the 5% level (P = .011). These results were robust to differences
in treatment,\textsuperscript{23} inoculation route, and additional infections.\textsuperscript{24} Thus, there is evidence that our proxy for virulence, increased initial maximum asexual density, is associated with an increased transmission probability, though the weakness of the correlation suggests that other factors are likely to play a significant role.

![Figure 6.6: Relationship between Maximum Asexual Density and Average Gametocytemia](image)

**Figure 6.6: Relationship between Maximum Asexual Density and Average Gametocytemia**

As the initial max asexual density increases (measured in the first 14 days), the average gametocyte density over the course of the infection also increases. Data was fit using locally-weighted least squares regression. Source: *Plasmodium falciparum* malaria therapy data of neurosyphilis patients in Georgia and South Carolina (as described in Collins and Jeffery 1999b). William E. Collins gave us permission to use the data and Klaus Dietz sent us the data.

We next examined the relationship between virulence and infection duration. Previous reports have suggested there is a link between asexual parasite density and infection duration in the field (Mackinnon and Read 2004b). In addition, at least one report of malariatherapy suggested a link between the virulence of a particular strain and the duration of infection (Jeffery and Eyles 1955), however, to the best of our knowledge, this relationship has not been extensively examined. As maximum initial asexual density was related to average gametocyte density, we hypothesized that this may also be related to the duration of an infection. We calculated the duration of infection

\textsuperscript{23} The goal of malariatherapy was to maintain infection, thus individuals that suffered severe clinical infections were ‘inadequately’ treated to lower parasite density.

\textsuperscript{24} In a very small number of cases, individuals were inoculated with either additional homologous or heterologous infections later in the infection period.
as the last day that parasites (either asexual parasites or gametocytes) were observed and plotted this versus the maximum initial asexual density. Then we used LOWESS to fit a smooth curve to the data (Figure 6.7). The results showed a striking pattern: as the maximum initial parasite density increased, the duration of infection increased. However, as the initial density increased further, the average duration starts to fall. Using ANOVA the results for the last day parasitemia was detected were significant at the 5% level ($P = 0.02$), while the last day gametocytes were detected was significant at the 1% level ($P = 0.003$).

**Figure 6.7: Relationship between Maximum Asexual Density and Infection Duration**

As the maximum initial parasite density increases, the duration of the infection, measured as the last day parasites were observed, increases. The relationship is qualitatively the same whether we count the duration as the last day asexual parasites were observed or the last day gametocytes were observed. Data was fit using locally-weighted least squares regression. Source: *Plasmodium falciparum* malaria therapy data of neurosyphilis patients in Georgia and South Carolina (as described in Collins and Jeffery 1999b). William E. Collins gave us permission to use the data and Klaus Dietz sent us the data.
While subject to a number of caveats, the relationship between maximum asexual density and infection duration is certainly suggestive of a tradeoff function. Increasing virulence is associated with an increase in the duration of infection, but too great a level of virulence results in a shorter duration of infection. This would be the relationship predicted by the virulence transmission tradeoff, except that duration is not cut short by host-mortality. One possibility for this result is that more virulent pathogens resulted in a greater likelihood of drug therapy cutting the parasite infection duration short. However, treatment was generally used only to suppress parasitemia and not clear the infection. In fact, while most infections were modified by treatment, in only a small percentage did treatment result in removal of the parasite in less than two weeks. Restricting our analysis to those patients did not modify the relationship.

Another possibility is that treatment may have kept people alive that may otherwise have died. This would suggest that increasing virulence is associated with increasing length of the infection, but too high a level of virulence results in death. Though this conforms with the classical definition of the virulence transmission tradeoff, as noted above, this generates problematic results at the population level, and doesn’t account for the generation of immunity that results from the parasite interacting with the immune system. A third option is that the primary tradeoff that impacts virulence in the system is not host mortality, but with the immune system. In other words, there is a balance in evading the immune system through antigenic variation: replicate too slow and the immune system response will rapidly clear the parasite before it can switch to a new antigenic variant; replicate too fast and the immune response is so overwhelming that even switching is not enough to evade the immune response. It has long been noted that increasing the inoculating dose of a pathogen increases the immune response (Lefford 1971). However,
recent evidence suggests that the strength and quality of the adaptive response is impacted by the innate immune response (Pulendran and Ahmed 2006). Thus, a higher parasite level would not only trigger a larger acquired immune response, but this response would likely be enhanced by the innate immune response. In other words, though mortality likely plays a role, the major tradeoff that impacts the virulence level of a malaria parasite is the role of the immune system. In the next section we examine how this assumption would impact the population dynamics.

Testing the Role of Immunity in Shaping the Virulence of Malaria

We used the individual-based model described in Chapter 5 to examine how virulence evolves in a population. Briefly, the model is a stochastic analogue of a deterministic malaria model similar to the one introduced in this chapter. Based on the Gillespie algorithm (Gillespie 1976; Gillespie 1977), it uses an efficient computational method developed by Allen and Dythan (Allen and Dytham 2009) to simulate a population of individuals. In its base format it produces statistically rigorous solutions to a complementary set of deterministic equations. Because the model is individual-based, it is possible to examine how changes in the assumptions of homogeneity impact the population dynamics. In this chapter we examine how the virulence of the parasite evolves over time.

We use a similar formulation as in the previous chapter. The mosquito population is made up of susceptible adult females and their dynamics are described by the following set of ordinary differential equations:
\[
\dot{M}_s = mN_H - gM_s - \frac{acI_H}{N_H}M_s
\]

(6.9) \[
\dot{M}_L = \frac{acI_H}{N_H}M_s - (g + n)M_L
\]

\[
\dot{M}_I = nM_L - gM_I
\]

where \( m \) is the emergence rate, \( g \) is the natural mortality rate, \( a \) is the biting rate, \( n \) is the rate that mosquitos become infectious, and \( \epsilon \) is the transmission efficiency of mosquitos biting infected humans \((I_H)\). \( N_H \) is the total number of humans.

Humans are either susceptible \((S)\) or infected, and their dynamics are described by the following equations,

(6.10) \[
\dot{S} = B - \left( \frac{abM_I}{N_H} (1 - \psi (\rho + \delta)) + \mu \right) S + \gamma I_1 + \psi \rho \sum_{j=1}^{n} \frac{abM_I}{N_H} I_j
\]

(6.11) \[
\dot{I}_j = \frac{abM_I}{N_H} (1 - \psi (\rho + \delta)) I_{j-1} - \left( \frac{abM_I}{N_H} + \mu + \gamma j \right) I_j + \gamma (j+1) I_{j+1}, \quad j \geq 1
\]

Where \( B \) is the birth rate, \( \mu \) is the background mortality rate, \( \gamma \) is the rate infections are cleared, \( \psi \) is the probability a new infection will become clinical, and \( \rho \) and \( \delta \) are the probabilities that a clinical infection will be treated and cleared or will kill an individual, respectively.
These equations describe the base version of the model. In the simulation we allow the virulence of the parasites to evolve. This impacts the duration of infection as well as the probability that an infection will become clinical. We used the malariatherapy data to fit a function for average duration that increases as virulence, $\omega$, increases, but after a point decreases,

\begin{equation}
    d = -46\omega^3 + 527\omega^2 - 1894\omega + 2188
\end{equation}

As the best fit to the duration data was a cubic spline, we assumed that when $\omega < 2.8$, we used a linearly increasing fit from zero for $\omega$ values below this. The expected recovery rate for a particular parasite is then equal to $1 / d$. The probability of transmission is also based on $\omega$, but accounts for the time since infection, which is assumed to exponentially decrease, thus, the probability is calculated as,

\begin{equation}
    \phi = \frac{1}{1 + 50\exp(-1.4v)}
\end{equation}

where $v = -0.5 \ln(\text{time since infection}) + \omega$. Equation 13 is based on the fit of the probability of transmission from the malariatherapy data.

Lastly, we account for immunity by assuming that the probability that an individual will become symptomatic after an inoculation is related to the number of infections that they have already received. Thus, with each infection their probability of becoming clinical falls to a baseline, after
which the probability is constant. This is then modified slightly by the virulence of the parasite. As virulence increases, the probability of becoming clinically sick increases, and with some probability clinical symptoms lead to death, otherwise with some additional probability the individual seeks treatment. Treatment is assumed to effectively clear all parasites immediately.

Transmission of the parasite occurs when mosquitoes bite humans. We assume that in each transmission event only a single parasite is transmitted, but that the parasite can mutate to become more or less virulent. Mutation increases or decreases are assumed to be normally distributed with a mean of 0.05 and a standard deviation of one.

Results
We first ran the model assuming that individuals could not be multiply infected. Thus, the only competition the parasite faced was between hosts. We simulated the model hundreds of times and calculated the mean and standard deviation of the mean level of virulence (Figure 6.8). Because of the long duration of infection, a large diversity of parasites virulence levels are supported, however, average virulence is fairly high, suggesting that transmission in this case is relatively more important than infection duration.

We then simulated the model with multiple infections. There was no assumption about the number of infections an individual could have, but the probability of transmission for any parasite was related to its probability of transmission, which as noted above was highest at initial infection and fell as the infection progressed. Similar to the single infection case, a large diversity of parasites was accommodated; however, the average virulence level was actually less than in the
single infection model (figure 6.9). This reflects an interesting paradox of within-host competition under this type of tradeoff: as the number of infections in an individual increases, the probability of any single one being selected at a particular transmission event goes down. Thus, even though being more virulent increases the probability of transmission, increasing the duration of infection is relatively more important than increasing the probability of transmitting at any single transmission events. This then leads to the surprising result that adding drug treatment actually increases the virulence of the pathogen (figure 6.10). This occurred, because most infections do not lead to clinical symptoms. As modest changes in virulence have little impact on the rate that infections become clinical (as most people are immune), the biggest impact of treatment is to lower the average number of infections in an individual. As reducing the number of infections increases the probability of transmission for each parasite, the result is that transmission becomes more important than duration relative to the non-treatment case.

A significant benefit of the individual based model is the ability to examine the impact of parasite evolution across multiple axes. Previous models have examined the impact of superinfection on drug resistance, but here we examine the impact of the emergence of drug resistance on the virulence of the parasite. Similar to the previous chapter, we allow the system to equilibrate, and then introduce resistance as the probability that an individual with an infection has one of their parasites become drug-resistant. Drug resistance is assumed to be mediated by a single mutation that confers complete resistance to drugs. Because the introduction of resistance is stochastic, we measured time from the introduction of resistance. While the initial virulence of the resistant parasite is random, as we select a random parasite from a random individual, the
average virulence of the resistant parasite quickly increases above that of the sensitive parasite (Figure 6.11). This makes sense for two reasons: (1) increased virulence increases the probability of clinical symptoms, which aids the resistant parasite by removing competitors; and (2) treated individuals with resistant infections will have fewer infections on average, which reduces the pressure to maintain a long infection. However, both of these are only advantages when resistant parasites are rare. Once they start increasing in frequency, the benefits of being more virulent decrease.

**Figure 6.8: Evolution of Parasite Virulence in Single Infection Model**
Parasite virulence rapidly evolves to an optimal level, though a large diversity of virulence levels are supported.
Parasite virulence evolves to an optimal value, however this level is below the single infection case as the duration of infection becomes more important relative to the probability of transmission at any single bite.

Treatment reduces within-host competition by decreasing the mean MOI. This results in a marginal increase in mean virulence.

Data is average virulence for drug-resistant and drug-sensitive parasites for each 1,000 runs once resistance emerges in population. As resistance emerges, on average, drug-resistant parasites are more likely to be more virulent than drug-sensitive parasites. However, as the proportion of the population with resistant parasites increases, the average virulence decreases.

If everyone in the population is assumed to have a base level of immunity, so that there is no non-immune individuals, the average virulence of the resistant parasite is higher when it emerges.
Discussion

The evolution of virulence is an important question in ecology and evolutionary biology. The emergence of the classical tradeoff theory between virulence and resistance was a significant advance as it suggested a reason why parasites may not become benign. However, empirical evidence for this tradeoff has been equivocal. A number of reasons for this have been advanced, but none have focused on the direct role of infection duration. The original theory, as described by Anderson and May (Anderson and May 1982) as well as Ewald (Ewald 1983), understood that the duration of infection was a key aspect of this tradeoff; however, the main mechanism assumed for limiting this duration was host-mortality. While Frank and Schmid-Hempel (Frank and Schmid-Hempel 2008) suggest that virulence may tradeoff against other factors, including immune modulation, in general the classical theory that host-mortality is the main tradeoff continues to hold sway.

However, a single point of tradeoff does little to explain virulent diseases that have long infectious periods. Malaria in particular poses a challenge as it is most virulent at the onset of infection, and yet maintains a long infectious period. This is contrary to suppositions on how parasites with long infectious periods should evolve (Frank and Schmid-Hempel 2008). Frank and Schmid-Hempel maintain that parasites gain by reducing the probability of clearance earlier in an infection in exchange for an increase in virulence later. Yet, both in an individual patient, where infections are most virulent at the outset, and across patients, where young children are most likely to die, malaria seems to have an opposite pattern. Using data from the purposeful inoculations of individuals with malaria, we suggest that increased virulence in malaria at the
onset of infection is related both to increased transmission probability (through the production of more transmissible stages) and increased duration of infection.

These results are subject to a number of caveats, yet, they point to a potentially significant area of research on the role of infection duration and virulence. In particular, it suggests that in some parasite host situations, the manifestation of virulence in clinical symptoms and possible death is a means to achieving longer infections by engaging the immune system. In other words, the larger the immune response triggered by the parasite, the longer the infection can persist. However, at some virulence level, the immune response is so great that it can actually overwhelm the parasite and the infection cannot persist for long periods. Thus, host mortality is more a side-effect of competition with the immune system rather than a direct regulator of virulence.

Simulations with this type of trade-off showed that for *P. falciparum*, an optimal level of virulence could be achieved fairly quickly. Importantly, we found that host morbidity and mortality rates had little impact on the results. Changes in the rate that clinical symptoms occurred also had no significant impact on the average virulence unless they were increased to levels that would lead to the extinction of either parasite or host. On the other hand, non-immune individuals, who have a greater likelihood of clinical symptoms, reduced the average virulence of the resistant parasite slightly when it emerged as compared to the case with all individuals having the same probability of clinical symptoms (Figure 6.12). This is because immune individuals have a higher MOI, which increases the benefit of causing clinical symptoms. The constancy of the population, in terms of immunity, also created a more constant reduction in the average virulence level.
The results were somewhat surprising though. We found that in the case where an initial parasite infection could exclude further infections in a host (single infection case), the parasite had a higher average virulence level. This is the opposite conclusion that is derived from the traditional hypotheses of the virulence transmission tradeoff (May and Nowak 1994; May and Nowak 1995; van Baalen and Sabelis 1995), where the assumption is that increasing within-host competition will increase the virulence of the pathogen because within-host competition becomes more important relative to host preservation. The different result though is due to the mechanism of trade-off. Because we assume that a single parasite can monopolize a host and exclude other parasites, that parasite will get all the transmission opportunities, whatever the length of the infection. Thus, increasing the probability of transmission at each bite becomes more important as there are fewer opportunities to transmit due to the limited number of susceptible individuals. The result was a duration that was nearly half of the multiple infection case (Figure 6.13). However, when the parasite can no longer exclude other parasites, and thus is engaged in within-host competition, the probability of transmission per bite goes down significantly. In this case, maximizing duration becomes relatively more important than the probability of transmission. The result is that increasing the number of infections in this context actually selects for lower virulence. The reverse of this is seen when treatment is added. In this case, treatment reduces the average MOI, thus parasites in multiple infections have less competition, which increases the benefit of each transmission opportunity and thus results in an increase in virulence. This difference in virulence between single and multiple infections suggests that parasites that can exclude other parasites, or limit their transmission opportunities, would select for higher virulence levels.
The evolution of drug resistance also had an impact on the average level of virulence. As drug resistance emerged in the population, on average, resistant parasites tended to be more virulent. This is due to two factors: 1) increased virulence leads to an increase in the probability of clinical symptoms; and 2) because drug treatment clears out all the sensitive parasites, the resistant parasite faces significantly less within-host competition for a bit. In the case of the former, this is beneficial when the drug-resistant parasite is in competition primarily with drug-sensitive parasites as it can clear out all competitors. With few or no competitors, the advantage of an increased virulence level is furthered because this increases the probability of transmission per bite. However, as the number of individuals harboring resistant parasites increases, these advantages quickly disappear, and the average virulence level begins to fall. This result suggests that when drug resistance begins to emerge in an area dominated by drug-sensitive parasites, morbidity and mortality is also likely to increase. This result is not just theoretical, field reports suggest that as CQ-resistance spread, there were increases in the number of clinical cases of malaria (Bødker, Kisinza et al. 2000; Shanks, Biomndo et al. 2000; Craig, Kleinschmidt et al. 2004) as well as increases in morbidity and mortality risk (Zucker, Lackritz et al. 1996; Trape, Pison et al. 1998b). Data from these areas suggested that these increases were not just due to a failure to clear infections, but that there was a linkage between the resistant parasites and the increased morbidity and mortality seen. However, once resistance has become widespread, resistant parasites may actually be less virulent than drug-sensitive parasites, as predicted (Giha, Elbashir et al. 2006).
Evidence from bacterial infections suggests that this result may be more general. For instance, the first reports of community-associated methicillin-resistant *Staphylococcus aureus* (CA-MRSA) were of young pediatric deaths (Centers for Disease Control and Prevention 1999), and increasing reports of virulent CA-MRSA infections led many to speculate that there was a link between drug resistance and virulence (Chambers 2005). And as CA-MRSA spread rapidly through the population, evidence linking specific factors, such as Panton–Valentine leukocidin (PVL), to virulence were reported (Chambers and DeLeo 2009). However, as the epidemic progressed, further testing suggested differing results, and no conclusive evidence for a link between them could be found. In addition, the majority of infections caused by CA-MRSA were likely non-invasive skin and soft-tissue infection (Klein, Smith et al. 2007; Klein, Smith et al. 2009). While numerous factors, including host immunity and susceptibility, play a role in the virulence of a pathogen, the numerous reports of highly virulent CA-MRSA strains that occurred primarily at the outset of the epidemic, when it was primarily in competition with drug-sensitive strains of *S. aureus*, conforms to the prediction that when drug resistance first emerges there is a benefit, when pathogens compete in a host, for the drug-resistant parasite to be more virulent. However, once the pathogen becomes more common, this benefit disappears.

Our results are of course subject to a number of caveats. In particular we have generalized virulence in malaria, the clinical nature of which encompasses a number of different possible conditions likely encoded by multiple genetic factors (Greenwood, Marsh et al. 1991; Marsh, Forster et al. 1995). In addition, we calculated our expected duration based on infections of naïve individuals. The development of clinical immunity, which is characterized by lower levels of
parasitemia and a reduced probability of clinical disease (Bull and Marsh 2002), is likely to play a major role in how duration and virulence interact. Thus, while we assume that infection duration is predicated on the virulence level of the parasite, factors such as prior exposure and clinical immunity likely make this relationship more complicated. However, as we noted, the probability of death in malaria is greatest on initial infection, as infections are long, and as individuals quickly develop immunity to severe disease (Gupta, Snow et al. 1999), we feel that host mortality is very unlikely to be a regulator of virulence in malaria. Thus, while the shape of the trade-off is certainly an open question, we feel that our results demonstrate the qualitative impact of such a trade-off within a host on the dynamics at the population level.

![Figure 6.13: Virulence and Duration](image)

**Figure 6.13: Virulence and Duration**
The curve is the assumed tradeoff between virulence level of parasite and the duration of infection. The average level of parasite virulence at equilibrium for the single infection case (■), the multiple infection case with no treatment (●) and the multiple infection case with treatment (▲), are shown on the curve.
Summary
Based on malariatherapy data, we suggested that the major trade-off for virulence in malaria is the duration of infection mediated by the interaction with the immune system. Using an individual-based model of malaria that is a stochastic analogue of a set of well-examined deterministic equations for malaria, we examined how this tradeoff may impact the virulence level of the malaria parasite. We then examined how such a trade-off might alter the dynamics of infection in a population, and suggest that some of the conventional wisdom associated with the virulence transmission tradeoff can be quite different when the tradeoff is primarily over duration, which helps to explain some of the contradictory findings when looking for evidence of the virulence transmission tradeoff. Lastly, we showed that when drug resistance is emerging in a population, this could increase the virulence of the pathogen, but once resistant parasites were common, the benefit of being virulent would be lost and average virulence would fall.
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