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POPULATION-SIZE DEPENDENCY OF ATOVAQUONE RESISTANCE IN *P. CHABAUDI* MALARIA

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ABSTRACT

In choosing treatment regimens that best manage drug resistance, it is important to understand the differences between the origin of resistance and the spread of resistance through microbial populations. It is well known that once resistance has arisen, strong drug use places selective pressure on microbes and leads to the proliferation of resistant phenotypes, often over short treatment periods. However, we know little about the factors that contribute to the origin of drug resistance, particularly within hosts. In this study using an *in vivo* rodent malaria model, we test the basic population genetics principle that mutational events occur more frequently in larger populations, and this contributes to increased rates of evolution (i.e. rate of resistance emergence). Mice were inoculated with equal parasite counts and subsequently drug treated at varying time points to simulate different population sizes, allowing us to examine the effects of population size on resistance emergence. Using the rodent malaria model and the antimalarial Atovaquone, we found that resistance emergence does depend on parasite population size: proportion of mice harboring resistant parasites increased with parasite densities at the onset of treatment. Thus, treatment of larger parasite populations is more likely to lead to resistance emergence. We hypothesize that this may be due to an increased genetic load found within large populations, increasing the probability of the parasite population containing a resistance-conferring allele.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

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1. Introduction

1.1 Malaria Overview

Malaria is a disease caused by a protozoan parasite of the genus *Plasmodium* that spends its life cycle alternating between the *Anopheles* mosquito vector and its host. The disease, intimately tied to the evolution of mammals (***1***), is a common topic of research due to its detrimental effects on human populations throughout much of Africa and Southeast Asia. In 2014 alone, there were almost 200 million cases of malaria diagnosed with many more unreported, and 600,000 deaths (***2***). Anopheles mosquitoes ingest haploid *Plasmodium* gametocytes in blood meals, which then progress through the mosquito midgut to the site of fertilization and sexual development. There, they fuse and mature into ookinetes that passage the midgut to the exterior midgut, causing potential damage to the vector (see Chapter 4 for pathogen effects on vector survival) (***3***). Ultimately, these ookinetes develop into oocysts, which then produce haploid sporozoites (the sexual transmission stage of the parasite) that travel to the mosquito salivary glands for transmission. Sporozoites are then transmitted upon taking a blood meal from the host and progress to the liver, where they replicate before developing into haploid blood-stage merozoites. This stage of the pathogen's life cycle is most often associated with malaria symptoms and the stage of drug treatment (see Chapters 2-3) before the merozoites develop into haploid gametocytes, completing the life cycle. With no effective vaccine to date, new drug development and combination therapies are in constant demand to combat the debilitating effects of malaria. However, due to widespread mismanagement of drugs, resistance has become rampant.

1.2 Emergence versus Spread

The introduction of antimicrobials in the early twentieth century started a revolution in the treatment of diseases previously thought to be terminal. However, as drug after drug has been invented and introduced into society, the emergence and spread of drug resistance in microbial populations has followed. While time to the emergence of drug resistance varies greatly across different drugs and their applications, the subsequent spread of resistance poses a formidable and frightening challenge for the future. Effectively analyzing the resistance patterns across different pathogens and drugs however requires a universal vernacular so that resistance can be better communicated across fields for developing resistance management strategies. In current literature about drug resistance, there exists a large misuse of two terms commonly used to describe drug resistance: emergence and spread. These words, to some degree, are used synonymously, yet they represent two vastly different and fundamental elements of resistance. Here, the specific differences between the two terms will be clarified as they are relevant in later chapters.

Emergence

Emergence is fundamentally the "initial genetic event which produces the resistant mutant" (***4***). This event, a mutation, is extremely infrequent and can be influenced by multiple factors. Often, it is simply correlated with the normal error rate of genome replication machinery. In malaria parasites, the frequency of the necessary mutations leading to resistance to the antimalarial Atovaquone is estimated at 1 in 10^{12} (***5***). In a population of microbes, the probability of obtaining a resistant individual is therefore scaled with the size of the population. If an infection reaches a peak density of 10^{13} parasites, that means that at that time, roughly 10 parasites will harbor

resistance mutations to Atovaquone. Any subsequent selection of the resistance based upon improved fitness in competition with susceptible pathogens is not related to emergence.

Several factors can affect the rate at which the emergence of resistance occurs. In a competitive or stressful environment, certain organisms, such as *E. coli,* are known to exhibit stress-inducible mutagenesis (***6***). For these organisms, the most common mode of inducible-mutation is through the SOS response as a result of DNA damage (e.g. UV radiation) or a stoppage of transcription. The mutagenesis results from the activation of error-prone DNA polymerases by the SOS response, leading to base pair substitutions during repair and both new proteins as well as new protein expression patterns should the changes occur in regulatory DNA regions. It has also been found that antibiotics such as ciprofloxacin can serve as mutagens to pathogens (***7***). Ciprofloxacin is a Fluoroquinoline that inhibits topoisomerases, which is known to induce the SOS response (***6***). The necessity of this pathway to the development of resistance was supported by an experiment where specialized *E. coli* cells in culture and the murine model carried an uncleavable transcription repressor that prevents induction of SOS genes (***8***). These *E. coli* cells were unable to develop resistance to ciprofloxacin, showing that in some cases antibiotics act as mutators.

Similar antibiotic-induced resistance has been found with *M. tuberculosis* resistance to rifampicin (***6***), *Pseudomonas aeruginosa* resistance to ciprofloxacin (***8***), as well as resistance to several beta-lactam antibiotics (***9***). In all of these instances, the SOS response leads to increased expression of error-prone polymerases, which supports the notion that the SOS response is important for the acquisition of antibiotic-resistance in bacteria. The experimenters thus

suggested that antibiotics be supplemented with drugs that inhibit the mutagenic process to prevent resistance emergence.

Spread

While emergence deals entirely with the evolution of resistance through mutation, the stage at which resistance becomes a larger issue is the spread of that resistance. In this process, the survival advantage conferred by resistance in the presence of a drug leads to the selection of those microbes and the spread of that phenotype in a population. Many factors affect the selection of resistance, including population size, drug concentration, specific drug resistance mechanisms, fitness benefits and costs of resistance, resistance stability, and host immunity. Once resistant pathogens emerge, they face increased competition for resources in order to spread, resulting in a tradeoff for selection dependent on population size.

Effects of drug dosage on the emergence and spread of resistance

A more controversial topic is that of drug dosage for the treatment of infections. There are two main ideologies on the course of drug treatment most likely to reduce the incidence of drug resistance (***4***) (***10***) (***21***) (***11***). The first is that an increased antimicrobial dose will prevent the spread of any resistant pathogens as a high dose will kill all pathogens, including those that are partially resistant. Such an approach is ideal for preventing the emergence of partial resistance as it kills off large populations of pathogens rapidly. However, if strong resistance is already present in a population even at low levels, strong drug selection could more rapidly lead to resistance spread. This has been found in the agricultural sector with fungicide applications as well as with malaria resistance to drugs in the murine model (***10***) (***12***) (***13***). The second idea

focuses on the use of moderate antimicrobial doses to combat that incidence of resistance already within a population. While a moderate dose of drug would not kill all pathogens (even sensitive strains), resistant organisms would be forced to compete with surviving sensitive microbes. Because resistance often comes at a fitness cost through the alteration of metabolic pathways, resistance could then be selected against and eliminated from the gene pool through normal competition for resources (***14***). The worrisome aspect of this approach is the potential for an increase in overall pathogen load resulting from insufficient drug treatment, leading to more severe symptoms for a patient, all in an attempt to decrease the spread of resistance. A final factor affecting resistance selection is host immunity, which can greatly alter the dynamics of an infection with resistant pathogens. In different scenarios, host immunity can suppress or enhance the selection of resistance (***4***).

The improper usage of emergence versus spread in published literature can be confusing and detrimental to the planning of resistance management strategies where in-depth and comprehensive understanding is a requirement, particularly with experiments examining resistance emergence in populations. That treatment strategies could be utilized to combat the emergence of resistance in itself is rather novel, as more common modes of treatment focus on controlling the spread of resistance. This will be a topic of focus in Chapter 3, and understanding drug dosing effects on resistance will further prove important for understanding the experiments discussed in this thesis.

1.3 The Anti-Parasitic Drug Atovaquone

A drug of specific focus throughout this thesis is the antimalarial Atovaquone. Atovaquone, a hydroxynapthoquinone derivative, is a common antimicrobial drug commonly in use today to treat malarial infections as well as AIDS-related protozoan and fungal infections (***15***). It was first developed over 60 years ago following the outbreak of World War II, when a prominent rise in microbial infections caused a widespread shortage of the drug quinine, which was used to treat many bacterial and parasitic infections at the time (***16***). Prior to the development of Atovaquone, only a few drugs dominated the antimalarial treatment landscape. The first prominent malaria drug was quinine, first identified by the early indigenous Quechua of Peru and Bolivia, and later brought to Europe by Jesuit explorers (***17***). Due to its importance in treating malaria, quinine is on the WHO List of essential medicines (***18***). Quinine modifications have resulted in several other prominent antimalarial compounds including Mefloquine and Chloroquine. Over 300 Quinone-derived drugs were synthesized alone, many of which were found to have antimicrobial activities of varying degrees *in vitro*. However, many of the drugs were found to be ineffective when administered orally to trial subjects, save for a couple of compounds (***19***). Due to a reliance on naturally occurring stocks of quinine derived from the cinchona tree as well as the development of resistance to many derivatives, further drug development was necessary. One of the drugs developed was Atovaquone. In addition to showing aggressive activity against malaria and other protozoans, Atovaquone was proven to be particularly promising as it was not reactive with liver proteins or endosomes and was also effective against several drug-resistant forms of *Plasmodium falciparum* malaria (***19***).

The spread of Atovaqoune was particularly important as the worldwide incidences of both HIV/AIDS and malaria have not decreased as rapidly as previously hoped, with infection rates only dropping approximately 30% for both diseases since the year 2000 (***20***). While Atovaquone was primarily used as an AIDS treatment early on in its development, today it has become a foundational drug for many modern antimalarial treatments, and is a component of the antimalarial of choice (Malarone) for over 70% of Westerners that travel to malaria-endemic regions (***16***). Unfortunately, Atovaquone resistance develops rapidly when Atovaquone is used as a mono-therapy in up to a third of patients due to the low number of SNPs required to achieve resistance (***5***) (***21***), and thus it is now commonly used in combination with the antifolate Proguanil (composing Malarone), drastically reducing the incidence of resistance emergence (***22***).

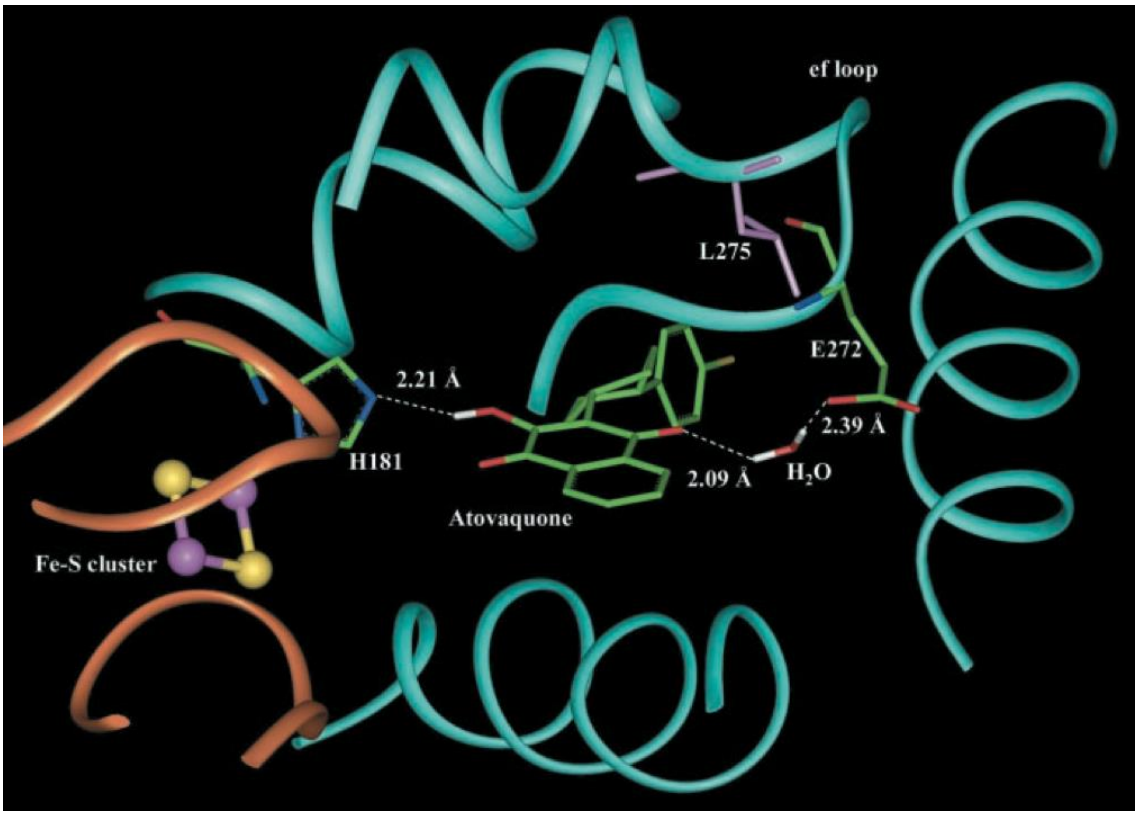

Figure 1. Atovaquone binding site in the cytochrome bc_1 complex **(***23***)**

Atovaquone acts by binding the cytochrome bc_1 complex of the electron transport chain, necessary for the production of adenosine triphosphate (ATP). This enzyme complex catalyzes the transfer of electrons between two components of the electron transport chain, ubiquinol and cytochrome *c*. Atovaquone binds to the Rieske iron-sulfur protein in the ubiquinol oxidation pocket at the center of the complex, acting as a competitive inhibitor as determined using a *Saccharomyces cerevisiae* competitive inhibitor called stigmatellin (***23***). By binding to the oxidation pocket, Atovaquone blocks the transfer of electrons through the chain, thus collapsing the electric potential of the electron transport chain, even at very low (nanomolar) concentrations (Figure 1). Specifically, the hydroxyl group of Atovaquone (Figure 2), hydrogen bonds to a histidine residue in the iron-sulfur protein, while its carbonyl group participates in hydrogen bonding with a glutamic acid residue on cytochrome *b* (***23***). In the intraerythrocytic stage of the malaria lifecycle, the mitochondrial respiratory chain is utilized to re-oxidize a complex involved in the pyrimidine biosynthetic pathway. When the membrane potential is collapsed by Atovaquone, pyrimidine biosynthesis is thus halted. While effective as an inhibitor against cytochrome bc_1 complexes for many primitive eukaryotes and thus killing the microbes, Atovaquone is not effective as an inhibitor for the complex in human cytochrome and does not kill human cells (***24***).

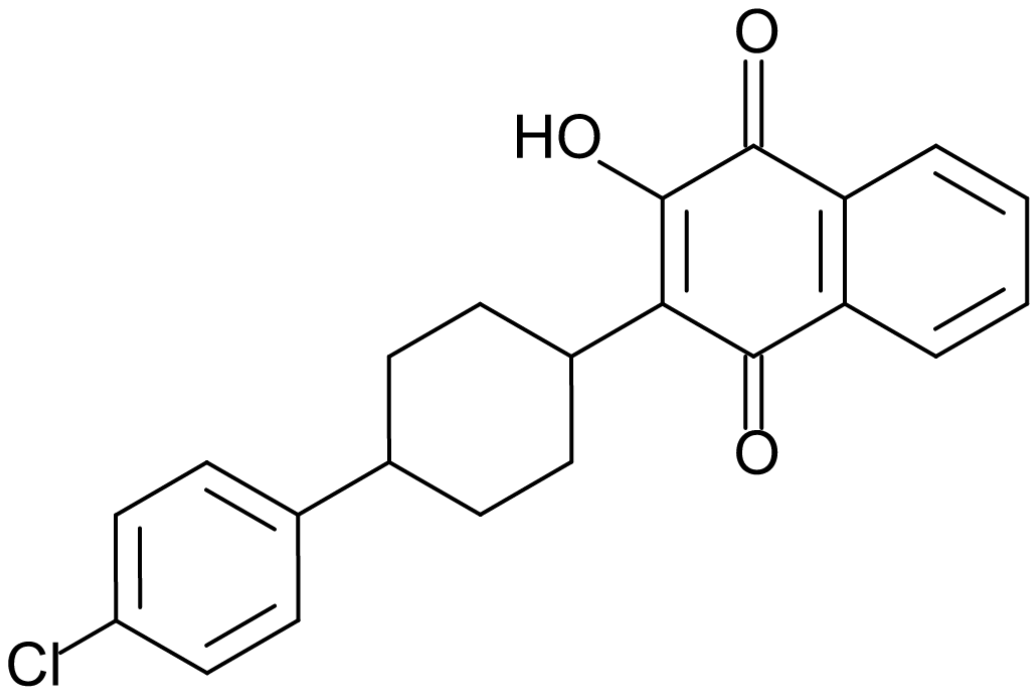

Figure 2. Atovaquone structure

Unfortunately, malarial resistance to Atovaquone, unlike the case with many other antimalarials such as Artemisinins and Antifolates (e.g. Pyrimethamine), arises rapidly in malaria parasites. This is potentially due to the simple molecular mechanism of resistance, as even a single nucleotide polymorphism (SNP) allows the parasite to survive in the presence of high levels of the drug (***25***). As a result, any small degree of resistance will provide a huge fitness benefit to the malaria parasite, allowing for rapid spread of the mutated parasite in any infection environment. There is still some debate as to why resistance to Atovaquone develops so quickly with a simple molecular mechanism of drug resistance, inadequate treatment levels, and low levels of immunity all suggested as potential explanations (***22***). It has even been suggested that Atovaquone itself could act as a mutagen that induces resistance emergence due to its mode of action in the mitochondria (***26***).

Through Atovaquone's mechanism of action in the mitochondria, the resulting collapse of the electron transport chain leads to the production of reactive oxygen species. Such molecules have the ability to mutate the resident mitochondrial DNA (mtDNA), which contains the genes necessary for transcribing the cytochrome bc_1 complex. The reactive oxygen species are produced as reduced proteins accumulate due to the stoppage of the electron transport chain, and thus molecular oxygen is reduced to form super-oxide radicals (***25***). The mode of replication of mitochondrial DNA in malaria is drastically different from that of humans, as gene conversion and recombination amongst all mtDNA copies present occurs (in contrast to humans where only one mtDNA genome is present per mitochondrion). Once resistant mutants arise, the conferred resistance makes those parasites more competitive and the mutant mtDNA will be selected for and proliferate throughout the population. This could explain the high sequence conservation of resistance SNPs in malaria populations (***22***).

Observed resistance to Atovaquone is concentrated in the cytochrome *b* gene of malaria parasites, with one or two base pair changes conferring resistance that can increase a mutant's fitness up to 25,000 fold over wild-type parasites (***25***). Mutations conferring Atovaquone resistance differ across different species of *Plasmodium* and further multiple SNPs have been found together in a single genotype, indicating the diversity of resistance that can emerge to Atovaquone. Of the 12 observed SNPs causing resistance, 11 were the result of a G:C base pair replacing an A:T base pair. This likely results from the production of 8-oxo-guanine nucleotides by reactive oxygen species present in the mitochondria that can base pair with adenine when present in DNA, resulting in the change (***25***). An aromatic amino acid at position 267 and a

lysine at position 272 are required for Atovaquone sensitivity. The observed changes conferring resistance alter the hydrophobic amino acid residue as well as the size of the normal Atovaquone binding pocket, thereby reducing the affinity of the complex for Atovaquone as well as the access of Atovaquone to the binding pocket. As a result of this observed resistance, Atovaquone was found to be unsuitable as a mono-therapy, and is only currently used in combination with Proguanil.

Experiments examining the emergence of resistance to Atovaquone based upon drug-treated population size as well as the potential ability of drug combinations involving Atovaquone to delay resistance emergence are discussed in chapters 2 and 3 respectively. While particular aspects of each experiment and their backgrounds are re-emphasized at the beginning of each chapter, an overall understanding of Atovaquone's target in pathogens as well as the specifics of Atovaquone resistance will help to better understand the context in which the experiments were designed. Additionally, distinguishing between the spread of resistance and the emergence of resistance is crucial when thinking about the results in each chapter.

2. Population-Size Dependency of Atovaquone Resistance in *P. chabaudi* Malaria

2.1 Introduction

Mathematical models have demonstrated that in larger populations, the greater genetic diversity present in that population has a propensity to generate resistant mutants at a faster rate than in smaller populations (***27***). Thus, the ability of larger populations to respond to selective pressures such as drug treatments is often increased due to a higher incidence of resistant mutants. In the absence of any stressors, normal replication errors are what lead to mutant emergence, which intuitively has a higher probability of occurring in larger populations simply due to an increased number of replication events. It can be explained perhaps more simply as there is an increased mutation supply rate as population size increases (***28***). An important term in such scenarios is evolutionary rescue, or the ability of a population to evade extinction following environmental stressors through subsequent adaptation or mutation (***29***). Several past studies have demonstrated an increasing rate of adaptation for both bacteria and viruses in larger populations versus smaller populations, fed by greater genetic diversity in those populations. In eukaryotic yeast (*Saccharomyces cerevisae*) exposed to salt stress, a clear population size threshold below which evolutionary rescue from extinction has been established (***30***), while in *Pseudomonas fluorescens* treated with antibiotics, similar results indicate that evolutionary rescue has a higher likelihood of occurring when higher levels of genetic diversity are present at the time of drug treatment (i.e. larger population size) (***31***). Important factors to consider when understanding the emergence of resistant mutants include population size, the initial number of resistant mutants in a population,

the ability of those mutants to establish in the environment (competition with sensitive strains and decreased fitness from resistance mutations), and the selective pressures placed upon the population by external stressors such as drugs.

Although it is well understood how resistance proliferates and spreads through populations, it is equally important to understand how environmental context affects resistance upon emergence. Previous studies have established population size as a critical factor in the ability of populations to evolve resistance, although no such study has been conducted in a more realistic *in vivo* system. Here, a study was conducted that examines such an effect on an *in vivo* rodent malaria model and whether or not a cutoff threshold might exist for a population's size before resistance can be established. Parasite population sizes were manipulated by drug treating at different times in order to alter the approximate total number of mutations that would exist in each population. The common antimalarial Atovaquone, for which the resistance genotypes and mechanism of resistance has been determined, was utilized to stress a population of parasites in mice. The experiment was designed to demonstrate whether underlying resistance profiles in the initial populations of infections are responsible for resistance emergence or if this is a result of increased genetic diversity as populations grow in size.

2.2 Materials and Methods

Experimental Design

AS clones (reference code AS13P) of *Plasmodium chabaudi* previously unexposed to Atovaquone were utilized to infect naïve Swiss Webster mice. These parasites were originally

collected from naturally infected thicket mice in the Central African Republic and maintained in liquid nitrogen at the Pennsylvania State University (***32***). Parasites were introduced via 100μL intraperitoneal injections into 35, 6-8 week old Swiss Webster mice, with inoculations prepared from donor mice and dissolved in citrate saline solution. Mice were also given 0.05% PABA drinking water to enhance parasite growth (***33***). Drug treatments containing 4mg/kg of Atovaquone dissolved in DMSO were administered via 100μL intraperitoneal injections. Five experimental groups composed of seven mice were drug treated on either days 3 and 4, 5 and 6, 7 and 8, 9 and 10, or 11 and 12 post initial infection to ensure treatment hit the parasites at different population sizes (Figure 3). Recrudescent infections were passaged at peak parasite density into naïve mice and denoted as secondary infections. Recrudescence is where parasite populations re-emerge at a later point, seeded by a few surviving pathogens, after an initial decline following drug treatment. This was done in an effort to understand the phenotypes of any resistance that had emerged following drug treatment. Secondary mice were drug treated with 10 mg/kg of Atovaquone on days 3 and 4 post-infection and monitored from day 2 post infection until day 7.

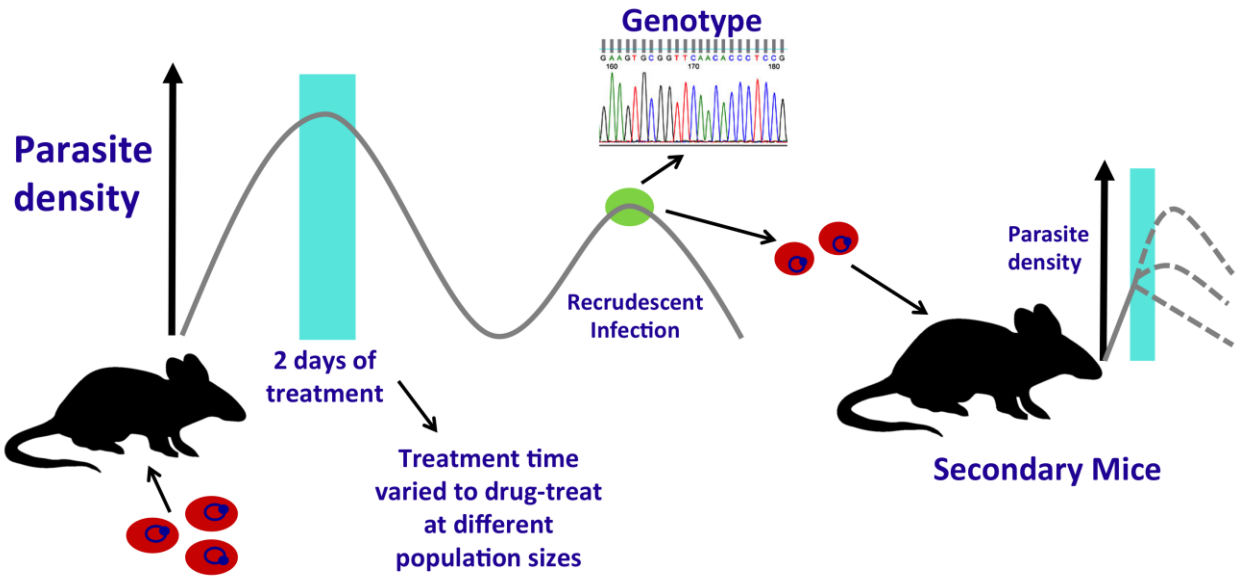

Figure 3. Experimental Design

Mice were infected with *P. chabaudi* malaria and drug treated for two days with 4mg/kg of Atovaquone on different days dependent upon treatment group to ensure drug treatment of different population sizes. Recrudescent infections were both genotyped at the cytochrome *b* locus for known Atovaquone resistance SNPs as well as passaged into naïve secondary mice and drug treated at 10mg/kg to determine the phenotypic strength of resistance.

Drug Treatments

Drug treatments were prepared from Atovaquone obtained from Sigma Aldrich (A7986) dissolved in DMSO and stored at -20˚C between treatments. All drugs were administered via intraperitoneal injections in the morning on the day of treatment.

Monitoring Experiment

Mouse red blood cell counts and weights were recorded daily starting on day 3 and continued until day 35. Parasite densities were determined via DNA extraction and quantitative PCR of 5µL of blood samples obtained during daily monitoring and frozen down at -80˚C. DNA extractions were performed on the ABI Prism 6100 Nucleic Acid PrepStation according to

manufacturer's instructions, utilizing the blood extraction protocol. DNA was eluted to a volume of 200µL and frozen down to -80˚C for PCR. PCR primers were designed to target the *P. chabaudi ama* gene and real-time quantitative PCR was run on an ABI Prism 7000 (see (***34***) for qPCR and analysis protocol). Red blood cell densities were determined using flow cytometry on a Beckman Coulter Counter. Daily blood smears were also taken to elucidate approximate parasite counts throughout the duration of the experiment.

Recrudescent Infection Genotyping

Peak recrudescent blood samples were utilized to determine the resistance genotypes of parasites present. A portion of the cytochrome b gene was sequenced using standard Sanger sequencing, as Atovaquone resistance has been observed previously in this region in a 422 base pair segment of the 1.3kb coding region (***25***). Upon obtaining results, the sequences were aligned utilizing MEGA version 6 (***35***) and electropherograms were viewed using 4Peaks to examine whether or not parasite mixed infections (different resistance mutations) were present in the blood samples. When one or more peaks were present in the electropherogram whose peak heights were greater than or equal to 25% of the strongest peak height, the infection was denoted as a mixed infection.

2.3 Results

Dependency of Resistance Emergence on Population Size

The experiment was designed to demonstrate whether increased parasite loads (and likely increased genetic diversity) are associated with a greater probability of developing a resistant infection. Eight recrudescent infections were observed in mice during the experiment: two in

mice drug treated on days 9 and 10 and six in mice drug treated on days 11 and 12 (see Figures 12-14). No recrudescent infections were observed in any of the other treatment groups where drug treated population sizes were smaller. The proportions of infections that resulted in recrudescent infections across the five treatment groups were compared utilizing a generalized linear model in R statistical computing software (version 3.2.3). The results show that the proportion of recrudescent infections increased as drug treatments were applied further from initial infection ($X^2 = 23.213$, $p << 0.001$, Figure 4). Thus, the proportion of mice infected with resistant parasites increased in mice drug treated at higher parasite population sizes.

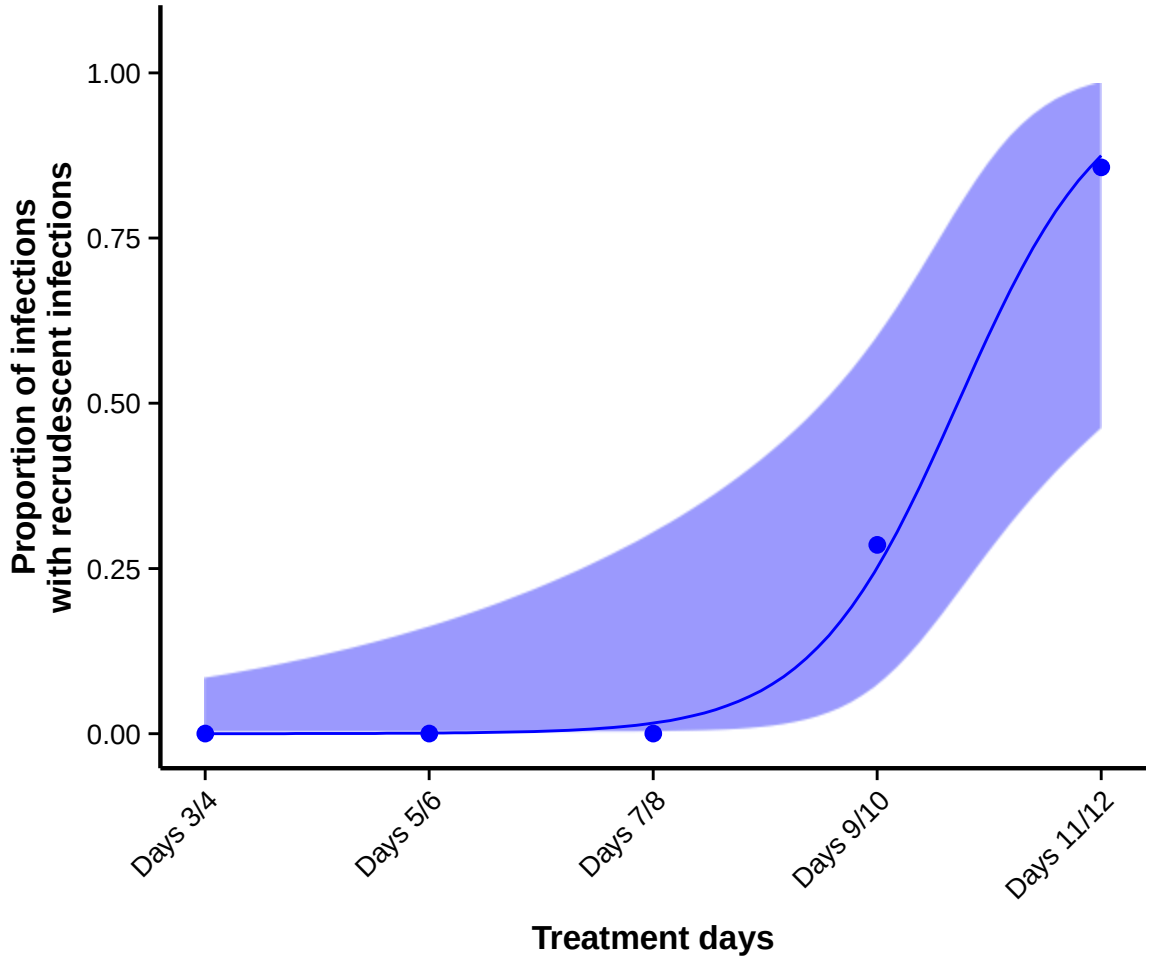

Figure 4. Proportion of Recrudescent Infections Based Upon Initial Population Size

Zero recrudescent infections were observed for mice drug treated on days 3 and 4, 5 and 6, or 7 and 8, while two recrudescent infections were observed for mice drug treated on days 9 and 10 and six recrudescent infections were observed for mice drug treated on days 11 and 12. The blue shading represents a 95% confidence interval, while the blue points are the actual proportions of recrudescence based upon treatment days. A line of best fit determined from the generalized linear model is plotted on the graph.

Recrudescent Infection Genotyping

Sanger sequencing of a roughly 400 base pair region of the parasite cytochrome b gene revealed mutations previously determined to be associated with Atovaquone resistance (***25***). In all recrudescent infections, one or more resistance mutations were found that confer varying degrees of phenotypic resistance to Atovaquone (Figures 5 and 6). Resistance mutations were found at codons 267-268 and 271-272, corresponding to specific, consistent protein alterations known to confer Atovaquone resistance. The following mutations were observed: codon 267 showed one phenylalanine to isoleucine mutation, codon 268 showed two mutations from tyrosine to either cysteine or asparagine, codon 271 showed one mutation from leucine to valine, and codon 272 showed one mutation from lysine to arginine. As explained previously, electropherograms were examined to determine whether or not recrudescent parasite populations contained dominant genotypes or were a mix of various resistance parasites. However, only one of the eight recrudescent infections harbored a mixed population.

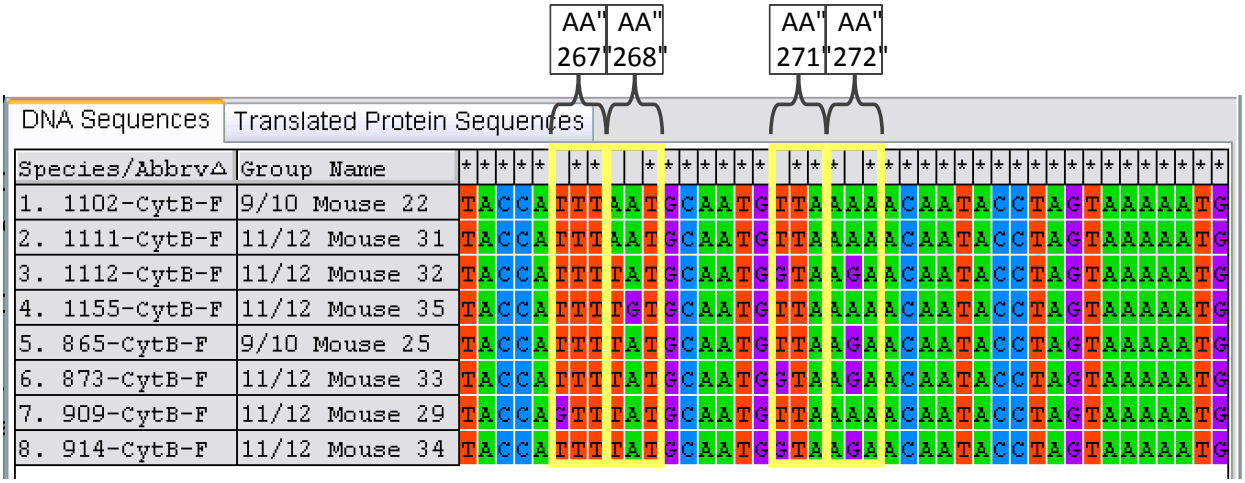

Figure 5. Cytochrome B Sequence Data

Recrudescent parasite DNA samples were sequenced at the cytochrome *b* gene, a spot of known Atovaquone resistance. SNPs conferring resistance were identified at codons 267, 268, 271, and 272.

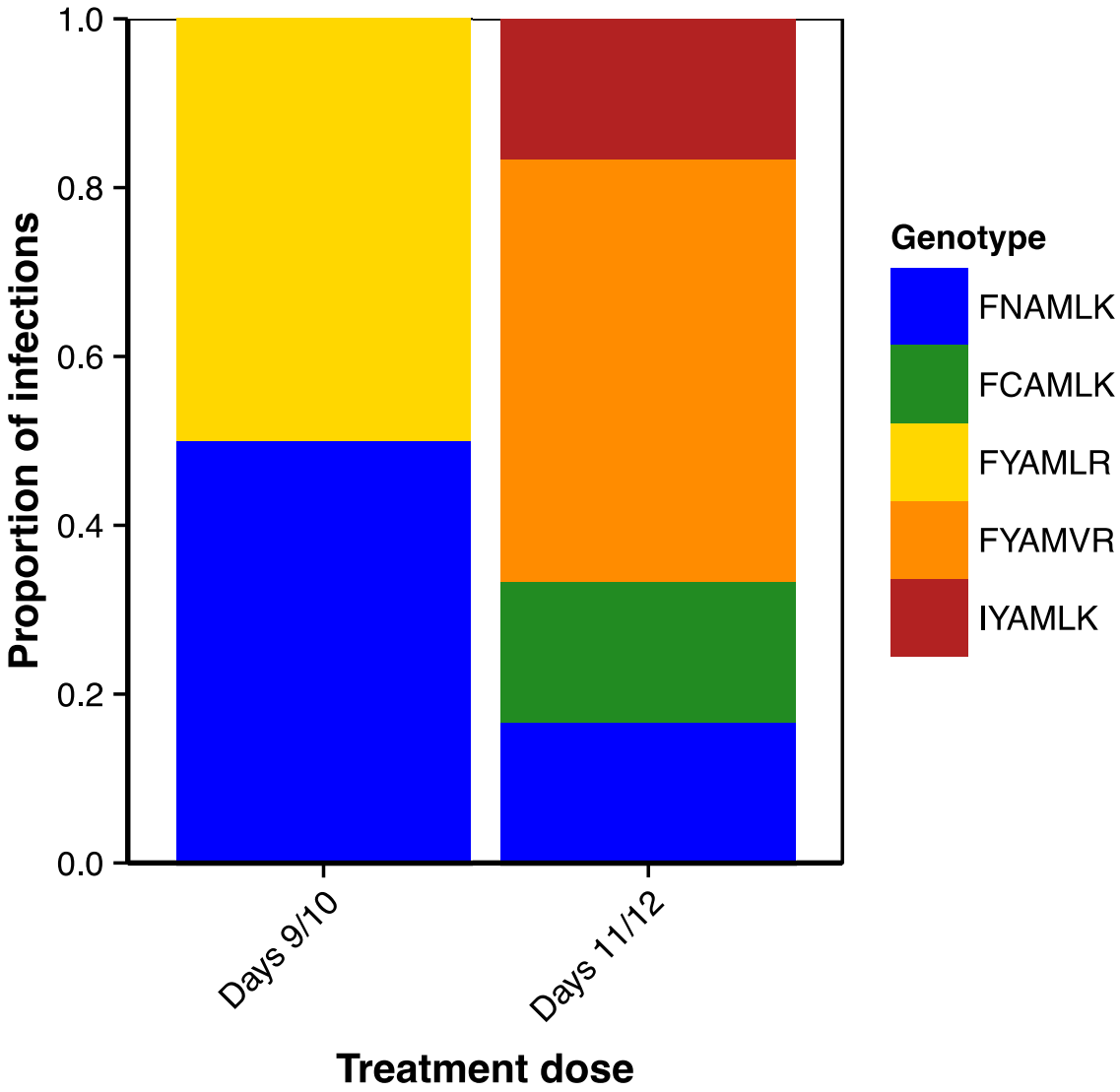

Figure 6. Resistance genotypes and relative proportions

Recrudescent infection genotypes (letters represent amino acids) are detailed above, with the wild-type genotype denoted as FYAMLK. Each recrudescent infection harbored at least one SNP resulting in an amino acid substitution.

2.4 Discussion

The results demonstrate that the emergence of resistance is dependent on the parasite population size at the time of drug treatment, as shown via the increased proportion of resistant,

recrudescent infections in populations drug treated at a larger size. This also indicates that drug treatment of larger parasite populations will lead to resistance emergence when genetic diversity is at a maximum and there is an increased probability of individual resistant parasites. At smaller population sizes, the genetic diversity of infections is therefore likely too low to harbor resistant mutants that would be selected for upon administration of drug. This holds particularly true as the infection sizes for many pathogens necessary to establish a full-blown infection are low. Here, it was only upon proliferation of the parasite population that resistance emerged. This is perhaps due to the number of random mutations that can accumulate as a result of replication machinery errors, or perhaps due to the mutagenic effects of the drugs themselves (***26***). These results mean that for Westerners taking prophylactic antimalarials such as Malarone, the probability of resistance emerging is drastically reduced due to low parasite populations throughout the duration of drug treatment. In individuals living in endemic malaria regions, parasite loads are often high at several points throughout life, indicating that resistance is more likely to emerge in such individuals particularly when compliance to drug regimens is often less than ideal.

Resistance is further enhanced due to the location of Atovaquone resistance mutations in the haploid mitochondrial genome, meaning that mutations are immediately expressed and can spread rapidly through strong selective pressures from drug treatments. Such a phenomenon represents an evolutionary advantage for malarial adaptation to environmental stressors. If this experiment were to be conducted using a diploid parasite where resistance mutations occur in the nuclear genome, complete resistance would require two independent mutations at the same locus. Therefore, resistance development would likely occur at reduced frequencies in comparison to

malaria. However, resistance would still scale with population size due to the higher probability of the two independent genetic mutations occurring in the same parasite.

Future directions for the project can go several ways. A follow-up experiment examining the effects of the host immune response on parasite resistance would be particularly interesting. Drug treatments would be applied on both sides of the parasite growth curve and the proportion of recrudescent infections examined. In the growth phase, as in this experiment, the host immune response is limited, while in the collapse phase following peak parasite density, the host immune response is in full swing. Such an experiment would allow for the examination of the effects of host immune factors on the emergence and spread of resistance. Other further experiments and analysis might examine the frequency of Atovaquone mutants as compared to other drugs in order to demonstrate that Atovaquone resistance is relatively common (estimated at 1 in 10^{12}) (***4***). This would provide insights as to whether or not Atovaquone might act as a mutagen, or whether this high incidence of resistance is more simply due to the few SNPs needed to confer resistance. Because Atovaquone resistance genes are located in the cytochrome b gene on the mitochondrial genome, superoxide radicals accumulating as a result of normal cellular processes might provide a mutagenic environment that causes the more rapid appearance of Atovaquone resistance as compared to some other drugs (***26***).

3. Potential to Delay Resistance Emergence Using Antagonistic Drug Therapy

3.1 Introduction

A specific topic of incredible importance for the delay of resistance development is the interaction of different drugs upon co-administration. Because many pathogens develop resistance to mono-therapies rapidly, drug cocktails are often utilized to delay resistance development (***36***). Combination therapies are not uncommon, and are perhaps best exemplified by HIV/AIDS anti-retroviral therapies (HAART therapies). The main principle relies on the idea that the probability of developing resistance to two separate drugs, often with differing sites or modes of action, is much lower. Such concoctions are often designed to include drugs that function synergistically, meaning that they complement each others mode of action by reducing the dose required to achieve the same curative level (***25***) (***37***). Recent studies have shown that while this strategy appears effective due to a lower required drug dose to cure patients, antagonistic drug interactions may in fact provide the best route for slowing or preventing resistance emergence (***38***) (***39***) (***40***). In such drug interactions, the drugs suppress each other's efficacy. This means that should a pathogen develop resistance to a focal drug in an antagonistic pairing, it may not face as strong of a selective advantage as compared to when arising in a synergistic combination of drugs. When development of resistance to one of the drugs occurs in an antagonistic combination, it removes the antagonistic interaction, effectively exposing the parasite to the full force of the secondary drug. In contrast, in a synergistic drug pairing, resistance to the focal drug will not only remove the efficacy of that drug, it will also remove the

additional inhibitory benefit of the synergistic combination, leading to strong selective pressures for those mutations.

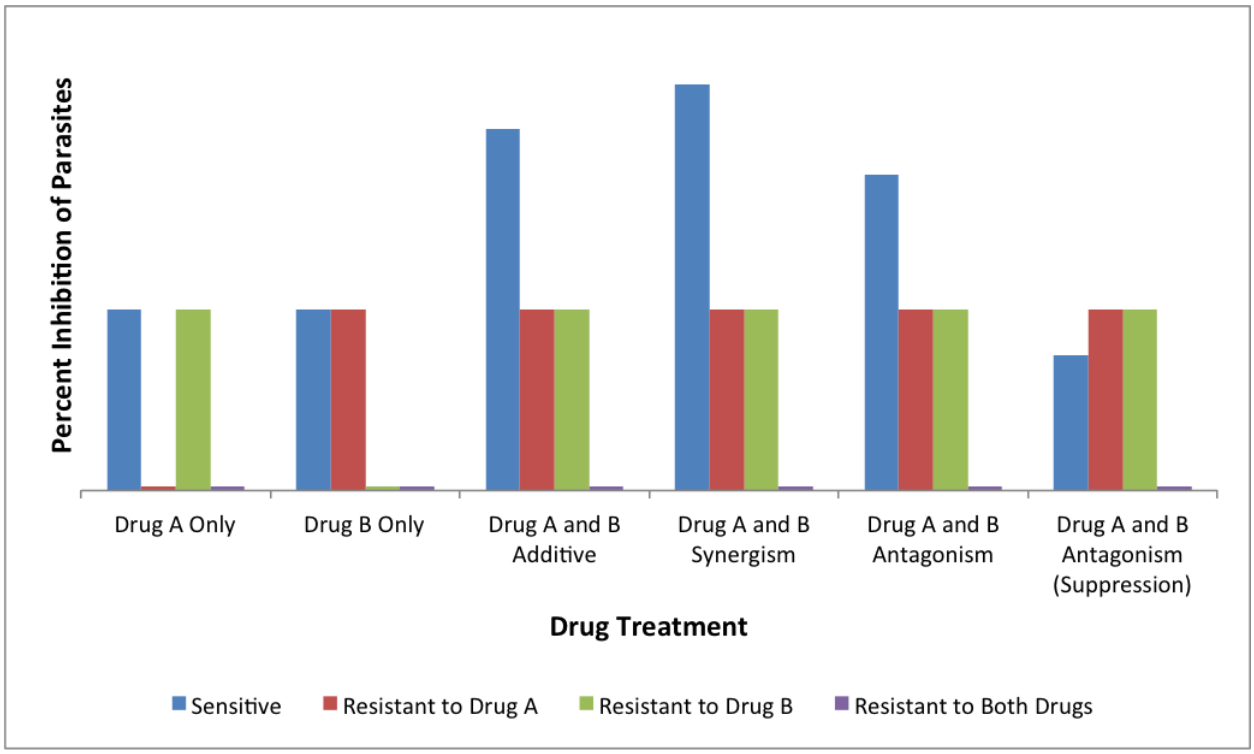

Figure 7. Differences between mono-therapies and varying combination therapies

Drugs often interfere with each other's modes of action. In scenarios where equal killing doses of drugs A and B are utilized against different populations of parasites, the above results will be observed based upon how the drugs interact.

It would thus be worthwhile to study these potential interactions *in vivo* to determine how drug combination therapies should be designed and implemented in order to minimize resistance emergence, rather than the traditional approach of reducing the required amount of drug. Antagonistic pairings have historically been avoided due to their decreased efficacy, but evidence suggesting that they may slow resistance emergence shows promise. In certain drug combinations, suppression can occur where the interaction of the drugs actually proves less

inhibitory than either of the drugs in use as a mono-therapy (see Figure 7). It is in this curious scenario, resistance can actually be selected against, although such interactions are rare (***40***). In a normal antagonistic interaction, the combination of drugs results in lower inhibition than would have been predicted by the addition of separate mono-therapies for the two drugs, still providing an environment where resistance development is less favorable than in additive or synergistic drug combinations. These resistance-slowing treatments have only been studied a few times *in vitro*, where *E. coli* was drug treated with a synergistic combination of doxycycline and erythromycin and a suppressive combination of doxycycline and ciprofloxacin (***40***). A small region of growth was generated in which only sensitive bacteria were able to grow and resistant bacteria were actually selected against. However, such a phenomenon has never been examined *in vivo*, and thus an experiment below has been devised to determine antagonistic combinations have the ability to delay resistance emergence in a more realistic environment using the murine malaria model.

A focal drug of particular interest is the common antimalarial Atovaquone. It is rarely used as a mono-therapy due to the high incidence of resistance to the drug, and thus it is now commonly used in combination with the antifolate synergist Proguanil, drastically reducing the incidence of resistance emergence (***22***). A comprehensive review of anti-malarial drug interactions was utilized to identify potential drug pairings for Atovaquone in this experiment (***37***). The combination of Atovaquone and several drugs such as Chloroquine, Mefloquine, and Ciprofloxacin are all known antagonistic interactions (Figure 8). However, the quinoline-derivatives quinine and Chloroquine show the highest the potential to delay resistance development, with demonstrated antagonistic interactions when administered to multi-drug

resistant malaria clones, those resistant to hydroxynapthogquinones (such as Atovaquone), and completely sensitive strains (***41***).

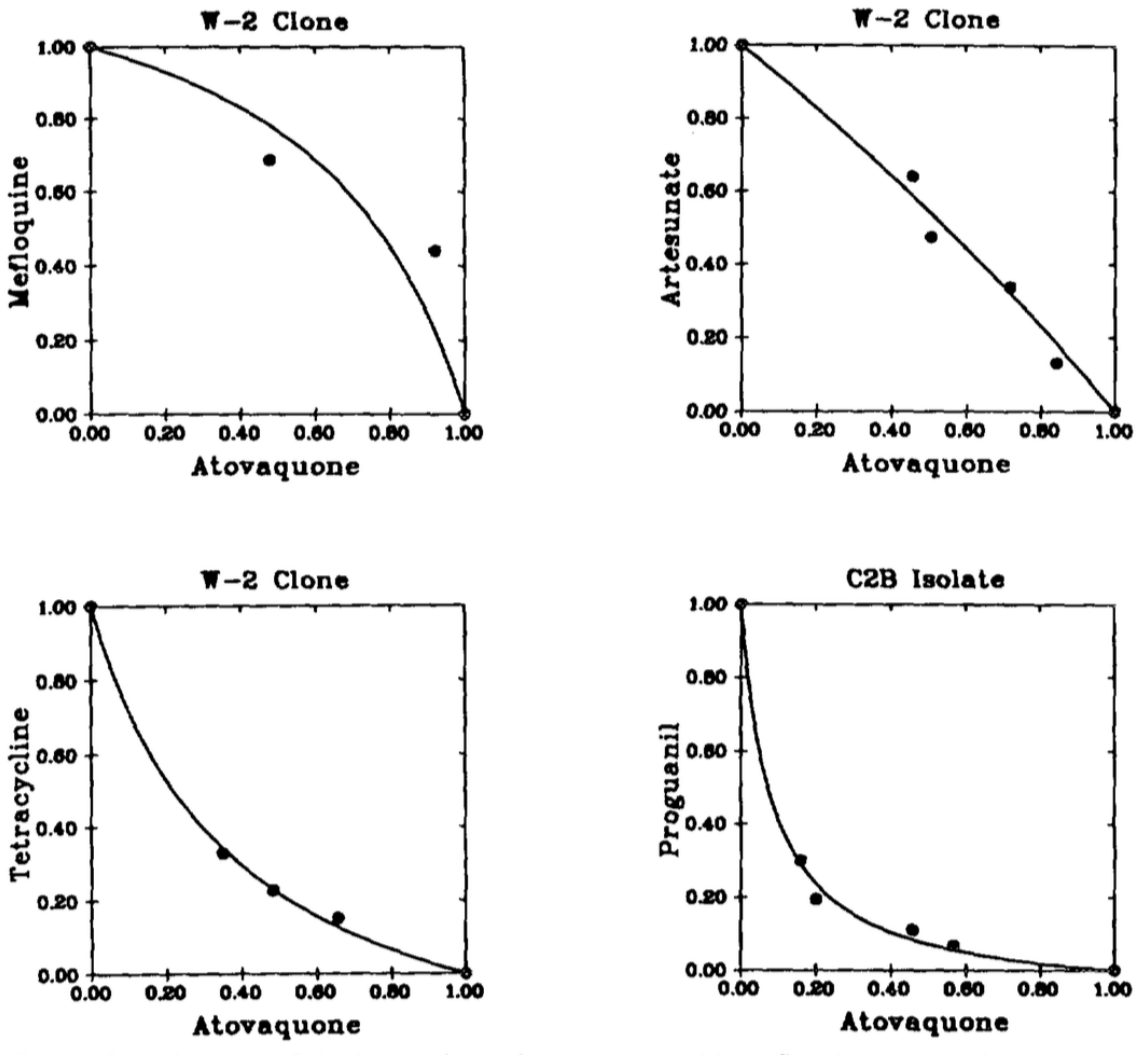

Figure 8. Isobolograms of Atovaquone drug interactions **(***41***)**

Isobolograms are graphical representations of the interactions of various chemical compounds. In this figure from Canfield, it is evident that Atovaquone has drastically different interactions with various drugs. A straight line would indicate an additive relationship, where the drug modes of action and dynamics are independent of one another, meaning that one dose of drug A plus one dose of drug B will equal two effective doses. When the line dips towards the origin in a synergistic interaction, the drug dose required to achieve the same curative level is reduced due to favorable interactions (i.e. Atovaquone and Proguanil). In an antagonistic pairing (i.e. Atovaquone and Mefloquine) the line bows outwards, indicating that the drugs are interfering with one another and that a higher dose of drug is required to achieve the same curative level.

While several antagonistic pairings exist for Atovaquone, many more synergistic pairings have been identified (likely because more is understood about how synergistic interactions occur) such as Atovaquone with Proguanil, Sulfamethoxazole, Clopidol, and Salicylhydroxamate. Of these, Proguanil on the surface presents a natural pairing for an experiment exploring resistance emergence due to its widespread use in combination with Atovaquone as the GlaxoSmithKline antimalarial drug Malarone. However, Proguanil as a mono-therapy has not been shown to have any effect unless converted to the active metabolite Cycloguanil, and thus is not a suitable partner for Atovaquone as a synergist in this experiment (***42***). Of the remaining drugs, Sulfamethoxazole is perhaps the best candidate synergist as its mechanism of action is the best understood by acting as a competitive antagonist of PABA, although Salicylhydroxamate remains a viable option (***41***). As described in chapter 1, the collapse of the mitochondrial membrane potential stops pyrimidine biosynthesis, and because PABA is a precursor in the pyrimidine synthesis pathway, Sulfamethoxazole is acts in a synergistic fashion with Atovaquone. Salicylhydroxamate inhibits oxygen consumption via an alternative pathway, thereby complementing Atovaquone's action on the cytochrome chain, but also allowing for resistance-development (***43***).

To test the potential to delay resistance development, an experiment below has been devised (but not yet conducted) where entirely sensitive parasite populations will be drug treated with either antagonistic or synergistic drug combinations. Recrudescent infection patterns will be analyzed and genotyped to determine whether or not resistance has emerged in the populations, and therefore if antagonistic drug pairings have the potential to delay resistance further than additive or synergistic therapies. Further experiments where mixed resistant and sensitive populations or

entirely resistant populations are drug treated will require a suppressive antimalarial drug interaction, which is as of yet not discovered.

3.2 Drug Dosing Trials

Before a full experiment examining the effects of antagonistic and synergistic drug combinations on the incidence of resistance can be attempted, the identification of the appropriate dosing of the three drugs that would be tested is of necessity. Examining the effects of Atovaquone at different concentrations on parasite death was a natural starting point for a pilot experiment. A small initial study composed of 10 mice split into three treatment groups and drug treated on days 3 and 4 post-infection with varying Atovaquone levels (the focal drug of a potential combination experiment) was attempted with monitoring occurring from day 3 until day 6. This was designed to demonstrate whether or not Atovaquone doses of 1mg/kg, 4mg/kg, and 8mg/kg resulted in significantly different parasite decline rates. Four mice were treated with 1mg/kg, three were treated with 4mg/kg, and another three were treated with 8mg/kg (Figure 9). Parasite monitoring was identical to that of chapter 2. Subsequent analysis using linear regression of the parasite slopes of decline showed that death rates across the three treatments were not significantly different and thus in a larger study would be indistinguishable ($F(1,8) = 0.303$, $p = 0.597$). This indicates that rather than utilizing Atovaquone doses within such a small dosage range for a larger experiment, dosing on a larger scale, perhaps orders of magnitude different from one another, is necessary to show significantly different parasite death rates. Atovaquone is known to be effective at very small concentrations, which might explain the failure here to differentiate between these gradients. Such results indicate the difficulty of determining inhibitory

concentrations *in vivo* due to the pharmacokinetics and pharmacodynamics of the drugs combined with host immune factors. However, such information is vital for comparing Atovaquone doses to other drugs such as Chloroquine and Sulfamethoxazole for future experimentation.

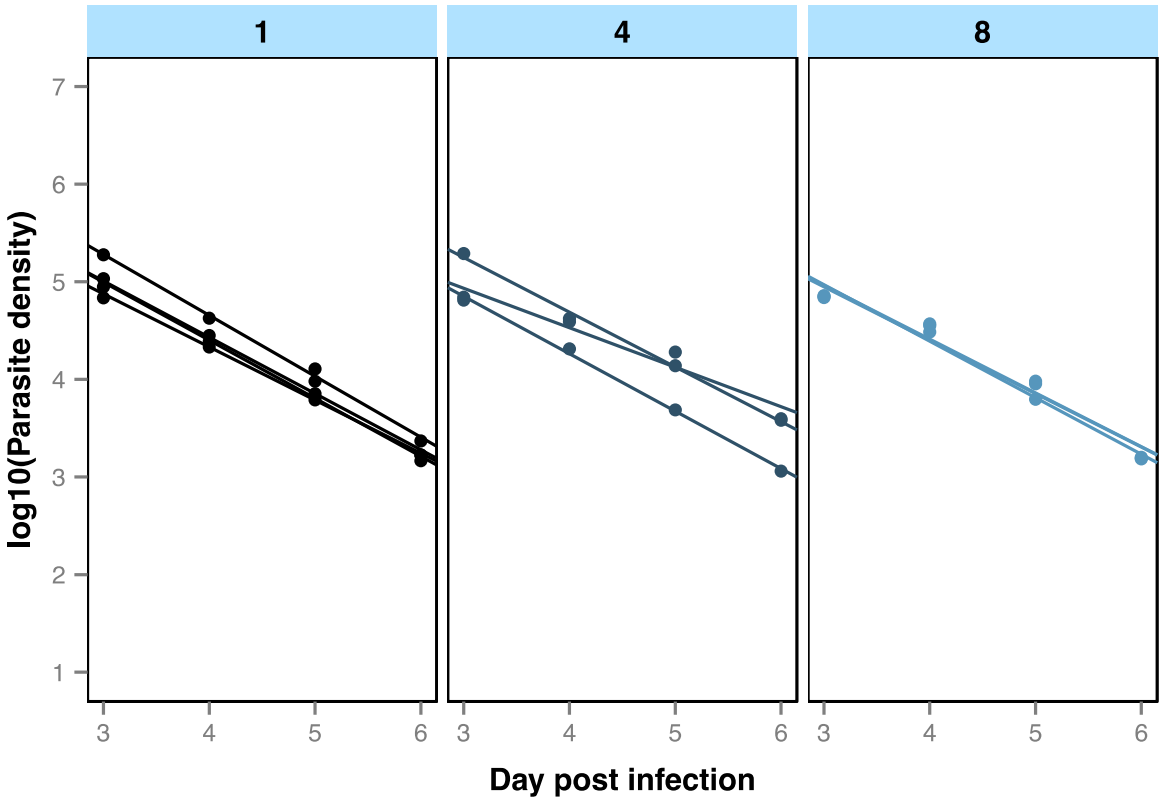

Figure 9. Effects of Atovaquone Dosing on Parasite Decline Rates

Parasite decline rates in mice drug treated with 1, 4, or 8mg/kg of Atovaquone exhibited no significant difference and thus would be indistinguishable in future experiments.

3.3 Combination Experiment Design

Drug Dosing

In order to design an appropriate experiment, equal effective killing doses for each drug must be determined so that the effects of the drugs on the emergence of resistance can be accurately assessed; otherwise one simply would be comparing the multiple drugs at different doses with different killing rates. Once this can be determined, an experiment examining the ability of synergistic and antagonistic treatments to delay resistance emergence can be attempted. It is hypothesized that antagonistic drugs would have a stronger ability to delay resistance emergence than synergistic drugs in a completely sensitive population because it is less beneficial from a competitive standpoint for parasites in an antagonistic setting to become resistant. Rather than removing the inhibitory effects of an entire drug plus the additional effects of the synergistic treatment, in an antagonistic setting, a fraction of one drug is removed as the two drugs were previously suppressing each other's mode of action and thus their efficacy. What this means is that resistant mutants in an antagonistic drug environment are less competitive compared to their wild type counterparts than are synergistic mutants (where sensitive strains are being hit with the full effects of the synergistic treatment). Thus, the ability of resistant mutants to outcompete sensitive clones becomes paramount for determining whether or not resistance can spread in the population. It is already known that Atovaquone mutants suffer from fitness drawbacks in the absence of drugs, further supporting the notion that sensitive strains may be able to outcompete resistant mutants if the selective advantage obtained upon development of resistance is not strong (***44***).

Potential Experimental Design

60 Mice (10 mice per treatment group) would be infected with 10^6 Atovaquone-sensitive, AS *P. chabaudi* malaria parasites. Drug treatment would occur via intraperitoneal injection on days

three and four following initial growth of the parasites: one with a combination of the two drugs Atovaquone and Chloroquine (antagonism) and one with a combination of Atovaquone and Sulfamethoxazole (synergism), where Atovaquone will serve as the focal drug in both treatments. Equal killing doses of all three drugs would be utilized. Three control treatment groups containing mono-therapies of the three drugs used in the experimental groups will serve as a baseline for determining the effects of the drug combinations on resistance emergence, while a final control group receiving no drug will also be utilized. Atovaquone dosing in the past has fallen between the values of 1mg/kg and 8mg/kg in the Read lab and thus similar dosages will likely be used in this potential experiment. Past Chloroquine intraperitoneal injections have utilized doses of roughly 1mg/kg to 10mg/kg (***45***) (***46***), while Sulfamethoxazole doses are expected to fall in a similar range.

Following drug treatment, mice will be monitored daily for red blood cell count, weight, and parasite growth. Recrudescence patterns and the genotypes of recrudescent parasites will be utilized to assess resistance emergence for each treatment group, with further phenotypic analysis of recrudescent parasite resistance occurring in secondary infections challenged with drugs. By comparing the parasite recrudescence patterns for the antagonistic treatment, synergistic treatment, and mono-therapies, the ability of specific drug pairings to delay the emergence of resistance will be elucidated.

4. Mosquito survival analysis in monoclonal and mixed malaria infections

4.1 Introduction

Although many humans in Africa are infected with one strain of malaria, evidence suggests that mixed-strain infections are relatively common at values as high as 30% of individuals in different regions of Africa (***47***). This is due to the fact that throughout a mosquito's lifetime, it will take multiple blood meals. The competition between strains in the mosquito has broad effects on the survival of the mosquito and transmission of the parasite (***48***) as strains evolve for increased virulence, their growth rates and transmission rates are maximized while also causing indirect host damage. When a more virulent and potentially deadly strain of *Plasmodium*, such as *P. falciparum*, has to compete with another strain *in vivo*, the competition should and does reduce the transmission rate of the more virulent strain.

Running parallel to a larger project examining mixed infection dynamics in mosquitoes, this experiment focused on the specific interactions of the malaria parasite and the mosquito vector. Over the course of the two weeks required for the development of *Plasmodium* in the mosquito, it is known that the parasite can cause substantial damage to the vector. As the ookinetes pass through the midgut, the parasites physically damage the mosquito body wall (***49***) (***50***). The parasites often also cause resource depletion (up to eight times as much glucose is used in an infected mosquito) and can elicit an immune response that draws resources otherwise utilized for the growth and development of the mosquito (***51***). Intuition would dictate that mosquito survival should be differentially affected by the malarial parasite evolutionarily, as evolution of the malaria parasite might lead to decreased virulence and thus harm to the mosquito (***52***). Previous

experiments have shown extreme parasite life cycle differences between the lab setting and the natural environment as well, where increased oocyst counts positively correlate with higher sporozoite counts in lab mosquitoes, whereas 'wild' mosquitoes often don't have this correlation (***53***). This adds to the complexity of the situation and makes it difficult to draw any serious conclusions.

Prior studies have demonstrated conflicting results as to the effects of parasite growth on the mosquito vector (***49***). Other work in the Read lab had indicated that the survival of the vector appeared to be detrimentally influenced by the presence of multi-clone infections, but those experiments were much smaller in scale and not focused on survival analysis. The goal of this study was thus to arrive at a conclusion regarding the effects of monoclonal and mixed infections on mosquito survival.

4.2 Materials and Methods

Parasites and Hosts

Two different clones of the malaria strain *Plasmodium chabaudi*, AJ and ER, were utilized for this experiment. *P. chabaudi* is a malaria strain that naturally infects thicket mice (*Thamnomys rutilans*). Strains used in this study were collected from mice in the Central African Republic in 1969 and 1970 and isolated from an area where mixed infections are found in high frequency (***54***). After being transported from the WHO Registry of Standard malaria Parasites at the University of Edinburgh, lines were maintained in liquid nitrogen at The Pennsylvania State

University. Mice utilized in the experiment were 6-10 week old female C57B1/6 mice that received 0.05% PABA containing water to help parasite growth (***33***).

Vector

The mosquito vector *Anopheles stephensi* was used to examine the transmission and competition of the two clones along with the survival effects of the clones on the mosquito. *Anopheles stephensi* is unlikely to be the natural vector host of *P. chabaudi*, but prior experiments have shown this mosquito species to be a good experimental host for *P. chabaudi*. Larvae were reared at 26˚C, 85% humidity using a 12L:12D photo-period, with eggs placed in trays filled with 1.5L of distilled water. *TetraFin* fish flakes were fed to the larvae and adults were fed 10% glucose solution containing 0.05% para-aminobenzoic acid (PABA) aside from the 24 hours before taking blood meals from mice. Male mosquitoes were discarded, and after feeding any unfed female mosquitoes were also removed. Mosquitoes during the experiment were fed *ad libitum* on glucose and PABA-soaked cotton balls, which were replaced on alternating days. The mosquitoes were housed at the Penn State insectary in six-inch cube mesh cages. Ovipositor bowls were placed in each cage for mosquito egg depositing after taking blood meals. The bowls consisted of small cups filled with distilled water and filter paper, which was consistently replaced weekly

Experimental Design

AJ and ER clones were injected into 84 mice; 42 were infected with AJ and 42 with ER, with 12 additional mice serving as uninfected controls. Each of the experimental mice was infected with 5×10^5 parasites. Red Blood Cell counts determined via flow cytometry of 2 μL of blood on a

Beckman Coulter Counter and parasite counts determined from blood smears were used to determine which mice would be used to infect the mosquito vectors. The mice with the highest gametocyte counts were utilized for transmission of parasites to mosquitoes. Mice were anaesthetized using 5 μL of Ketamine (100mg/kg) and Xylazine (10mg/kg) and set atop the mosquito cages for 30 minutes so that the mosquitoes had sufficient time to obtain a blood meal (see (***55***) for further methods).

Seven different experimental groups were examined, with three replicates each for a total of 21 mosquito cages. 100 mosquitoes were placed in 15 of the cages and 150 in 6 other cages (for the mixed infections) for a total of 2400 mosquitoes. The mice were infected with the clones fourteen or fifteen days before the first transmission day to the mosquitoes. The seven experimental groups (denoted by first treatment and then second treatment) were Control-Control, Control-AJ, AJ-Control, Control-ER, ER-Control, AJ-ER, and ER-AJ. The mixed infection cages were each populated with 150 mosquitoes versus the 100 mosquitoes used for every other treatment in anticipation of potentially higher death rates (totaling six cages of 150 mosquitoes each). The second transmission occurred four days after the first transmission. The first mosquito dissections to examine oocyst counts and to obtain parasite DNA samples as part of the larger experiment occurred on day seven after the first transmission, and the second set of dissections occurred on day eleven. For the first dissection, 30 mosquitoes were removed from treatment cages AJ-ER, ER-AJ, AJ-C, ER-C and dissected. Oocyst counts for each mosquito midgut were recorded. For the second dissection, 30 mosquitoes were dissected from each cage from the treatments C-AJ, C-ER, AJ-ER, and ER-AJ. Additionally, 25 mosquitoes were removed

for sporozoite dissection from AJ-ER and ER-AJ treatment cages on day 21 after the first transmission, all accounting for the drastic reduction in mosquito cage populations.

Table 1. Experimental treatment groups and sample sizes

1st feed	**2nd feed**	Number of mosquitoes	Dissected day 7	Dissected day 11
Control	**Control**	232	0	0
Control	**AJ**	212	0	90
Control	**ER**	195	0	86
AJ	**Control**	222	91	0
ER	**Control**	202	90	0
AJ	**ER**	294	90	88
ER	**AJ**	256	90	91

Mortality monitoring

For the duration of the experiment, cages were monitored twice per day for dead mosquitoes. All dead mosquitoes were removed from the cages and stored in 1.5 mL eppendorf tubes in a -20˚C freezer. The number of dead mosquitoes per cage was recorded, as was a cause of death, as several mosquitoes drowned in ovipositor bowls, escaped when the cages were opened, or were removed for dissection. Only those mosquitoes whose deaths were deemed natural (or caused by parasite burden) were used in the mortality analysis.

DNA Extraction and PCR

Dead mosquito DNA extraction to determine presence or absence of parasites was performed using the Qiagen DNeasy® Blood & Tissue Kit according to the tissue protocol. All samples were then stored at -80˚C until PCR for one or both clones was performed, depending on the treatment group. PCR was run using an Applied Biosystems 7500 Fast Real-Time PCR System (FAM protocol). The PCR mix was setup with 12.5 µL Fast mix, 4 µL dH_2O, 2.5 µL BSA, 1.5

µL forward and reverse primers, and 1.0 µL DNA probe. Bovine Serum Albumen (BSA, 10mg/mL Bovine Serum Albumin, New England BioLabs Inc.) was included in the mix because a pilot study confirmed previous researchers results (***56***) indicating that when whole-body DNA extraction from mosquitoes are run through PCR, a mosquito eye pigment can interfere with DNA amplification during PCR (see Figure 10). The BSA has been shown to eliminate this interference in a separate experiment run in the Read lab on Merek's disease in chickens as well.

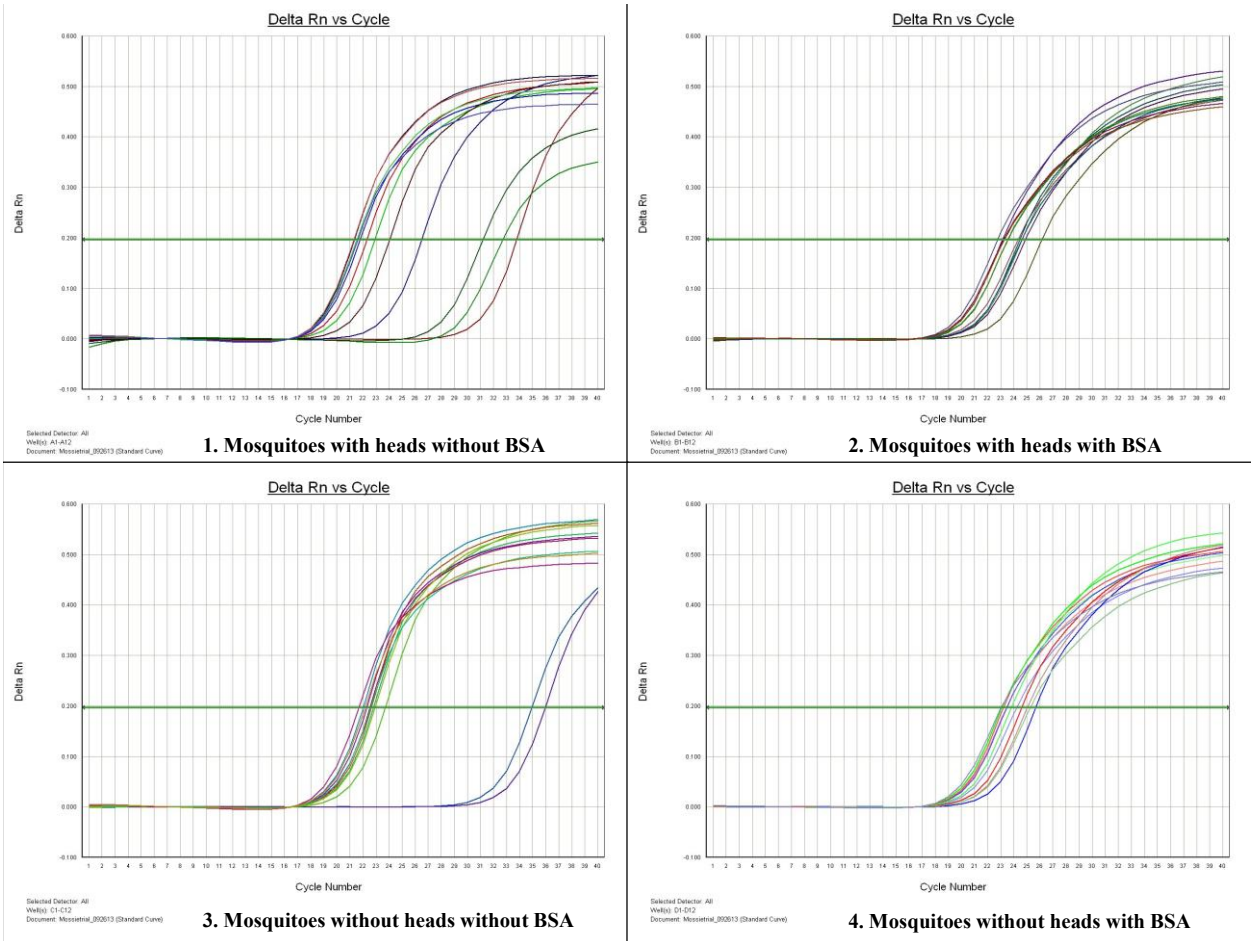

Figure 10. Mosquito Extraction and PCR Trial Results

1. Mosquitoes extracted with heads and PCR without BSA; 2. Mosquitoes extracted with heads and PCR with BSA; 3. Mosquitoes extracted without heads and PCR without BSA; 4. Mosquitoes extracted without heads and PCR with BSA

Statistical Analysis

Statistical analysis was performed using R version 3.0.2 statistical software. Analysis of mortality data was conducted using a cox proportional hazard mixed effect model. Experimental cage was set as a random effect, while infection status, estimated total red blood cells in blood meals and mean oocyst density from mosquitoes dissected from the same cage were set as fixed effects.

4.3 Results and Discussion

In total, 1631 mosquitoes were included in the analysis of vector survival, which spanned 72 days of twice daily monitoring (Figure 11). After an initial analysis ($X^2_{1,615} = 0.003$, $p = 0.96$) indicated no difference between uninfected mosquito and exposed but uninfected mosquito survival outcome, the groups were pooled to generate four groups: uninfected, infected with AJ, infected with ER, mixed infections (see (***55***) for further analysis and background information about the full experiment). Analysis again was not conducted for mosquitoes that were censored and removed for dissection, those that escaped the cages, or those that drowned. Overall, there was a significant effect of infection status on survival rates (4 level factor; uninfected, AJ infection, ER infection, mixed infection; $X^2_{3,891} = 9.53$, $p = 0.024$), although the only significant pairwise comparison was between AJ infected mosquitoes and those that were uninfected (AJ and uninfected: $X^2_{1,673} = 6.5$, $p = 0.01$; ER and uninfected: $X^2_{1,810} = 1.05$, $p = 0.31$; AJ and ER: $X^2_{1,253} = 0.24$, $p = 0.62$; mixed and uninfected: $X^2_{1,638} = 0.002$, $p = 0.99$; mixed and AJ: $X^2_{1,81} = 0.15$, $p = 0.70$; mixed and ER: $X^2_{1,218} = 0.002$, $p = 0.97$).

This experiment indicates that there is some effect of malaria parasite infection on the survival of mosquitoes, although curiously mixed infections had no greater an effect on survival than single infections with either AJ or ER clones. Initially it was predicted that increased parasite load resulting from multiple clones present would lead to lower vector survival rates. This experiment however represents a laboratory scenario with regular access to food for the mosquitoes, and the low parasite densities obtained here may not have enabled the parasites to have a large effect on mosquito mortality.

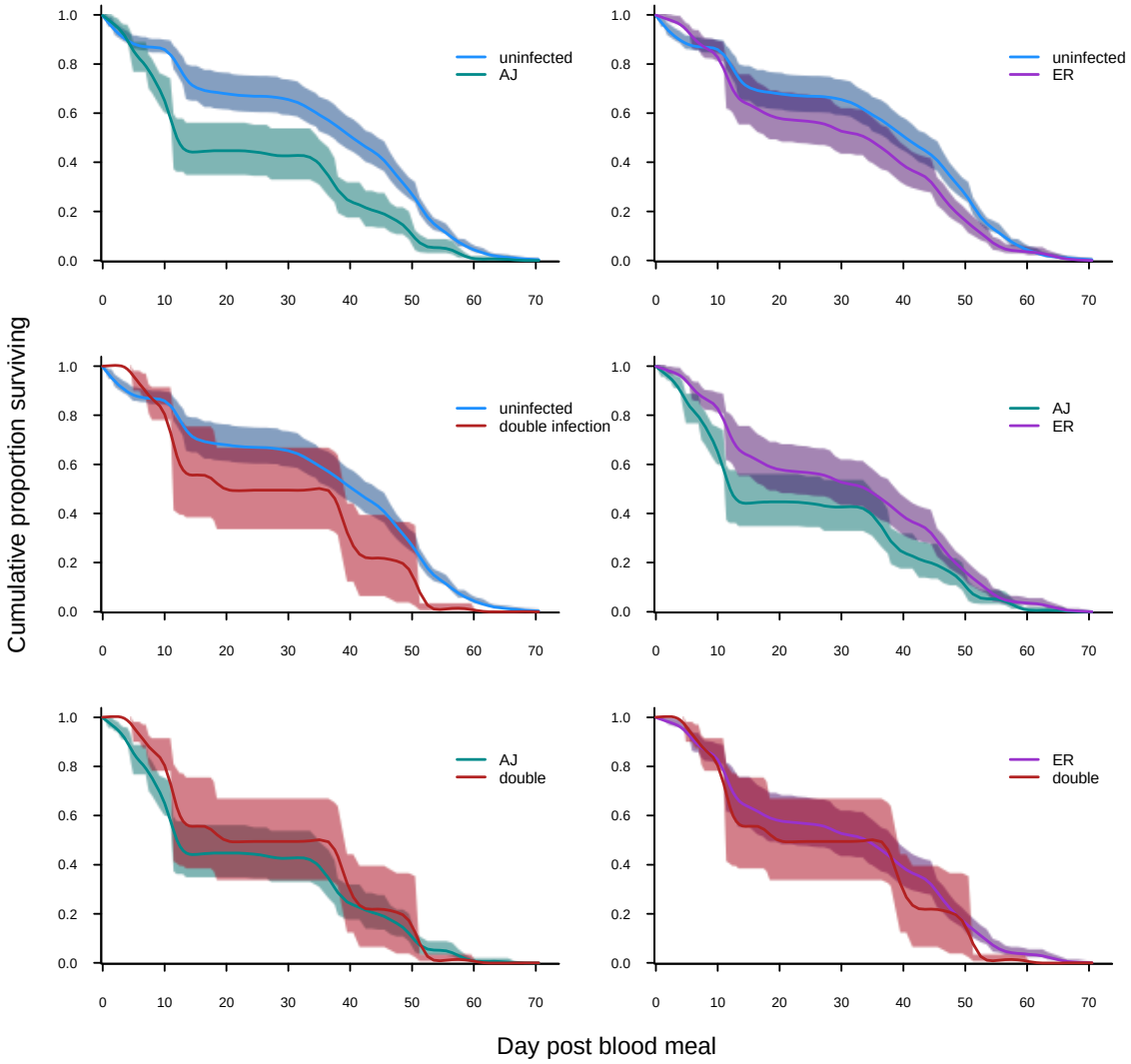

Figure 11. Vector Survival Results

Vector survival results indicate an overall significant effect of malaria infection status on vector survival, although the only significant interaction was observed between uninfected mosquitoes and those infected with clone AJ.

Appendix A: Parasite Growth Dynamics and Mouse RBCs/Weights

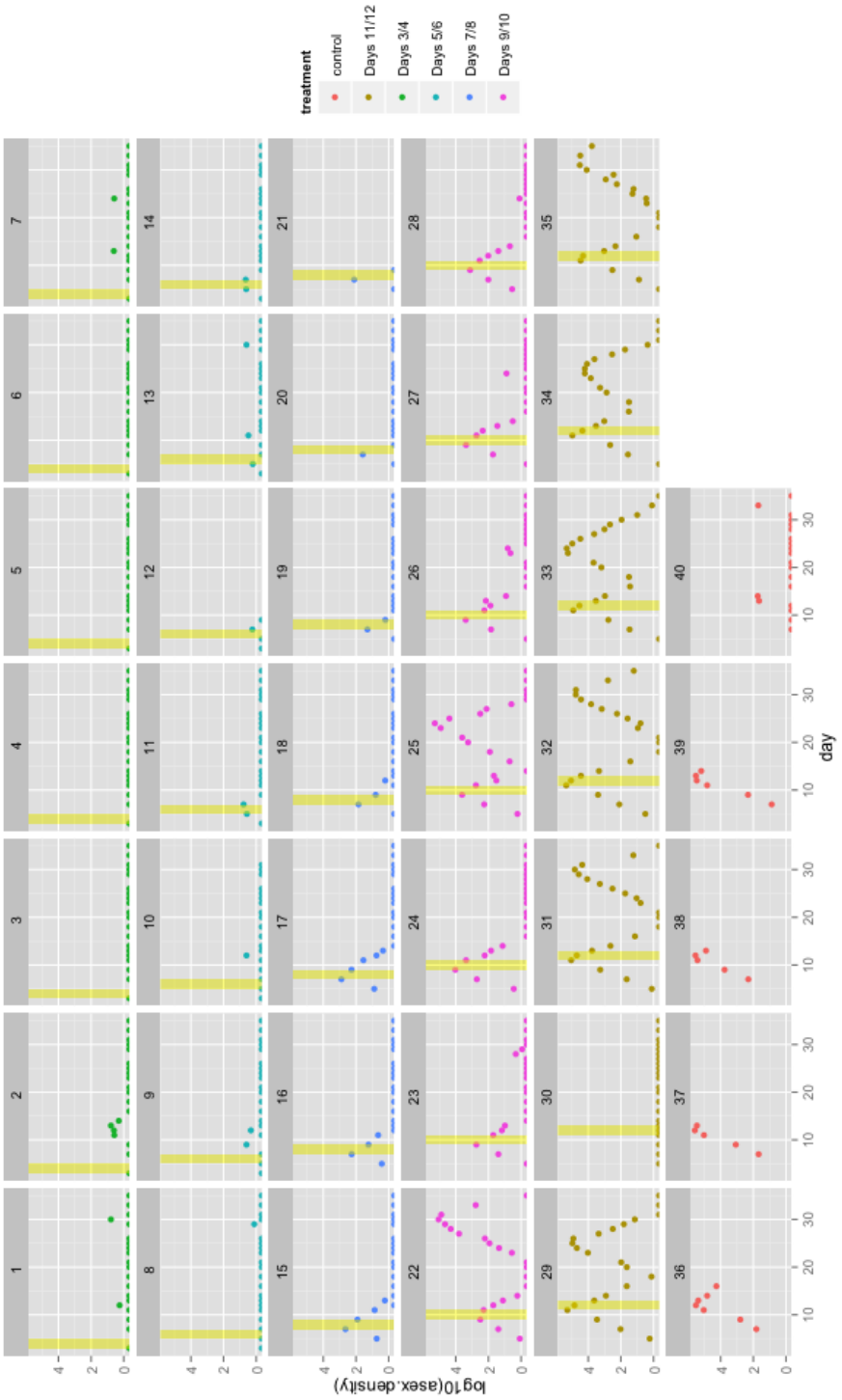

Figure 12. Parasite growth dynamics in mice (see chapter 2)

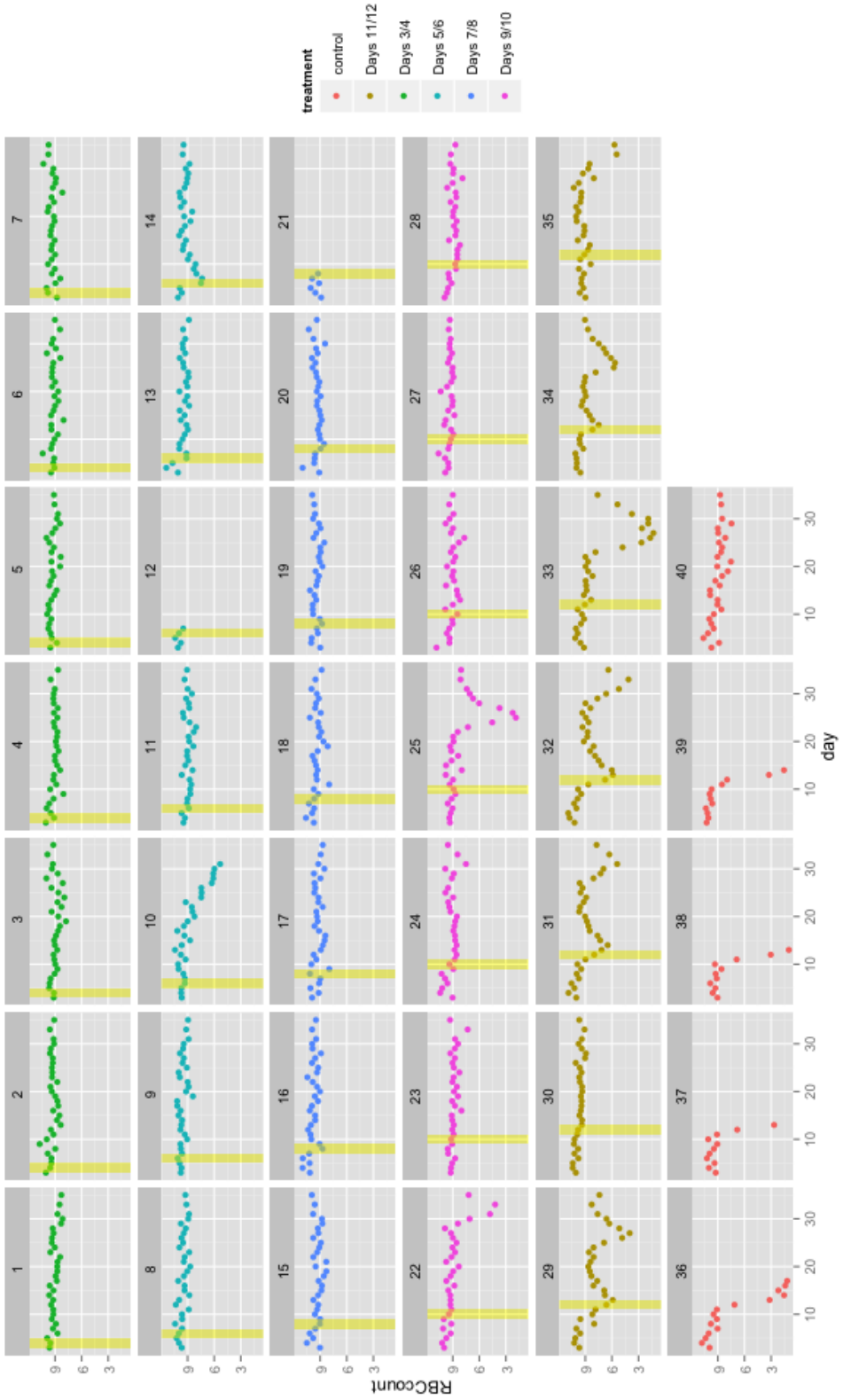

Figure 13. Mouse red blood cell counts (chapter 2)

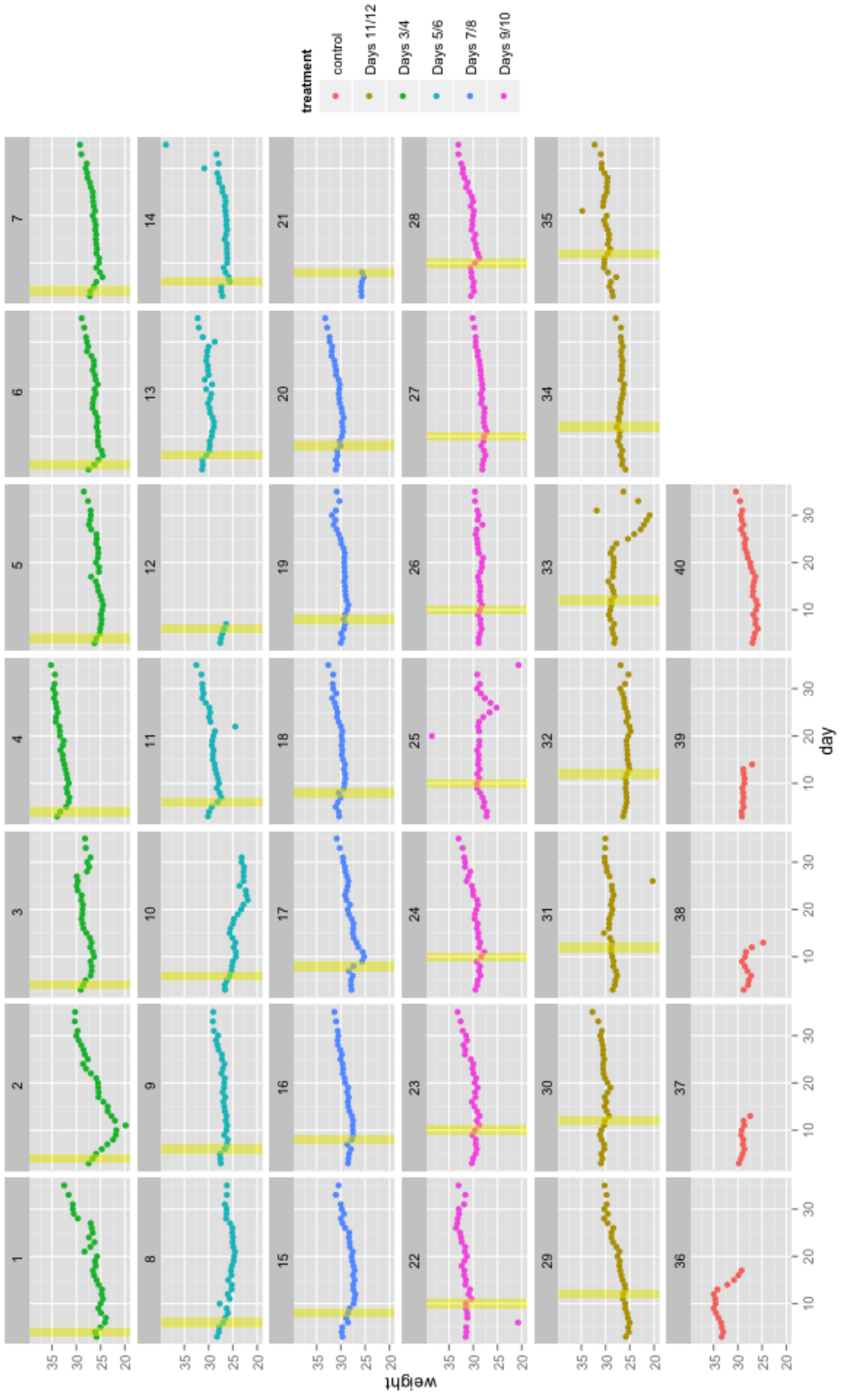

Figure 14. Mouse weights (see chapter 2)

Appendix B: Determining the Competitive Ability of Drug-Resistant Parasites

B.1 Background

Understanding fitness costs of drug-resistant parasites within an infection is important for understanding how within host ecology can dictate transmission success in the presence or absence of drugs. The goal of this study would be twofold: to evolve genetically distinct drug-resistant *Plasmodium chabaudi* malaria parasites, a model system for studying human malaria, and to use those strains for a pair-wise competition experiment looking to determine if the cost of malarial drug resistance is common across multiple different genotypes, and whether that cost manifests itself in competitive interactions. The experiment would hopefully answer long-standing questions surrounding the fitness of drug-resistant parasites in competition with drug-susceptible parasites. The initial phase of the experiment would focus on generating the drug-resistant parasites needed for the larger experiment, with a specific focus on the time required for drug-resistance development across the different parasite lineages used in the study. The second phase of the experiment would constitute a larger pair-wise competition study that examines the fitness cost of drug-resistance in malaria parasites of various genetic backgrounds to bring clarity to the issue of drug-resistance fitness cost.

B.2 Introduction

Over the past half century, with massive deployment of anti-malarial medications, the evolution of drug-resistant malaria parasites has become a major challenge facing countries across the globe. When challenged with a single drug, the parasites rapidly develop drug-resistance due to

the massive selection pressure the drug imposes. This has led to an increasing need for effective multi-drug regimens and a need to better understand how the evolution of drug resistance proceeds in order to generate better management strategies.

It is also well understood that fitness costs are a common byproduct of drug-resistance. Therefore, as management strategies are developed, an important area of research deals with understanding the evolutionary forces surrounding the emergence and spread of drug-resistance and how within host ecology affects the transmission of resistant parasites, particularly early on in the spread of resistance when fitness costs of resistance are thought to be higher. Later on, surviving resistant parasites are thought to have developed compensatory mutations that allow them to better compete with drug-susceptible parasites in the absence of drugs (***14***). These mutations allow the parasites to retain drug-resistance and lead to increased transmission success to new hosts. Evidence suggests a majority of malaria infections are multi-strain (***57***), so experiments focusing on the in-host dynamics of drug-resistant parasites are particularly relevant. Parasites inhabit a dynamic environment within the host, dealing with immunity and with competition from other strains. Understanding how these interactions change depending on the resistance status of the parasites is important when predicting how quickly resistance will spread or in developing new control measures.

Previous studies have shown that resistance to the drug Pyrimethamine in malaria leads to an increased requirement for *p*-aminobenzoic acid, an intermediate in the folate pathway, amongst drug-resistant parasites in competitive scenarios (***33***) (***58***). However, this fitness tradeoff has not been extensively explored. Previous limited experiments have shown that drug-susceptible

clones outcompete drug-resistant clones in the absence of drugs (***14***). These experiments mimicked competition scenarios across only two distinct malaria clones with small genetic and phenotypic changes. This study would seek to focus on competition scenarios across a wide range of parasite genotypes with widely varying genetic identities, much more accurately reflecting the in-host dynamics of human malaria infections. By evolving a suite of genetically distinct, drug-resistant malaria clones along with the completion of the competition experiment, the question of drug-resistance fitness cost in the absence of drug treatment can be more readily resolved.

B.3 Materials and Methods

Evolving Drug Resistance and Time to Resistance Development

Resistance to Pyrimethamine, a common anti-malarial, consistently appears as a result of a combination of single nucleotide polymorphisms (amino acid position 106) in the gene encoding the enzyme dihydrofolate reductase (DHFR), which is a key enzyme in the folic acid pathway and metabolism of nucleic acids in *P. chabaudi* (***59***). For this reason, along with the inherent associated fitness cost of resistance already mentioned, Pyrimethamine would be used to evolve the resistant lineages. The specific malaria clones to be used in the study are denoted AT, ER, and AS. These clones represent parasites representing an array of virulence and infectivity, with AT and ER expected to be more virulent than AS. Among the clones used in the study, cross-lineage comparisons of the time required for resistance development will be monitored to determine if specific phenotypic traits that are unique to each clone lead to faster drug-resistance development. This will be measured as the average amount of time after drug treatment required

for the reappearance of parasite densities. Subsequent genotyping of the parasites would be utilized to determine the actual presence of resistance. Mice would be infected with susceptible strains of AT, ER and AS and become exposed to gradually increasing doses of Pyrimethamine until resistance is developed.

Pair-wise Competition

The competition experiment would consist of twelve treatments, focusing on singular infections of the resistant clones of AT, ER, and AS, the susceptible clones of AT, ER, and AS, as well as the six pair-wise infections of AT_R-ER_S, AT_R-AS_S, ER_R-AT_S, ER_R-AS_S, AS_R-AT_S, and AS_R-ER_S (R denotes resistant and S denotes sensitive). Infections would be monitored daily for a total of 30 days. Drug dosages would be determined following the resistance development protocol to see which drug dose would be most appropriate. By examining the pairwise interactions of sensitive and resistant parasites and comparing the outcomes to control singular infections, the dynamics of resistance evolution in the absence of drug treatments can be elucidated.

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Disease Transmission. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **1999,** *92,* 580-585.

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ACADEMIC VITA

JOSHUA T. BRAM

EDUCATION

The Pennsylvania State University | The Schreyer Honors College — **University Park, PA**
The Eberly College of Science | Bachelor of Science in Biology — Class of May 2016
The College of Agricultural Sciences | Bachelor of Science in Immunology and Infectious Disease
The College of Engineering | Humanitarian Engineering and Social Entrepreneurship Program Certificate

State College Area High School — **State College, PA**
Class of June 2012

RELEVANT EXPERIENCE

Center for Infectious Disease Dynamics, Andrew Read (Director) — **University Park, PA**
Undergraduate Researcher — *September 2012 – Present*

- Study the development of drug resistance and virulence evolution in *P. chabaudi* malaria
- Examine the effects of antagonistic and synergistic drug pairings on the emergence of drug resistance
- Run transmission and clone competition experiments in mosquito vectors and murine hosts
- Write grant proposals and research papers for publication

Eberly College of Science, Biology Department — **University Park, PA**
Laboratory Teaching Assistant – Biology 110, Biology 230M, Biology 110S — *Fall 2013, 2014, 2015; Spring 2016*
Classroom Teaching Assistant – Biology 322

- Instructed honors cellular biology and introductory biology laboratory sections of 22 students
- Taught innovative freshman research initiative introductory laboratory course section of 20 students
- Held office hours and assisted students through genetics problems and solutions
- Wrote quizzes and lesson plans, graded assignments, and prepared weekly laboratory experiments

HESE Program, College of Engineering — **University Park, PA**
Teaching Intern and Research Assistant — *Fall 2014 – Fall 2015*

- Wrote and edited research manuscripts for publication about mobile payments and mobile health programs
- Coached the next generation of HESE students through research paper writing and venture development
- Traveled to Sierra Leone (June 2014) and Zambia (May 2015) to conduct research studies for the program

Mount Nittany Medical Center — **State College, PA**
Emergency Department Volunteer — *June 2009 – Present*

- Safely transport patients and visitors to CT scans, radiation oncology, and X-Ray (350+ hours)
- Train new volunteers monthly and assist both doctors and nurses in the delivery of medications

LEADERSHIP EXPERIENCE

Springfield, Benefiting THON (Penn State IFC/Panhellenic Dance Marathon) — **University Park, PA**

- THON is the largest student-run philanthropy in the world with over 15,000 student volunteers
- Since being founded in 1973, THON has raised $128 million with over $13 million last year alone

President — *March 2015 – Present*

- Oversee an executive board of ten directors and an organization of approximately 250 members
- Work with other organization presidents and directors to implement innovative fundraising strategies
- Plan strategy and goals for the organization moving forward and run general and board meetings

ACADEMIC VITA

JOSHUA T. BRAM

LEADERSHIP EXPERIENCE (CONTINUED)

Donor and Alumni Relations Director — *March 2014 – March 2015*

- Coordinated corporate and individual donations benefitting THON
- Partnered with other fundraising organizations and directors to develop policies for solicitation
- Led the organization to increase our fundraising total by over 15% to more than $270,000

Skull and Bones Senior HAT Society — **University Park, PA**

Vice President — *March 2015 – Present*

- Selected to join an honor society recognizing 1/1000 members of the senior class for leadership and service to Penn State
- Responsibilities include coordinating projects to improve the University and working with administrators
- Lead a group of student leaders through the discussion and implementation of University-wide initiatives

Science LionPride — **University Park, PA**

Alumni Relations Director — *April 2013 – April 2014*

- Served as the student ambassador to the Eberly College of Science Alumni Board in board meetings and conference calls
- Interacted with Eberly College of Science alumni and faculty and coordinated alumni events for club

HONORS AND AWARDS

Eberly College of Science Marshal for Spring 2016 Commencement	*March 2016*
Evan Pugh Senior Scholar Award	*February 2016*
Eric A. Walker Award	*February 2016*
Eberly College of Science Undergraduate Research Grant	*September 2013, 2015*
Student Representative on Penn State Homecoming Court	*September 2015*
American Society of Microbiology Undergraduate Research Fellowship	*Summer 2015*
Schreyer Ambassador Travel Grant	*May 2013, 2014, 2015*
Rosenthal Ambassador Scholarship	*April 2013, 2015*
Phi Beta Kappa Society	*March 2015 – Present*
Evan Pugh Junior Scholar Award	*March 2015*
First Prize in the Life Sciences – Eberly College of Science Research Poster Session	*September 2014*
Bayard D. Kunkle Academic Achievement Scholarship	*August 2014*
Pennsylvania State Undergraduate Summer Discovery Grant	*May 2014*
Phi Kappa Phi Society	*March 2014 – Present*
Gamma Sigma Delta Society	*March 2014 – Present*
President's Freshman Award	*March 2013*
Eberly College of Science Braddock Scholar	*August 2012 – Present*
Schreyer Academic Excellence Scholarship	*August 2012 – Present*

PUBLICATIONS

Bram, J.T., Warwick-Clark B., Obeysekare E., and Mehta K. Utilization and Monetization of Healthcare Data in Developing Countries. *Big Data* 3(2), 59-66 (2015).

Pollitt L., Bram, J.T., Blanford S., Jones M.J., and Read A.F. Existing Infection Facilitates Establishment and Replication of Malaria Parasites in their Mosquito Vector. *PLoS Pathogens* (2015). DOI: 10.1371/journal.ppat

Wenner G., Bram J.T., Marino M., Obeysekare E., and Mehta K. Organizational Models for Mobile Payment Systems in Low-Resource Environments. [In Review in *ITD*].

Gorski I., Bram J.T., Sutermaster S., Eckman M., and Mehta K. Typology of M-Health Project Value Propositions. [In Review in *JMET*].

Gorski I., Bram J.T., Suffian S., Lackey J.D., Dzombak R., and Mehta K. How to Set Up a Community Health Program: Lessons from 10 Years in Kenya [In Review in *IJSEI*].