RESOURCE COMPETITION AND THE EVOLUTION OF DRUG RESISTANCE IN MALARIA PARASITES

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Biology
by
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Parasites extract resources from their hosts. Since hosts have little interest in supplying resources to parasites and parasites often live at high densities, resources can be in short supply. As a result, competition between parasites within-hosts can be intense. The important role that within-host competition plays in the evolution of drug resistance, which is considered to be the greatest threat to human health in the 21\textsuperscript{st} century, has lately been recognized. Drug resistance is often associated with costs, such that drug resistant parasites lose in competition with their drug susceptible conspecifics in untreated environments. Only when drug treatment removes their susceptible competitors from the within-host environment can drug resistant parasites acquire enough resources to proliferate. So, within-host competition for resources could play a key role in the control of drug resistant parasites and understanding the nature of within-host competition is essential.

Here we use a mouse model of malaria infection, \textit{Plasmodium chabaudi}, to investigate how the availability of within-host resources impacts both the population dynamics of parasites and the emergence of drug resistant parasites. We demonstrate that a micronutrient, para-aminobenzoic acid (pABA), limits the growth of parasites and mediates competition between pyrimethamine resistant and susceptible parasites. We show that pyrimethamine resistant parasites require more pABA than susceptible parasites, apparently as a cost of the mutation that confers pyrimethamine resistance. As a result, pyrimethamine resistant parasites suffer intense competitive suppression from susceptible parasites in hosts depleted of pABA. Indeed, depleting pABA from the host causes the competitive exclusion of pyrimethamine resistant parasites and prevents their emergence following drug treatment. pABA depletion has this effect irrespective of the genetic background of either drug resistant or susceptible parasites. We propose depleting resources required in excess by resistant parasites as a novel, potentially ‘evolution-proof’ resistance management strategy, which could be used in a wide variety of systems. Motivated by the observation that the availability of pABA within-hosts appears to change how drug resistant parasites interact with the immune system, we develop a modeling framework with which to investigate the nature of the antimalarial immune response and its drivers. The model illuminates that the nature of the antimalarial response changes with the availability of pABA within-hosts, as well as with the genetic background of parasites, and suggests a common mechanism for
these observations. It also shows that the immune response may have an important role in determining the availability of another within-host resource required by malaria parasites, namely red blood cells.

Our work shows that changes in within-host resource availability can alter parasite-parasite and host-parasite interactions and can be harnessed in the management of drug resistance.
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3.1 Number of mice per treatment in Experiment 1. Numbers in brackets indicate the number of mice that were removed from some or all analyses because they were inadvertently treated (triangle superscript, removed from all analyses); misinoculated (circle superscript, removed from all analyses); they died (cross superscript, removed from analysis of total/peak parasite density) or because the initial growth trajectory of parasites was non-linear (square superscript, removed from analysis of growth rate). .......... 49

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5.1 Details of experiments from which we took data to fit to our model. All infections were left untreated and consisted of just the strain specified. Since all AS strains are resistant to pyrimethamine, their growth dynamics are expected to change with the pABA concentration administered to mice. Hosts in all experiments were female C57BL/6J mice. .............................. 95
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Dedication

To my Mum and Dad, Bindu and Christopher Wale, for giving me all the opportunities they could muster and the strength and encouragement to enjoy them.
Parasites, like all organisms, require resources to survive and reproduce. Host individuals can be thought of as habitats that contain both resources and predators (the immune system), which promote and constrain the growth of parasite populations, respectively. Understanding what elements of the within-host environment parasites use as resources and how the availability of these resources impact the population dynamics of, and competitive interactions between, parasites is the work of ecologists. In an age when parasites are evolving resistance to drugs, thwarting our attempts to control them, this work has never been more important. For if we can understand what resources limit the growth of parasites, and mediate competition between them, we might modify the within-host environment to make it less favorable to drug resistant parasites, and so prevent their emergence and spread.

1.1 Resources & Competition

No population of organisms can grow indefinitely. This is because the availability of resources, broadly defined as consumable factors that promote population growth rate [1], is finite. The capacity to acquire resources thus determines an organism’s fitness. When resources are scarce, the acquisition of resources by one organism reduces the amount of resources available to another and so the fitness of one organism comes at the expense of another. The negative impact of one organism on another is called competition [2].

Competition has had a central, but fiercely debated, role in both evolutionary biology and ecology, and has been hypothesized to have driven the evolution of biodiversity [3] (reviewed in [4]) and to structure ecological communities [5, 6]. Competition occurs at all biological levels, from genes [7] to species [8] and has
been observed in a wide variety of biological systems [8,9]. Organisms compete in a number of ways, all of which involve restricting the access of other organisms to resources: via exploitation competition, by which organisms consume resources, thereby making them unavailable to others; via interference competition, by which organisms directly interfere with each other’s access to resources, for example by aggression; and via apparent competition [10], by which an organism indirectly prevents another’s acquisition of resources by sustaining a population of predators or parasites, that it shares with its competitor [2].

The intensity with which a pair of organisms compete is predicted to be proportional to the extent to which their resource requirements overlap, at least when resources are in short supply [11]. Theory predicts that no two organisms can share the same single resource and coexist at equilibrium in a constant habitat, and that the resource level at which an organism can maintain its population size (its R*) determines the outcome of competition - the organism with the lowest R* wins [6]. An organism may have a smaller R* by more efficiently converting resources into offspring or by requiring fewer resources to maintain its survival [12]. Resource competition theory further predicts that organisms coexist at equilibrium only when they compete over multiple resources, which they require in different ratios (i.e. the capacity to compete for one resource comes at the cost of the capacity to compete for another). Though resource competition theory was developed to explain exploitation competition between plants and plankton for nutrients, it has been extended to the scenario where competition between organisms is mediated by a shared predator (i.e. apparent competition) [13]. Again, the organism that is least vulnerable to the shared predator, that is it requires the least amount of the resource called enemy-free space, competitively excludes the other. Enemy-free space can thus be considered a resource in the same way as a nutrient. Nutrients and enemy-free space are qualitatively different, however, in that the space that an organism uses to hide from predators or parasites only increases the fitness of that organism when a predator or parasites is present, whereas a limiting nutrient always increases an organism’s fitness [14], all else being equal.

While classical resource competition theory concentrates on the mechanistic basis of competition between organisms (and has been criticized for doing so [2]),
most studies of competition in nature focus on detecting the response of organisms to competition. The experimental examination of competition (in particular interspecific competition) tends to involve either the addition of competitors or their removal, with a focal organism being said to experience competition when its fitness (as measured by the growth rate, abundance etc.) decreases and increases, respectively. Both of these experimental designs have been used to study competition among parasites (e.g. [15, 16]), with the removal of parasite species/strains achieved via the use of antimicrobial drugs [16,17].

Parasites compete for resources at both the between- and within-host scales. First, parasites compete for access to susceptible hosts. We harness this form of competition between parasites in our efforts to control them - by vaccinating hosts with a benign or attenuated strain of an invading parasite, we reduce the availability of susceptible hosts for the competing, disease-causing strain. Second, parasites compete with the host for resources [18] and a number of vertebrate traits have evolved in response, including the transferrin molecules that transport iron within vertebrates [19], as well as other metal chelators [20]. Anemia, which we think of as a symptom of infection, may also represent an attempt by the host to constrain the access of parasites to iron and/or red blood cells [21, 22]. Selection may not favor parasites that maximally extract host resources, however, since the supply of resources for parasites is dependent on the continued survival of the host, which is in turn contingent on the host’s capacity to acquire resources. That said, maximal extraction of resources will be favored when parasites compete within-hosts with other parasites [23]. Competition among parasites has been observed numerous times (see examples referenced in Chapter 2) and is thought to have driven a number of parasite traits, including antigenic diversity [24] and virulence [23]. Bacteriocins, for example, are thought to have evolved as weapons with which bacteria might clear the within-host environment of competitors sharing its niche [25]. We now use these toxins, which evolved as weapons of ‘bacterial warfare’ [26, 27], as drugs. Unfortunately, as a result of their previous role in mediating between-bacteria competition, many bacteria harbor genes that confer resistance to bacteriocins [28].
1.2 Drug Resistance

The specter of drug resistance appeared almost as soon as antimicrobial drugs were discovered in the early twentieth century [29]. Today, antimicrobial drug resistance is responsible for the deaths of 23,000 people in the United States of America alone [30] and threatens efforts to control the world’s biggest infectious disease killers, including HIV, tuberculosis and malaria. By 2050, drug resistant pathogens are projected to kill 10 million people a year, more than die of cancer, today [31].

Here, we define drug resistance as a heritable reduction in sensitivity to a drug (though non-heritable drug resistance has been observed [32]). Like any heritable trait, the evolution of drug resistance is determined by the forces of mutation, selection and drift. As such, evolutionary biology may play an important role in understanding and preventing the emergence of drug resistant pathogens [33].

The drug resistance crisis can be staved off in two ways. The first is to develop new drugs to which microbes are not (yet) resistant, the second is to design ways of using the drugs that are still effective to slow the evolution of resistance to them. These options are not mutually exclusive - indeed, doing the latter will increase the time available to achieve the former [34] - and incorporating evolutionary thinking could benefit both pursuits. With the failure of the traditional screening techniques used to identify antimicrobial compounds from natural sources and of synthetic drug design [35], decreasing economic incentives for drug discovery (which themselves arise from the inevitability of drug resistance) and regulatory hurdles, few believe that the old model of drug discovery will yield the number of novel compounds necessary [36]. Expanding both the definition of a drug, to include phages and metals [37], and the types of environments in which we seek them, could yield more novel candidate compounds. Technological advances that permit once unculturable bacteria to be grown in the lab [38], might also open up new frontiers. Incorporating evolutionary thinking into the process of drug design could speed the process of selecting candidate drugs and might stimulate the drug development industry: the slower resistance emerges, the greater the economic benefits of developing drugs. Evolutionary biology tells us that we should target the process by which traits arise, mutation, and by which they rise in frequency, selection. To reduce the input of drug resistance mutations, we might choose
compounds to which resistance is conferred by several mutations or a mutation that is difficult to acquire [39]. Alternatively, we might design drugs to which resistance is weakly favored by selection, such as those that target pathogen public goods (compounds produced by an individual pathogen that benefit the wider pathogen population) [40–42]. Even better would be to design drugs to which resistance confers no selective advantage, for example drugs that decompose into compounds that are lethal to resistant parasites [43].

Before novel compounds arrive on the market, however, the only option we have is to develop ways to thwart the emergence of mutants resistant to the drugs that remain useful. Using drugs of different classes in combination is perhaps the simplest way to prevent the emergence of mutants carrying de novo resistance mutations, since the probability of a parasite acquiring multiple resistance mutations simultaneously is smaller than the probability of it acquiring just one mutation. A further advantage of combination therapy is that the interactions between the drugs used can be utilized to manage the selection of any resistant mutants that do appear. Using an antagonistic pair of drugs, whereby the action of one inhibits that of the other, can maintain selection for susceptible rather than resistant parasites, for example [44, 45]. When resistance mutations are associated with costs, such that resistant mutants have lower fitness relative to their susceptible conspecifics in untreated environments (as is common [46]), these costs might be maintained or exacerbated in the absence of treatment such that resistant parasites lose in competition with their susceptible conspecifics [47, 48].

Competition is increasingly recognized as a force that plays an important role in preventing the emergence of drug resistant parasites [49,50]. Some of the best evidence we have for competition in parasite systems is from experiments involving drug susceptible and drug resistant parasites, where drug resistant parasites are almost invariably found to experience significant competition from susceptible conspecifics (reviewed in [51–54] but see [55, 56]). These experiments suggest that one reason why we do not observe drug resistance at high frequencies in untreated environments is that they are competitively suppressed by susceptible parasites. Only when their susceptible competitors are removed from the within-host environment by drug treatment do drug resistant parasites flourish. It has thus been proposed that competitive suppression be harnessed to slow down the
emergence of drug resistance [57]. For example, one might use a lower dose of drugs than is normally recommended to maintain the population of susceptible competitors in patients and so maintain competitive suppression of resistant parasites. Experiments using a mouse model of malaria infection have shown that low dose drug treatment can indeed prevent the emergence of resistant parasites and, importantly, that it comes at no cost to the health of hosts [58–60].

1.3 Malaria Parasites

Malaria parasites are vector-borne, protozoan parasites that infect an array of taxa and threaten both wildlife and human populations [61, 62]. Five species of malaria parasites infect humans. Together in 2013 they exacted a toll of 198 million cases and 584,000 deaths, most of which were of children [62]. Anemia, the characteristic symptom of malaria, is caused by blood-stage parasites invading and reproducing within red blood cells. After maturing, the progeny (known as merozoites) burst out of the red blood cells, destroying them as they go. Along with anemia, malaria infection can cause fever, loss of consciousness, convulsions and pulmonary edema [63], with the severest of symptoms associated with infections of the \textit{Plasmodium falciparum} parasite. In addition to the mortality and morbidity it causes, malaria infection can increase a person’s susceptibility to other infectious diseases [64, 65] and restricts the economic development of the countries in which it is endemic [66].

Efforts to control malaria have focused both on the parasites that cause the disease and the vectors that transmit them. Drug treatment plays a role both in the treatment of infected, sick individuals and in preventing vulnerable groups, such as children and pregnant women, from contracting the disease [63]. With an effective vaccine still not available, only drugs can fulfill the latter role. Though there are a number of drugs recommended for the treatment of malaria [63], in total they belong to just four drug classes (artemisinins, quinolones, antifolates and lincosamide antibiotics). With the emergence and spread of resistance to artemisinins [67,68], the last class of antimalarials that are approved for frontline use, there are now parasites resistant to every class of antimalarials in at least some part of the world.
Selection, rather than frequent mutation, underlies the widespread distribution of drug resistant malaria parasites. This is because mutants carrying a *de novo* mutation are extremely unlikely to reach transmissible densities within the hosts in which they originate [69], as the fact that all the world’s sulfadoxine-pyrimethamine resistant parasites are descended from just four parasites [70], demonstrates. So, while it is laudable that the World Health Organization (WHO) recommends that all antimalarials be given in combination with at least one other antimalarial of another chemical class [63], the WHO’s recommendations do little to address the dominant force in the evolution of drug resistant malaria parasites. There may be great potential to manage the selection of drug resistant malaria parasites, however, since the mutations that confer resistance to antimalarial drugs, including chloroquine, atovaquone and artemisinin, are associated with costs *in vitro* [71–74] and these costs likely play out in the field, as is evidenced by the observation that drug resistant mutants disappear upon the withdrawal of drugs [75, 76] (reviewed in [53]).

These observations suggest that competition between conspecific malaria parasites within-hosts might be intense, as has been observed in experiments of the mouse malaria model *P. chabaudi* [77–79]. Observational studies of the dynamics of the malaria species that infect humans also suggest that there is competition between different species of malaria parasites. The increasing incidence of the zoonotic malaria species *P. knowlesi*, following efforts to control *P. vivax* and *P. falciparum* has been interpreted as evidence that the latter species had hitherto competitively suppressed *P. knowlesi* [80, 81]. That mixed species infections are found less often than expected and infections of different species peak in sequence rather than simultaneously has also been taken as evidence of competitive interactions of different species of malaria [82,83].

The question of what mediates competitive interactions between malaria parasites within-hosts has been the subject of extensive theoretical, but limited experimental, investigation. Red blood cells have been the focus of mathematical models aimed at understanding competitive interactions between malaria parasites. These models suggest that parasites of both humans [84] and rodents [85] competitively suppress each other via their depletion of red blood cell resources and that the age range of red blood cells that a parasite infects determines its
competitive ability. It is possible to imagine that there might also be interference competition for red blood cells, whereby parasites are prevented from invading already-infected red blood cells. Little support for this mechanism has been found [86, 87], though Greischar [87] suggested that multiply-infected red blood cells might be more vulnerable to clearance in the spleen, such that there is spleen-mediated apparent competition. The host immune system might also mediate apparent competition between parasites via its production of antibodies, since antibodies are capable of targeting multiple parasite strains [88, 89] and species [83]. Indeed, Bruce et al. [82] have proposed that strain-transcending immunity caps the number of parasites within a single individual, so that competition for immune-free space is intense. In *P. chabaudi*, where the potential for CD4+ T cells to mediate apparent competition has been examined experimentally [90, 91], the evidence for apparent competition is equivocal, having been found in one study but not the other. A mathematical model fit to data from the study where evidence for apparent competition was found [91], suggested that clone-specific differences in the susceptibility of merozoites to the innate immune system determines their competitive ability [92]. Though both exploitation competition and apparent competition have been hypothesized to be important in the lives of malaria parasites, only Antia et al. [85] have attempted to assess which type of competition best explains the dynamics of malaria infections that we observe [85, 93]. They compared different theoretical models of within-host competition and found little evidence that one type of competition was more important than the other.

## 1.4 Thesis Objectives

In this thesis, we investigate which elements of the within-host environment limit populations of *P. chabaudi* and whether within-host resources might be manipulated for the control of drug resistant malaria parasites. In Chapter 2, we investigate the role of the micronutrient, para-aminobenzoic acid (pABA), as a resource for pyrimethamine resistant *P. chabaudi* and in mediating within-host competition between susceptible and pyrimethamine resistant parasite strains. In Chapter 3, we investigate the extent to which pyrimethamine resistant and susceptible parasites have differential requirements for pABA and whether the
availability of pABA within-host might be manipulated to intensify the competitive suppression of pyrimethamine resistant parasites and so prevent their emergence. In Chapter 4, we investigate whether the effect that manipulating the availability of pABA on the emergence of pyrimethamine resistant parasites changes with the genetic background of the parasites competing within a host. We also use a novel assay to investigate whether drug treatment changes in efficacy with changes in the within-host availability of pABA. In Chapter 5, we return to the investigation of elements of the within-host environment that limit the growth of malaria parasites, this time concentrating our focus on the immune system. We develop a modeling framework with which to investigate the amount of parasite mortality caused by the immune system in malaria infections. We use this to investigate how the shape and magnitude of the immune response changes with the availability of pABA within-hosts, among other variables. In Chapter 6, we draw these chapters together and discuss future directions for research. These chapters were written as isolated units, intended for publication, and so inevitably there will be some, limited amount of repetition.
Chapter 2

A micronutrient, para-aminobenzoic acid, mediates within-host competition between malaria parasites

2.1 Abstract

Parasites, by definition, extract resources from their hosts. Competition among parasites for host resources is thought to be intense, since parasites often live at high densities. Competition among malaria parasites of the species *Plasmodium chabaudi* has been the focus of much theoretical but limited experimental investigation. Here, we investigate the role of para-aminobenzoic acid (pABA) in mediating competition between two strains of *P. chabaudi*, by varying the supply of pABA to their rodent hosts. We confirmed earlier findings that pABA is a limiting resource for pyrimethamine resistant malaria parasites, in infections from which a competitor was absent. In infections in which a drug susceptible competitor was present, the intensity of competition that the pyrimethamine resistant parasite experienced changed with the concentration of pABA supplemented to the host, indicating that pABA mediates interstrain competition. Parasites in unsupplemented hosts experienced more competition from susceptible parasites than those in hosts supplemented with a low concentration of pABA, in accordance with classical resource competition theory. However, the intensity of competition increased with further increases in pABA concentration. We discuss hypotheses that might explain the non-linear relationship between competition intensity and resource supply. This work represents the first time that a nutrient has been demonstrated to mediate competition between *P. chabaudi* parasites.
2.2 Introduction

Communities of parasites within-hosts can be extremely large and diverse. As a result, the availability of host resources may be severely limited and competition between parasites, rife. In the last decades, evidence for interspecific and intraspecific competition between parasites, including the causative agents of malaria and sleeping sickness, has accumulated (interspecific [17, 83, 94–96], intraspecific [77, 97–100]) and within-host competition has been implicated as a driver of the evolution of several parasite traits, including virulence [23, 26], antigenic diversity [24, 101] and drug resistance [55, 56]. With the recognition that competition has an important role in the within-host ecology of parasites has come interest in harnessing within-host competition for their control [57,102,103]. Yet what mediates competition between parasites within hosts is still little understood and rarely investigated experimentally (but see [90,91]). A mechanistic understanding of within-host competition is required if we are to fully understand the role it plays in the dynamics of parasite populations and communities and if we are to manipulate competitive interactions to our advantage.

Organisms can compete directly, reducing the quantity of resources available to each other by consuming resources (exploitation competition) or by obstructing access to them (interference competition), or indirectly, via the action of common enemies (apparent competition, a special kind of interference competition) [2]. The importance of exploitation and immune-mediated apparent competition has been explored in the rodent malaria parasite system, *Plasmodium chabaudi*, which has several advantageous properties as a system with which to begin studying the mechanistic basis of competition between parasites. Firstly, intraspecific competition between different strains of *Plasmodium chabaudi* has been observed many times and can be intense [15, 77–79, 104]; second, different strains of *P. chabaudi* vary in their competitive ability and the outcome of competition between them is relatively predictable [79]; third, much is known about *P. chabaudi*'s biology because of its importance as a model of human malaria and, for the same reason, experimental tools are available for its manipulation. Indeed, tools borrowed from biomedicine were employed in two experimental studies of the importance of CD4+ T cell-mediated apparent competition in *P. chabaudi* [90,91].
Unfortunately, these studies made conflicting conclusions about the importance of immune-mediated apparent competition in malaria parasites. Studies of exploitation competition in malaria parasites, meanwhile, have focused on the role of red blood cells. Parasites invade and reproduce within red blood cells, which are destroyed when parasites’ offspring emerge, and so red blood cells might reasonably be considered to be limiting resources for malaria parasites. The assumption that red blood cells mediate intrastrain competition between parasites is implicit in theoretical studies of \textit{P. chabaudi} infection dynamics, in which parasite densities are typically modeled as a function of red blood cell density [85, 105, 106] and the immune system is only sometimes incorporated as an additional regulatory force [107–109]. Red blood cells are also thought to mediate competitive interactions between different strains of malaria parasites (interstrain competition). That the maximum number of red blood cells that a parasite strain destroys, when it is alone in a host, predicts a strain’s success in competition with other strains [79,110] has been taken as evidence that red blood cells mediate interstrain competition. The results of a mathematical model bore out this hypothesis [85]. According to this model, the competitive ability of a parasite strain is a function of its capacity to infect red blood cells of different ages (i.e. how much of a ‘generalist’ the strain is) [85]. A recent model by the same authors suggested that the competitive ability of a parasite strain might equally be explained by its growth rate or susceptibility to innate immunity [93], however, and the importance of red blood cells in mediating within-host competition has never been assessed experimentally. Despite the advantages of this system, then, it has proven difficult to demonstrate that any single aspect of the within-host habitat mediates competition between \textit{P. chabaudi} parasites.

Fortunately, malaria parasites require more than one resource to survive and reproduce. The red blood cells in which malaria parasites make their home are, compared to other cell types, metabolically inert. While red blood cells provide hemoglobin, a rich source of some amino acids, they do not provide other nutrients in great quantities [111]. Thus parasites must manufacture nutrients or import them from the serum to meet their resource requirements. It may be more helpful, therefore, to imagine the red blood cell not as the sole resource over which malaria parasites could compete but rather as a patch of relatively poor-quality habitat.
We may then ask whether the resources that must be brought into these habitat patches to facilitate parasite reproduction mediate competition between parasites. Here, we explore whether one such nutrient, the folate precursor para-aminobenzoic (pABA), mediates competition between malaria parasites. While their mammalian hosts obtain folate from their diet, *Plasmodium* parasites are able to synthesize it from pABA and 6-hydromethyl-7,8-dihydropterin pyrophosphate, via the folate pathway [112]. Malaria parasites use the folate they produce for pyrimidine synthesis and methionine metabolism [113]. Maximal growth of *Plasmodium* parasites (including the rodent malarias *P. chabaudi* [114], *P. berghei* [115–117] and *P. yoelii* [118] and the primate malarias *P. knowlesi* and *P. cynomolgi* [115]) *in vivo* is achieved only when pABA is present in the diet of experimental hosts, which themselves do not require pABA [119]. So, it is routine practice to supplement the drinking water of experimental animals with pABA [120, 121]. We take advantage of this simple way of manipulating the availability of pABA within-hosts to experimentally explore the role of pABA in mediating within-host competition. To confirm that pABA is a limiting nutrient, we first investigate whether the growth of a focal parasite strain changes with the concentration of pABA administered to hosts. To investigate pABA’s role in interstrain competition, we then assess how the negative impact of a competitor on the performance of the focal strain, which we call competitive suppression, changes with pABA concentration.

### 2.3 Materials & Methods

Hosts were female 6-8 week old C57BL/6J mice, maintained on 5001 Laboratory Rodent Diet (LabDiet, USA). PABA was administered to mice via drinking water at a concentration of 0.05% (high treatment), as is standard in experiments involving *P. chabaudi*, 0.01% (medium treatment), 0.005% (low treatment) or 0% (unsupplemented treatment). Ten mice were assigned to each pABA treatment and further allocated to either the single or mixed infection treatment (Table 2.1). Mice in single infection treatments were infected with $10^6$ parasites of the pyrimethamine resistant AS44p strain (hereafter, AS$_{pyr}$), which was derived by passage from 47AS, a strain made resistant to pyrimethamine by a single round of drug selection [122].
Mice in mixed infection treatments were simultaneously infected with $10^6$ AS$_{pyr}$ and $10^6$ AJ22p (hereafter, AJ) parasites. Competition between AJ and AS$_{pyr}$ has been well characterized and is intense and asymmetrical - while the performance of AS$_{pyr}$ is greatly depressed in AJ’s presence, AS$_{pyr}$’s presence has little impact on the performance of AJ [79]. Note that mice in the single infection treatments received half the number of parasites as those in mixed infection treatments. Since increasing the inoculum of a single strain of parasites by an order of magnitude has a negligible impact upon the dynamics of infections [123], doubling the number should have little impact, in and of itself. Any differences in the dynamics of single and mixed infections can therefore be attributed to the effect of interstrain competition.

Infections were monitored daily from days 3 to 21 post-inoculation (PI). Each day, 7 μl of blood was taken from the tail: 2 μl for the quantification of red blood cell (RBC) density via flow cytometry (Beckman Coulter); 5 μl for the quantification of parasite density by quantitative PCR (qPCR), as previously described [58]. As an additional measure of morbidity, mouse weight was measured. Experiments were conducted in accordance with the protocol approved by the Animal Care and Use Committee of the Pennsylvania State University (Permit Number 44512).

Statistical analysis was performed using R (version 3.2.0). For each mouse, we calculated total parasite density and total red blood cell density, the sum of the daily measurements of parasite density and red blood cell density, respectively. Prior to analysis, measurements of parasite density (total parasite density or parasite density at a single time point) were log transformed. The temporal dynamics of infections were analyzed using linear mixed effects (LME) models, with day fitted as a factor to allow for nonlinearity in infection dynamics and individual mouse fitted as a random effect, following [60]. The initial replication rate of the parasite population was analyzed in a similar way but with day included as a numeric rather than a factorial variable. Since the growth rate of parasite populations in some mice had slowed by the fifth day, only data from days 3 and 4 were used for the analysis of initial replication rate. When the variance of measurement variables differed between treatment groups, generalized least squares (GLS) models were used, following [124]. Both GLS and LME models were run using the \textit{nlme} package. Model simplification was performed by sequentially
dropping the least significant term, until all terms were significant. Least significant terms were identified using likelihood ratio tests, for GLS and LME models, and F-tests for standard linear regression models. To test for differences between treatment groups, Bonferroni posthoc tests were performed using the lsmeans package. We report the adjusted p-values ($p_{adj}$) of these tests. Three mice received a parasite inoculum lower than was intended and were removed from all analyses (Fig. A.1, A.2). All mice in the high pABA mixed infection treatment eventually succumbed to infection (Fig. A.2) and day 8 was the last day on which all mice were alive. The effect of pABA concentration on the magnitude of competitive suppression was therefore assessed during the period between days 3-8 PI, using data from all treatment groups, and then again during the period from days 3-21 PI, with the high pABA treatment excluded. In the analysis of the magnitude of competitive suppression on total parasite density, one mouse was identified as having a particularly large impact on the results. In the main text, we report the results of the analysis with this mouse excluded. The results of the same analysis, with the outlier included, are reported in the Appendix A.

2.4 Results

**pABA is a limiting resource for $A_{pyr}$ in single infections.** Both the size and dynamics of $A_{pyr}$ infections changed with the concentration of pABA (Fig. 2.1AC; total density pABA $\chi^2_{2} = 22$, $p < 0.001$; parasite density day*pABA $\chi^2_{54} = 177$, $p < 0.001$). $A_{pyr}$ produced significantly more parasites in the supplemented treatments (low, medium & high treatments) than in the unsupplemented treatment (pairwise comparisons between individual supplemented treatments vs. unsupplemented treatment $p_{adj} < 0.001$). The number of parasites produced by $A_{pyr}$ in the high treatment was significantly greater than that produced in either the low ($p_{adj} = 0.01$) or unsupplemented treatment ($p_{adj} < 0.001$), but did not differ from the number produced in the medium pABA treatment ($p_{adj} = 0.28$). So, while supplementing hosts with pABA boosted the productivity of $A_{pyr}$ parasites, it only did so up to a point. The initial replication rate of $A_{pyr}$ was also impacted by pABA treatment (Fig. 2.1B; parasite density day*pABA $\chi^2_{2} = 16$, $p = 0.001$). $A_{pyr}$ grew almost twice as fast in the high treatment as in the unsupplemented
treatment, multiplying at a rate of 8.2 fold per day (6.5-10.4 95% CI) as compared to 4.5 fold per day (3.7-5.6 95% CI), respectively. Though the replication rate of AS<sub>pyr</sub> appeared to increase with the concentration of pABA (Fig. 2.1B), differences in the replication rate of AS<sub>pyr</sub> in the different supplemented treatments were not significant (all pairwise comparisons among supplemented treatments p<sub>adj</sub>=1). The dynamics of infection in the different pABA treatments varied considerably (Fig 2.1C; parasite density day* pABA $\chi^2_{14}= 177$, p<0.001). In the supplemented treatments, following the initial crash in parasite numbers, parasite numbers temporarily plateaued or, in some cases, grew. In the unsupplemented treatment, by contrast, parasite numbers fell at a relatively continuous rate after reaching their peak (Fig 2.1C).

**The virulence of single AS<sub>pyr</sub> infections changes with pABA concentration.** Over the duration of the experiment, pABA supplementation altered the virulence of AS<sub>pyr</sub> infections, as measured by total red blood cell density but not total weight (Fig. 2.2AB; total RBC density pABA $F_{3,14}= 6.3$, p=0.006; total weight pABA $F_{3,14}= 0.5$, p=0.7). Mice administered high and medium concentrations of pABA were more anemic than mice administered unsupplemented water (high vs. unsupplemented p<sub>adj</sub>=0.03; medium vs. unsupplemented p<sub>adj</sub>=0.007), but mice in the low and unsupplemented treatment suffered equally (p<sub>adj</sub>=0.13). The acuteness of disease also varied with pABA treatment (Fig. 2.2CD; minimum RBC density pABA $F_{3,14}=22$, p<0.001, minimum weight pABA $F_{3,14}= 9$, p=0.001). The maximum degree of anemia and weight loss experienced by mice in pABA supplemented treatments was significantly greater than that experienced by mice in the unsupplemented treatment (all pairwise comparisons between supplemented and unsupplemented groups p<sub>adj</sub>=<0.01), but among mice in the supplemented treatments disease was equally acute (all pairwise comparisons among supplemented treatments p<sub>adj</sub>=0.2). So, while supplementing hosts with pABA increased the severity of disease, how much pABA was administered to them had no impact.

**pABA mediates competition between AS<sub>pyr</sub> and AJ.** Consistent with previous findings, AS<sub>pyr</sub> was competitively suppressed by AJ (Fig. 2.3, 2.4). The
impact of competition on the size of $AS_{pyr}$ infections between days 3-8 varied among pABA treatments (Fig. 2.3A; total density pABA*competition $\chi^2_3 = 14$, $p= 0.004$), though the statistical significance of the interaction between pABA and competition was conditional on the exclusion of an outlier (see Appendix A). Competitive suppression appeared to be least intense in the intermediate treatments (low, medium) and most intense at the extremes (unsupplemented, high treatments) (Fig. 2.3B), but only among the low and high pABA treatments was the intensity of competitive suppression significantly different ($p_{adj}=0.008$). Over the whole experimental period, pABA treatment had a marginal impact on the intensity of competitive suppression (Fig. 2.3CD; total density pABA*competition $\chi^2_3 = 5.4$, $p= 0.07$). Again, competitive suppression appeared to be most intense in the unsupplemented treatment (Fig. 2.3D).

The dynamics of competition differed among pABA treatments (Fig. 2.4; parasite density, days 3-8 day*paba*competition $\chi^2_15 =80$, $p<0.001$; parasite density, days 3-21 day*paba*competition $\chi^2_36=110$, $p<0.001$). Competition attenuated the pre-peak phase of infection in all pABA treatments but this phase was most shortened in the unsupplemented treatment (Fig. 2.4). The initial replication rate was not affected by the presence of a competitor (parasite density day*pABA*competition $\chi^2_3 = 6$, $p= 0.1$).

The concentration of pABA administered to mice had a minimal impact on AJ (Fig. 2.5). The number of parasites produced by AJ was not affected by pABA treatment (total density of AJ, days 3-8 pABA $F_{3,15}=1.7$, $p=0.2$; total density of AJ, days 3-21 pABA $F_{2,11}= 1$, $p= 0.4$). The dynamics of AJ infections were not impacted by pABA between days 3-8 PI but, over the duration of the experiment, pABA significantly altered susceptible parasite dynamics (Fig. 2.5; density of AJ, days 3-21 day*pABA $\chi^2_36= 82$, $p<0.001$). The steepness of the post-peak decline in parasite density and the time between the primary and secondary peaks, appeared to increase as the concentration of pABA decreased (Fig. 2.5).

### 2.5 Discussion

Which elements of the within-host environment mediate competition between parasites has been the subject of much speculation but few experimental
investigations. Here, we experimentally demonstrate that pABA can mediate competition between malaria parasites. So far as we are aware, this is the first time that it has been experimentally demonstrated that a nutrient mediates competition between *P. chabaudi* parasites.

Over the gradient of pABA supplementation, the intensity of competitive suppression changed, but it did not do so in the way that one might expect. Classical resource competition theory states that competition between organisms should be most intense when resources are scarce [1]. Here, however, the relationship between the intensity of competitive suppression and pABA concentration appears to be U-shaped (Fig. 2.3B). Initially, as we would expect, competition weakened in intensity as pABA concentration increased. However, with subsequent increases in the concentration of pABA, the intensity of competition increased. Indeed, the only significant difference in the intensity of competitive suppression was observed between the low and high pABA treatments, where competitive suppression was least and most intense, respectively.

What could explain this U-shaped pattern in the intensity of competition? First, to address the reason why competitive suppression increased in intensity with pABA concentration, between the low and high treatments. It is possible that pABA is not a limiting resource for pyrimethamine susceptible AJ (at least not at the concentrations at which it was supplemented) and so the parasite strains do not engage in competition until AS$_{pyr}$ reaches high densities in the medium and high treatments. Jacobs [116] found that, while pABA was a limiting resource for *P. berghei* parasites susceptible to pyrimethamine, they required an order of magnitude less pABA than pyrimethamine resistant parasites to sustain a given level of parasitemia [116]. Parasites susceptible to pyrimethamine also have a greater capacity than pyrimethamine resistant parasites to acquire folate from molecules other than pABA [125]. For AJ the low pABA environment may represent an environment in which folate is available in excess. As a result, AS and AJ might operate effectively in isolation of each other in the low pABA treatment. Only at high pABA concentrations, where AS$_{pyr}$ is freed from pABA limitation and flourishes, might AS$_{pyr}$ begin to compete with AJ over resources other than pABA (including immune-free space). Due to AS$_{pyr}$’s inferiority as a competitor [79], however, it loses in competition with AJ. Explanations similar to this have been
invoked to explain the observation that the intensity of competition increases with environmental fertility/productivity in multi-species plant communities [126, 127]. What could explain the other side of the competition-intensity curve, the increase in the intensity of competition that occurs between the low and unsupplemented treatment? The first explanation is that of classical resource competition theory. In unsupplemented mice, pABA becomes limiting for AJ. Therefore, AJ engages in direct competition for pABA with AS_pyr and, by dint of its competitive superiority, acquires most of the available pABA. An alternative hypothesis is that the increase in the intensity of competitive suppression in the unsupplemented treatment vs. the low pABA treatment is a result of an increase in apparent competition. If the size of the immune response is proportional to the amount of antigenic stimulation the host receives, the immune response is likely to be larger in mixed than single infections. It has been suggested that there is a threshold population size below which parasites cannot escape the impact of immune killing [108]. The dramatic reduction in population size that occurs with the removal of pABA might push the population of AS_pyr below this threshold. As a result, in unsupplemented mice, AS_pyr experiences high levels of immune-mediated competition, whereas in the low pABA treatment AS_pyr escapes it. Measuring the concentration of pABA within-hosts would help to establish whether competition between AS_pyr and AJ in the unsupplemented treatment takes the form of direct competition for pABA or immune-mediated apparent competition. Keddy [2] notes that ‘interference competition does not change resource levels, only access to them’ (pg. 319). If the immune system mediates competition between strains in the unsupplemented treatment, therefore, the concentration of pABA in the blood of mice in the unsupplemented, mixed infection treatment should not differ much from the concentration of pABA in the blood of mice in the unsupplemented, single infection treatment. To further investigate the validity of our hypotheses about the ways in which pABA mediates competition, an experiment in which AJ is grown in single infections across a pABA concentration gradient should also be conducted. This will help us to establish at what concentration, if any, pABA limits the growth of AJ.

While AS_pyr and AJ may not directly compete for pABA, our data suggest that there is likely to be competition among AS_pyr parasites for pABA. The
finding that pABA is a limiting resource for *P. chabaudi* is consistent with earlier investigations [114]. Both the number of parasites produced and the growth rate of AS pyr were promoted by pABA supplementation, as might be expected given that pABA is involved in DNA synthesis (and hence the production of offspring). However, the response of AS pyr to the availability of pABA was a saturating one. This makes sense in light of resource ratio theory [1] - at some point a resource other than pABA will begin to limit the growth of parasites - and will be reassuring for laboratory investigators. Since it is standard laboratory practice to administer pABA at a concentration of 0.05% (as per the high pABA treatment) [120, 121], pABA is is unlikely to limit parasite growth in laboratory experiments of *P. chabaudi*. That said, reducing the concentration of pABA given to experimental animals to 0.01% (as per the medium treatment) might reduce the variability of parasite dynamics among experimental hosts (Fig. 2.1C). Parasites in the medium treatment might encounter pABA more reliably than parasites in other treatments, since their hosts are being supplemented with pABA but the density of intrastrain competitors, which could reduce the density of pABA molecules in the blood, is not maximal.

Our data suggest that, as well as altering interactions among AS pyr parasites, changes in pABA supplementation might change the interaction of the population of AS pyr with the immune system. PABA treatment altered the dynamics of parasite growth in the period after parasite density had peaked, a period during which the dynamics of infection are thought to be governed by the immune system [85, 106, 108]. PABA is unlikely to have a direct impact on the immune system, since mice do not require it [119]. Furthermore, the removal of pABA from the host diet has a similar inhibitory impact on parasite growth in intact mice as in immune-deficient mice [118]. Instead, the absence of pABA in the unsupplemented treatment might alter the capacity of parasites to deal with the immune response. The growth rate of parasites in supplemented treatments may be large enough that the number of parasites produced balances out, or perhaps even exceeds, the number lost as a result of immunity-induced mortality. As a result, we observe a plateau, and in some cases an increase, in parasite numbers during the post-peak phase. We refer to the hump in the dynamics of parasite growth in supplemented treatments as ‘the shoulder’ (see Chapter 5). By
contrast, the growth rate of parasites in the unsupplemented treatment may be too low to compensate for the impact of immune pressure and so the shoulder is missing from these infections. An alternative explanation is that parasites in supplemented treatments stimulate a qualitatively different immune response than those in unsupplemented treatments and it is the difference in the nature of the immune response that determines whether the shoulder is present or not. The antimalarial immune response consists of two components: the TH1 response provide early protection, while the TH2 response is involved in the clearance of parasites [128]. Inflammatory cytokines of the TH1 response come at the expense of the TH2 response and can cause immunopathology [128, 129]. Taylor-Robinson and Phillips [129] found that as the dose of parasites that a mouse receives increases, so too does the number and rate at which TH1 cytokines are produced (note that parasite dose and growth rate were confounded in their study). Indeed, it has been suggested that a threshold amount of antigen is required before T cells produce significant quantities of the TH1 cytokine IFN-γ [130]. Since parasites grow faster and produce a greater number of parasites in supplemented than unsupplemented mice, their population size will exceed any such threshold sooner and for longer in supplemented than unsupplemented mice. Perhaps, then, parasites in supplemented treatments stimulate an immune response characterized by a large TH1 response and a small TH2 response, which causes them to be cleared more slowly than parasites of unsupplemented mice and allows them to produce the shoulder. By contrast, parasites in unsupplemented treatments might stimulate a more balanced response, which causes them to be cleared rapidly. This explanation also fits with our observation that infections of unsupplemented mice were less virulent than those of supplemented mice, since a balanced immune response would cause less immunopathology than one skewed toward the TH1 response.

Since modulating the availability of pABA to hosts altered the virulence of infections, it is tempting to ask whether pABA’s availability might be manipulated for the promotion of patient health. The scientists that originally observed that pABA limited the growth of malaria parasites shared this thought and speculated about administering malaria-infected patients dairy-based diets, which contain very low concentrations of pABA, to limit patients’ parasitemia [115,131].
Today, the availability of pABA for malaria parasites is reduced via the drug sulfadoxine, a pABA antagonist that competes with pABA for the folate-pathway enzyme dihydropteroate synthase and which is administered in combination with pyrimethamine as sulfadoxine-pyrimethamine (S/P). Dietary manipulation of pABA may still have some relevance, however. *In vitro*, the efficacy of S/P is inversely related to the concentration of pABA in the medium, because pABA competes with sulfadoxine for its dihydropteroate synthase binding site [132–134]. Kicksa *et al.* [118] recently proposed that diets low in pABA be administered to patients receiving S/P treatment, to boost S/P’s efficacy. As the efficacy of S/P, and almost every other drug used to treat infectious diseases, is threatened by the evolution of drug resistance in parasite populations, there is mounting pressure to find ‘evolution-proof’ treatment strategies. Targeting host resources that are required for parasite growth might represent such a strategy [135]. Mechanisms used by parasites to resist drugs, such as increases in the numbers of efflux pumps or changes in enzyme conformation, would be of little help in resisting an intervention that involved the depletion of pABA from hosts, for example.

Indeed, because pABA mediates competition between parasites, it might be a particularly good target for ‘evolution-proof’ therapies. Susceptible pathogens competitively suppress resistant pathogens (as was observed here). Drug treatment removes these competitors, allowing resistant parasites to flourish [50]. Day and Read (*in prep*) have suggested that, since susceptible parasites inhibit the proliferation of resistant parasites via their impact on the availability of resources in the within-host environment, one might prevent the emergence of resistant parasites by reducing the availability of within-host resources over which parasites compete. Having thus mimicked the impact of susceptible competitors on the within-host environment, one could remove susceptible parasites using drugs without causing the emergence of resistant pathogens. Here, removing pABA from the water of mice had a larger impact on parasite proliferation than competition did (Fig. 2.3AC). This experimental system might thus present a good opportunity to test the ideas of Day and Read. If, however, susceptible parasites do not require pABA for growth, a possibility that warrants further investigation, pABA depletion will be a poor way of mimicking the impact of a susceptible parasite on the within-host environment. Nonetheless, depleting pABA from the host
environment could be used to intensify the competitive suppression of resistant parasites by their susceptible conspecifics. Theory predicts that when an organism requires a resource more than its competitor, reducing the availability of that resource can increase the organism’s suppression by its competitor [136]. We saw this phenomenon here (the left side of the competitive suppression curve, Fig. 2.3BD). If pyrimethamine resistant parasites require pABA in excess of susceptible parasites, then depleting pABA from the water of mice could intensify competitive suppression of pyrimethamine resistant parasites, perhaps to the extent that they go extinct before they have the opportunity to emerge.

A mechanistic understanding of within-host competition between parasites can thus open up opportunities for their control. Despite a resurgence of interest in within-host parasite communities (e.g. [137,138]), and in the idea of harnessing the interactions that govern them, there have been few attempts to empirically study the forces that structure parasite communities. Early interest in competition as a force structuring infracommunities sprang from the observation that there were striking divisions in spatial use by different helminth species in the gut [139,140]. This pattern was interpreted as a signal that competition in the past was so intense that it had driven the evolution of tissue tropism [139], a hypothesis that was, regrettably, almost untestable. Since then, experiments investigating the response of helminths to competition have been conducted (reviewed in [140]). Advances in technology have further facilitated similar experiments in microparasite systems (e.g. [77,96–100]). Yet, while the number of studies investigating the response of parasites to competition has grown, the factors that mediate competition have largely been the subject of speculation - we could not find a single study in which competition was studied over a resource gradient. Perhaps this is unsurprising given that, because of the nature of the parasitic lifestyle, the resources used by parasites are also likely to be host resources, making their manipulation difficult. Though understandable, the dearth of experimental investigations of the drivers of competition in parasite communities threatens to render the community ecology of parasites a descriptive rather than a predictive science. *P. chabaudi*, and other model systems of human diseases, might facilitate a move from phenomenological descriptions of competition to explanations of it. In these systems, one is most likely to have tools with which to manipulate the within-host environment and
detailed knowledge of the parasite’s resource requirements, acquired through efforts to grow parasites in culture and/or metabolomic investigations. Here, we used knowledge of the nutrient requirements of malaria parasites to expand our imagination of the parasite niche beyond the two most obvious axes, the cells that parasites infect and the immune system, and to identify a resource that might mediate competition. We then used well-established ways of manipulating this resource to experimentally demonstrate that pABA mediates competition between malaria parasites.

2.6 Acknowledgements

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2.7 Notes

This work was conducted in collaboration with Andrew F. Read, with technical assistance from Derek G. Sim. It will be published with these persons as coauthors.
### 2.8 Figures & Tables

<table>
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<tr>
<th>pABA concentration</th>
<th>Single Infection (AS\textsubscript{pyr} alone)</th>
<th>Mixed Infection (AS\textsubscript{pyr} &amp; AJ)</th>
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<td>5 (1\textsuperscript{o})</td>
<td>5 (5\textsuperscript{+})</td>
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<tr>
<td>Medium (0.01%)</td>
<td>5 (1\textsuperscript{o})</td>
<td>5 (1\textsuperscript{o}, 1\textsuperscript{*})</td>
</tr>
<tr>
<td>Low (0.005%)</td>
<td>5 (1\textsuperscript{o})</td>
<td>5 (1\textsuperscript{o}, 1\textsuperscript{*})</td>
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<tr>
<td>Unsupplemented (0%)</td>
<td>5</td>
<td>5</td>
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Table 2.1: **Number of mice in each treatment.** Numbers in brackets indicate the number of mice that were removed from some or all analyses because they were misinoculated (circle superscript, removed from all analyses), they died (cross superscript, removed from analysis of total parasite density) or they were identified as an outlier (asterix superscript, removed from analysis of total parasite density).
Figure 2.1: **pABA is a limiting resource for AS<sub>pyr</sub> parasites.** Total density from days 3-21 (A), initial replication rate (B) and infection dynamics (C) of single infections of AS<sub>pyr</sub> parasites in unsupplemented (blue), low (green), medium (pink) and high (orange) pABA treatments. In A and B, model estimates of the mean and 95% confidence interval are shown; treatments that were not significantly different from each other share the same letter annotation. In C, the star represents the number of, and the time at which, parasites were administered; the dot represents the density of parasites detected in an instance when parasites were not detected the day before or after. n specifies the number of mice plotted and included in the analysis.
Figure 2.2: The impact of pABA concentration on the virulence of infections. Total and minimum red blood cell density (A, C) and total and minimum weight (B, D) of mice infected with AS_{pyr} parasites only and administered unsupplemented water (blue) or low (green), medium (pink) or high (orange) concentrations of pABA as drinking water. Model estimates of the mean and 95% confidence interval are shown. Treatments that were not significantly different from each other share the same lowercase letter annotation. n specifies the number of mice plotted and included in the analysis.
Figure 2.3: **pABA mediates competition between AS$_{pyr}$ & AJ.** Total density of AS$_{pyr}$ (A,C) in single (filled circles) and mixed (open circles) infections and the intensity of competitive suppression experienced by AS$_{pyr}$ (B, D) during days 3-8 (A,B) and days 3-21 (C,D) of infection. Model estimates of the mean and 95% confidence intervals are shown. $n$ specifies the number of mice plotted and included in the analysis. In A and C, the significance of the difference in the total density of AS$_{pyr}$ in single vs. mixed infections is indicated by the number of stars as follows - one ($p<0.05$), two ($p<0.01$), three ($p<0.001$). In B and D, treatments that were not significantly different from each other share the same lowercase letter annotation. These results are from an analysis where an outlier was removed, see Fig. A.3 for the results of the same analysis with the outlier included.
Figure 2.4: The intensity of competitive suppression of $A_S^{pyr}$ changes through time and with pABA treatment. Dynamics of $A_S^{pyr}$ alone, in single infections (solid lines), and in competition with AJ (dashed lines) in unsupplemented (blue), low (green), medium (pink) and high (orange) pABA treatments. Each line represents the dynamics of infection in a single mouse. The dynamics of $A_S^{pyr}$ in competition in the high pABA treatment (dashed, orange lines) are attenuated because the mice died. The stars indicate the number of, and the time at which, parasites were administered. Dots represent the density of parasites detected on a particular day, in instances where parasites were not detected the day before or after. n specifies the number of mice plotted and included in the analysis of parasite dynamics.
Figure 2.5: **In mixed infections, pABA treatment has no impact on the growth of AJ.** Dynamics of AJ in mixed infections in unsupplemented (blue), low (green), medium (pink) and high (orange) pABA treatments. Each line represents the dynamics of infection in a single mouse. The star represents the number of, and the time at which, parasites were administered. Dots represent the density of parasites detected on a particular day, in instances where parasites were not detected the day before or after. n specifies the number of mice plotted and included in the analysis.
Chapter 3  

Resource depletion can prevent the emergence of drug resistant parasites

3.1 Abstract

Preventing the emergence of drug resistant pathogens is a major challenge for evolutionary biologists and pharmacologists, alike. In nature, competition from susceptible pathogens keeps resistant pathogens in check. Drug treatment removes these competitors, allowing resistant parasites to flourish. Ecological theory states that the intensity of competitive interactions changes with resource supply - when an organism requires more of a resource than its competitor, depleting that resource will increase its suppression by its competitor. We tested whether resource depletion could cause the competitive exclusion of resistant parasites, before they were released from competition by drugs, using pyrimethamine resistant *Plasmodium chabaudi* parasites, which require more para-aminobenzoic acid (pABA) than susceptible parasites. Depletion of pABA prevented the emergence of resistant parasites and so boosted mouse health. The impact of pABA depletion was competition-mediated, since it did not prevent the growth of resistant parasites in infections where competitors were absent. In a second experiment using artesunate resistant parasites, which do not require pABA in excess, pABA depletion did not prevent resistance emergence. Resource depletion as an intervention has several desirable properties. Target resources could be rationally identified, given knowledge about the mechanisms conferring resistance. Since their action is competition-mediated, resource-depleting interventions need not be lethal to be effective, relaxing a constraint that applies to traditional drugs. As resource depletions leave susceptible pathogens largely unscathed, resistance to them may be slow to arise. In an age where new treatments emerge rarely but resistance to them emerges at speed, resource depletion represents an exciting, new
3.2 Introduction

Antimicrobial resistance poses a major threat to public health, causing thousands of deaths a year in the US alone [30]. Yet as the number of deaths from drug resistant microbes is expected to rise [31], the rate at which novel antimicrobials are discovered declines [141]. Developing interventions that slow the evolution of drug resistance is therefore essential. Here, we demonstrate that depletion of a within-host resource required in excess by drug resistant pathogens can prevent their emergence by intensifying competitive interactions between resistant and susceptible pathogens. As a resistance-management tool, resource depletion represents an intervention that has potential in a variety of systems and may be, itself, ‘resistant to resistance’.

Utilizing the forces that keep resistant parasites in check in untreated environments represents an intuitive, and possibly evolutionarily robust, resistance management strategy. The frequency of resistant parasites in untreated environments may be limited by competitive suppression. Resistant parasites often emerge into an environment in which there is already a large susceptible population, for example when they arise by de novo mutation or horizontal gene transfer or when they superinfect an already-infected individual. In such cases, the susceptible parasite will have consumed much of the pool of within-host resources, such as nutrients or immune-free space. Few resources will thus be available to the resistant population and its growth rate and size will be limited - it is said to experience competitive suppression [50]. Only when drug treatment removes their susceptible competitors, allowing host resources to rebound, can the population of resistant parasites grow, a phenomenon known as competitive release [50, 58, 59, 142, 143]. If the resistant population could be driven to extinction before host resources rebound, the emergence of resistant parasites via competitive release could be prevented. Intensifying competitive suppression represents one way of achieving this.

The strength of competitive interactions between organisms can be altered by changing the supply of resources into the environments that they inhabit [18,136].
When an organism is competitively inferior because it requires more of a resource than its competitors for growth, reducing the supply of that resource can intensify its suppression by competitors. Resistance mutations are commonly associated with costs [46], which often take the form of increased resource requirements. Glucose is at a premium for cancer cells that resist drugs by pumping them out of their cells [48]. Chloroquine resistant malaria parasites have impaired abilities to catabolize hemoglobin, the parasite's major source of amino acids, and so suffer more than drug susceptible parasites in conditions of amino acid scarcity [72]. Pyrimethamine resistant malaria parasites require more of the folate precursor para-aminobenzoic acid (pABA) for growth than susceptible parasites [116, 117], because the resistance mutation impairs folate acquisition and/or production [125].

Here, we explore whether reducing the supply of a resource required in greater quantities by resistant than susceptible parasites can prevent the emergence of resistant parasites from an infection dominated by susceptible parasites. We do so by manipulating the supply of pABA to mice infected with pyrimethamine resistant Plasmodium chabaudi, a mouse model of malaria. First, we establish the extent to which susceptible and resistant parasites have differential pABA requirements. Then we examine the impact of depleting pABA from the within-host environment on the magnitude of competitive release. In a second experiment, similar in design, we test the hypothesis that depleting a resource for which susceptible and resistant parasites have similar requirements will not prevent the emergence of resistant parasites. For this we use parasites resistant to artesunate, a drug related to artemisinin, which is the last drug approved for the treatment of malaria. Neither the pathway targeted by, nor the gene conferring resistance to, artesunate are associated with folate acquisition. In accordance with expectations, pABA depletion prevented the emergence of parasites resistant to pyrimethamine but not those resistant to artesunate.

### 3.3 Results

**Pyrimethamine resistant parasites are disproportionately impacted by pABA depletion.** Pyrimethamine resistant parasites are expected to require pABA in excess of susceptible parasites and should thus perform worse than
susceptible parasites in conditions of pABA scarcity. To test this hypothesis, we initiated single infections of either $10^6$ susceptible or $10^5$ resistant parasites in mice. Prior to the experiment’s initiation we confirmed that resistant, but not susceptible parasites, carried the mutation that confers resistance to pyrimethamine (Fig. B.1). Mice were administered a 0.05% pABA solution as drinking water (pABA abundant treatment) or distilled water (pABA depleted treatment). Administering pABA to hosts is standard practice in experimental studies of rodent malaria [120,144] because pABA optimizes parasite growth [115–117]. Resistant parasites suffered more than susceptible parasites from pABA depletion. pABA depletion reduced the total number and peak density of parasites produced by both resistant and susceptible strains but it had a greater impact on resistant parasites (Fig. 3.1AB; total parasite density pABA*strain $\chi^2=10.1$, p=0.002; peak parasite density pABA*strain $\chi^2=10.5$, p<0.001). Depletion of pABA also reduced the initial replication rate of resistant parasites from 6.1 (95% CI 5.2-7.2) to 3.95 (95% CI 3.3-4.7) fold per day (Fig. 3.1C). Curiously, pABA depletion increased the initial replication rate of susceptible parasites from 5.7 (95% CI 4.7-6.7) to 8.6 (95% CI 7.3-10.3) fold per day (Fig. 3.1D). As predicted, the requirements of pyrimethamine resistant parasites for pABA were greater than those of their susceptible competitors.

**Depletion of pABA prevents the competitive release of resistant parasites.** Having established that resistant and susceptible parasites have differential pABA requirements, we explored the impact of pABA depletion on the competitive release of resistant parasites. Mixed infections were initiated by inoculating mice with $10^6$ susceptible parasites. A resistant mutant is most likely to arise when the susceptible parasite population is large and growing. To model this circumstance, $10^5$ resistant parasites were inoculated on day 5, near the point where the susceptible population peaks. The density of a resistant mutant upon arising would be several orders of magnitude smaller than $10^5$, so this model is highly conservative. Since $10^4$ and $10^5$ parasites burst out of the malaria-infected liver to initiate a blood stage infection [145], our experiment also mimics the circumstances by which a resistant parasite infects an already-infected individual, as is likely when a resistant mutant is spreading. A subset of the mice were administered
drug treatment at a dose capable of clearing susceptible parasites, between days 6-12.

Consistent with previous studies [16, 58, 59, 143], resistant parasites were competitively suppressed by susceptible parasites in untreated infections (Fig. B.6). Following their release from competition with susceptible parasites by drug treatment, resistant parasites in the pABA abundant treatment reached densities of over a million parasites per microliter of blood (Fig. 3.2A). Depletion of pABA completely prevented the competitive release of resistant parasites (Fig. 3.2B).

pABA depletion did not prevent the growth of resistant parasites in treated single infections (Fig. 3.2C) - continuous growth of resistant parasites was observed in eight of ten mice. Thus pABA depletion interacts with the susceptible competitor to contain resistant parasites in the very environment in which they should have an advantage.

By preventing competitive release, pABA depletion increases host health & prevents transmission. The health of hosts with mixed treated infections was improved by pABA depletion. Depletion of pABA made hosts less anemic (Fig. 3.2DE, total red blood cell (RBC) density pABA F_{1,17}=29, p<0.001). This effect was a result of pABA depletion’s impact on resistant parasites - the dynamics of red blood cells in the two pABA treatments were the same, except between days 18-25 (RBC density pABA*day interaction days 18-25 p<0.01), when the second wave of anemia that accompanied competitive release occurred in the pABA abundant treatment. Depletion of pABA had no impact on the weight of mice (total weight pABA F_{1,18}=0.9, p=0.35).

Depletion of pABA reduced to zero the probability that resistant parasites would be transmitted from mixed treated infections (Fig. 3.2 H). At no point were transmission stages detected in pABA depleted conditions and so at no point was the transmission of resistant parasites probable [146]. In contrast resistant parasites were transmissible for an average of 10.5 (± 1.2) days in the pABA abundant treatment, with the probability of successful transmission to mosquitoes reaching ~0.4 at its highest.
Depletion of pABA does not prevent the competitive release of resistant parasites that do not require pABA in excess. To examine whether pABA depletion could prevent the emergence of resistant parasites that do not require pABA in excess, we performed a second experiment using artesunate resistant parasites, which do not possess the pyrimethamine resistance mutation (Fig. B.1). Artesunate resistant parasites were no more impacted by pABA depletion than susceptible parasites (Fig. B.7; replication rate pABA*strain $F_{1,14}=2.8$, $p=0.12$, total parasite density pABA*strain $\chi^2=1.29$, $p=0.26$; peak parasite density pABA*strain $\chi^2=0.73$, $p=0.39$).

pABA depletion had no impact on the competitive release of artesunate resistant parasites, resistant parasites emerged following artesunate treatment irrespective of pABA treatment (Fig. 3.3AB). Neither the total number nor peak density of resistant parasites was impacted by pABA treatment (total parasite density pABA $F_{1,7}=0.23$, $p=0.65$; peak parasite density pABA $F_{1,7}=0.23$, $p=0.65$).

The dynamics of infections in each individual mouse can be found in figures B.2-B.5.

3.4 Discussion

We have demonstrated that, when a within-host competitor is present, depleting a resource for which drug resistant parasites have elevated requirements can completely prevent the emergence and onward transmission of drug resistant parasites. By thwarting the emergence of resistant pathogens, pABA depletion also promotes host health. We propose pABA depletion as a model for a novel class of resource depleting interventions that could be used with traditional drugs as combination therapy.

The extinction of resistant parasites is a consequence of the intensification of competitive suppression in pABA depleted environments, as evidenced by the fact that resistant parasites are not prevented from emerging by pABA depletion in single infections. Susceptible parasites could drive resistant parasites to extinction in pABA depleted environments via their impact on nutrients, the immune system or both. Firstly, susceptible parasites might consume so much pABA during their initial growth phase that they leave a pool of pABA too small to sustain
a population of resistant parasites. Secondly, susceptible parasites might alter the host environment by priming the immune system, such that resistant parasites are detected and targeted by the immune system as soon as they emerge. With their growth rate reduced by pABA depletion, pyrimethamine resistant parasites would be unable to cope with this immune pressure. These explanations are not mutually exclusive. Depletion of pABA may intensify resource and apparent competition in tandem.

The intensity of competitive suppression experienced by de novo mutants would be greater than that experienced by the resistant mutants used in our experiments. So our work likely underestimates the potential of pABA depletion as a strategy for the control of de novo mutants. The intensity of competitive interactions between organisms is expected to change with their ecological similarity, for example their antigenic relatedness [89]. A de novo mutant will share its genetic background, and likely its niche, with its susceptible competitor. By contrast, the resistant parasites used here are genetically different from their susceptible competitors, to facilitate their measurement using qPCR. Second, competitive suppression varies with the relative population size of resistant parasites to susceptible parasites [78, 104]. When it arises, the population size of a de novo mutant will be orders of magnitude lower than the resistant inoculum used here. Third, a de novo mutant will not have had time to acquire compensatory mutations that could offset the fitness costs of pyrimethamine resistance, whereas the strain used in our experiments has been passaged a number of times (in mice not administered pyrimethamine). With the competitive suppression of de novo mutants already being so intense, they might be expected to be particularly vulnerable to the further intensification of competitive suppression that comes with pABA depletion. As a result, pABA depletion may cause de novo mutants to go extinct earlier than the pyrimethamine resistant parasites used here or in more ‘favorable’ conditions (for example, in an environment with less susceptible parasites). The flip side of this logic is that any change in the within-host environment that alleviates the intensity of competitive suppression could reduce the efficacy of pABA depletion as a resistance management strategy. Consistent with this prediction, pyrimethamine resistant parasites emerged in the pABA depleted treatment when the susceptible parasites were inoculated at a density lower than was intended (Fig. B.4).
We hypothesize that the containment of resistant parasites by pABA depletion is dependent on their having a single mutation in the dihydrofolate reductase (DHFR) gene, which confers an elevated requirement for pABA. Malaria parasites acquire folate in two ways: by producing it de novo from pABA salvaged from the serum, their preferred method [147]; and by salvaging it from the extracellular environment [147–150]. In the process of de novo production, pABA is converted to dihydrofolate, which is in turn reduced to tetrahydrofolate by DHFR, the target of pyrimethamine. The mutation that confers pyrimethamine resistance in *P. chabaudi* causes both a reduction in the affinity of DHFR for pyrimethamine and a six-fold reduction in the activity of DHFR, as compared to its susceptible ancestor [151]. This mutation is also associated with a reduced capability to salvage folate, at least in *P. falciparum* [125], which could cause an increased reliance on the endogenous pathway and so an increased requirement for pABA (John E Hyde, personal communication). Alternatively, to compensate for the inefficiency with which parasites can convert dihydrofolate to tetrahydrofolate, parasites may have to produce dihydrofolate in larger quantities to produce a unit of tetrahydrofolate than wild-type parasites, which would require larger quantities of pABA. Whatever the mechanism, pyrimethamine resistant parasites carrying this single resistance mutation require more pABA than their susceptible counterparts, which presumably diminishes their competitiveness. The ability of pyrimethamine resistant parasites to compete in the first few days after inoculation is also probably impeded by their enhanced vulnerability to pyrimethamine in pABA depleted conditions, which delays their growth (Fig. B.8). As parasites did emerge to transmissible densities in spite of this delay, however, the emergence of resistant parasites was prevented by pABA depletion because of its impact on competitive suppression not its potentiation of pyrimethamine.

Our experiment shows that, in principle, manipulating resource supply can turn the selective tables against resistant parasites in drug-treated environments. The combination of an antimicrobial drug and resource depletion may retard the evolution of resistance over both short and long timescales. Resource depletion will slow the emergence of resistance to the antimicrobial drug, as we have shown. Importantly, resource depletion will have this effect even if it has some negative impact on susceptible parasites - all that is required is that resistant parasites
require the depleted resource more than susceptible parasites. The boon that comes from pairing a drug with an intervention like pABA depletion, which has almost no impact on susceptible parasites, comes in the long term. Traditional combination therapies consist of ‘primary’ and ‘partner’ drugs, both of which are lethal to susceptible parasites. There is therefore selection for resistance to both drugs in the population of parasites as a whole. By contrast, selection for resistance to the depletion of a resource that is only required in significant quantities by resistant parasites is limited to the (very small and highly disadvantaged) resistant population. Depletions of resources of this nature may thus prove to be relatively ‘evolution-proof’ partner interventions, lending long-term viability to an antimicrobial drug-resource depletion combination. Vaccines [152] and resistant-specific drugs [153, 154] have also been proposed as ways of selectively targeting resistant organisms. The combination of chemotherapy and resource depletion also has similar properties to an ‘antagonistic’ drug combination [45], whereby resistance to a first drug confers an increased susceptibility to a second. Unlike the partner drugs used in antagonistic combinations, however, resource depletion does not reduce the efficacy of the primary drug and so its use does not come at the cost of having to use very high (sometimes unachievable) drug doses.

Intriguingly, pyrimethamine may have been used in combination with a drug to which resistant parasites have greater susceptibility, for many years. Our work provides insights into ways that the useful lifetime of this treatment combination could have been extended. Pyrimethamine is administered in combination with the drug sulfadoxine, an analog of pABA, as sulfadoxine-pyrimethamine (S/P). On its own, sulfadoxine inhibits the growth of pyrimethamine resistant P. chabaudi but only depresses that of susceptible parasites [155]. Similarly, pyrimethamine resistant P. falciparum parasites carrying one DHFR mutation are sensitive to sulfadoxine, whereas pyrimethamine sensitive parasites are not [156, 157]. Since pyrimethamine resistance emerges before sulfadoxine resistance [158, 159] (but see [160]), it is possible that sulfadoxine has acted as a resistant-targeting drug, suppressing the emergence of single DHFR mutants. Interestingly, single mutants are almost absent in the field [159, 160]. That said, S/P is a synergistic drug combination - together the potency of pyrimethamine and sulfadoxine against wild-type parasites is greater than the sum of their individual effects - and this mode of
action can favor the evolution of resistance [45]. Our work suggests that the lifetime of S/P could have been extended had the dose of sulfadoxine in the combination been reduced. A lower dose of sulfadoxine would diminish the synergistic action of S/P [161], which would cause a reduction in selection for resistance to S/P, yet reduce the access of pyrimethamine resistant parasites to pABA, and so enhance the competitive suppression of resistant parasites.

Whereas the pairing of pyrimethamine with a drug that targets resistant parasites was apparently accidental, pairings of antimicrobials and resource depletion interventions can be designed rationally. Much work has been done to work out the mechanisms of resistance to old and, increasingly, new drugs [162]. It should thus be possible to identify the metabolic costs associated with resistance (where they exist) and the resources used by resistant parasites to compensate for these costs. Bespoke interventions to target these resources might then be developed to either protect already-deployed drugs or those in the pipeline. Resource depleting interventions could take various forms including dietary interventions (à la pABA depletion), compounds that block the recipient enzyme (à la sulfadoxine), resource-degrading molecules or molecules that temporarily bind to host resources. Compounds that could be used to limit resource availability may be relatively easy to find, having remained untapped because of their sub-lethality (they would not show up in traditional toxicity screens) and concerns about hosts suffering because of resource depletion. These concerns are understandable but may be unwarranted. Parasite resources are not necessarily required by the host, as is the case with pABA [119]. Furthermore, the availability of resources to parasites could be restricted by means other than resource depletion or destruction, as described above. Even where target resources are required both by host and parasite, and their restriction is achieved by depletion from the host, there may be little to arouse concern. Small reductions in host resources, that do not harm the host, may be enough to affect parasites, since parasites may have higher nutrient demands than hosts. This is because parasites reproduce more frequently and rapidly than their hosts but at the same time have a comparatively smaller capacity to store resources. Small reductions in resources might also suffice because resources need only be depleted to the extent that competition is intensified - we are not proposing to starve out parasites.
The idea of using resource manipulations to alter organismal interactions has a long history in the management of plants [163, 164] and their pathogens [165–167] and, almost two decades ago, it was proposed as a method managing human disease. Using the framework of classical resource competition theory, Smith and Holt [168] proposed ‘superinfection therapy’, whereby a patient is inoculated with a wild-type parasite with the intention that it outcompetes drug-resistant parasites. They even suggested manipulating resource supply to maximize the probability of resistant pathogens being competitively excluded by superinfecting pathogens. Since many patients are infected with both resistant and susceptible pathogens (in areas of both high and low transmission of malaria, many infections consist of multiple malaria strains [169]) there may be no need to give susceptible parasites to patients. Instead, it has been suggested that the susceptible population could be maintained via low-dose drug treatment [58, 59], though this comes at the cost of an increase in the input of resistant mutations and possible negative health outcomes [57, 170]. We have demonstrated that by intensifying competitive suppression, rather than simply maintaining it, we can reap the advantages of high dose treatment while still preventing the emergence of resistant pathogens. Restricting within-host resource availability may thus have a new and important role in the fight against drug resistance.

3.5 Materials & Methods

Hosts & Parasites. Hosts in all experiments were inbred C57BL/6 mice, maintained on 5001 Laboratory Rodent Diet (LabDiet, USA). *P. chabaudi* parasites, originally isolated from thicket rats (*Thamnomys rutilans*), were used. The impact of competition on parasites was explored by comparing their performance alone (single infections) with their performance in competition with a genetically distinct parasite strain (mixed infections). Mice in mixed infection treatments were inoculated with 10^6 susceptible parasites on day 0 and 10^5 resistant parasites on day 5. Mice in single infection treatments were inoculated with either 10^6 susceptible parasites on Day 0 or 10^5 resistant parasites on day 5.

Experiment 1. Experiment 1 was performed in two blocks.
Block One. To explore the impact of pABA concentration, pyrimethamine treatment and competition on the dynamics of pyrimethamine resistant and susceptible parasite infections, we performed a fully factorial experiment (Table 3.1). A drug susceptible clone, AJ22p, and a pyrimethamine resistant clone, AS123p, were used. In untreated mice AJ22p competitively suppresses AS123p [110]. Pyrimethamine (PYR) dissolved in DMSO was administered to mice at a dose of 8mg/kg, 11 times between days 6-12 (twice daily, days 6-9; once daily, days 10-12). To reflect that patients seek treatment upon feeling sick, treatment was started on day 6, when mice start to exhibit symptoms. DMSO can be harmful to mice. Human patients that do not receive drug treatment are spared its toxic side effects, so mice in untreated groups were given an injection of saline equal in volume to that given to treated mice. Each treatment group contained five mice, with three exceptions. Eight mice were allocated to the pABA abundant, mixed infection group that did not receive pyrimethamine treatment, as these mice were expected to experience higher than average mortality. Eight mice were allocated to each of the pABA depleted, mixed infection groups (one of which received pyrimethamine treatment), to guard against resistant parasites failing to take hold in these treatments, as had occurred in a previous experiment (unpublished data).

Block Two. To replicate key findings from Block One, we repeated a subset of the treatments from Block One in Block Two (Table 3.1). Here, the drug susceptible clone, AJ35p, was used in place of AJ22p, from which it is derived. To confirm the impact of pABA depletion on competitive release, we repeated both the pABA depleted and pABA abundant, treated mixed infection groups. To confirm that pABA does not in and of itself kill treated resistant parasites, we repeated the pABA depleted, treated single resistant infection group. As in Block 1, the treated, pABA depleted, mixed infection group contained more mice (7) than the other groups (5). In Block One, mortality was higher in treated groups than untreated groups. We reasoned that this was because of the toxic effects of DMSO. We thus used a smaller injection volume (0.03ml, as compared to 0.05ml) of DMSO-pyrimethamine solution in Block 2. No mortality was observed in Block 2.
Experiment 2. To determine whether pABA depletion prevents the emergence of resistant parasites that do not require pABA in excess, we conducted a second experiment using artesunate resistant parasites (Table 3.2). The drug susceptible clone AJ34p and an artesunate resistant clone AS117p were used. AS117p was derived by serial passage from a susceptible parasite of the AS genetic background and is competitively suppressed by susceptible parasites in untreated mixed infections [60]. Artesunate (ART) treatment was administered twice daily from day 6-10 at a dose of 32mg/kg, with the exception of a single treatment on day 9 when all mice were inadvertently treated at a dose of 64mg/kg. Since we had investigated the effect of pABA concentration on parasite strains of the AJ and AS genetic background in untreated conditions in Experiment 1, untreated groups were excluded from Experiment 2.

Sequencing. In P. chabaudi, pyrimethamine resistance is conferred by the S106N mutation in the dihydrofolate reductase (DHFR) gene [151, 171, 172]. We investigated the presence of this mutation in the parasite strains used in Experiments 1 and 2. Primers were designed using Primer3 software [173, 174]. Primers were DHFR-F, AAG GGA CTT GGG AAT GAA GG, and DHFR-R, CAG ATG CAC CTC CTA TAA CAA AA and were used at a final concentration of 400nM. PCR was performed using the Taq PCR Core Kit (Qiagen) under the following conditions: 94C for 2 minutes, 40 cycles of 94C for 30 seconds, 54C for 30 seconds, 72C for 1 minute, 72C for 10 minutes. The resulting product of 400 base pairs was purified using the E.Z.N.A. Gel Extraction Kit (OMEGA Bio-tek), before being sequenced by the Penn State Genomics Core Facility, University Park, PA. Sequencing results were analyzed using SeqMan Pro and MegAlign software (DNASTAR).

Monitoring infection dynamics & virulence. In Experiment 1, 5µl of blood was taken from the tail of each mouse daily from days 3-33, after which blood was taken every other day until day 40. In Block 1, an additional 10µl of blood was taken for the quantification of transmission-stage parasites (gametocytes) until day 30. In Experiment 2, 5µl of blood was taken from the tail of each mouse daily from days 3-30. The density of asexual parasites and gametocytes of each strain were
quantified using quantitative real-time PCR (qPCR), as previously described [58]. In all experiments, an additional 2μl of blood was taken for the quantification of red blood cell density, which was measured by flow cytometry (Beckman Coulter) as an indicator of host health. Mouse weight was also measured. Experiments were conducted in accordance with the protocol approved by the Animal Care and Use Committee of the Pennsylvania State University (Permit Number 44512).

**Statistical analysis.** Statistical analysis was performed using R (version 3.2.0). We calculated two measures of parasite population size, total parasite density (the sum of parasite densities observed in a single mouse over time) and peak parasite density. These measures were log transformed for analysis. The probability of parasites transmitting to mosquitoes was calculated using the gametocyte density-infectivity functions derived experimentally by Bell *et al.* [79]. The rate at which drug treatment cleared susceptible parasites was determined by the slope of the regression of the log of parasite density against time, in the period of drug treatment, following [60, 175]. Initial replication rate was calculated as the slope of the regression of the log of parasite density against time using the first three consecutive positive parasite counts or, where the growth of the parasite population had already slowed by the third day, the first two counts. The $r^2$ of the linear regression of log of parasite density against time was less than 0.9 in three mice, suggesting the parasite population in these three mice was not growing exponentially. These mice were therefore excluded from the analysis of replication rate (Fig. B.2-B.5, subplots marked with a square).

On several occasions we observed heterogeneity in the variance of the measures of parasite growth, among treatment groups. To account for this, we incorporated the variance structure into generalized least squares (GLS) models using the *nlme* R package following the protocol outlined by Zuur *et al.* [124]. Linear regression was used when the assumptions of normality were met. To assess the affect of treatment on the temporal dynamics of red blood cells, linear mixed effect models were employed, following [60]. Where relevant, we tested for the impact of experimental block and included it in the final model if it improved model fit. In the analysis of the initial replication rates, the impact of inoculum size was investigated by using parasite density on the day after inoculation as a proxy,
where possible.

Mice that died, received a dose of parasites lower than was intended or were inadvertently given drug treatment were excluded from the analysis (see Tables 3.1 & 3.2, Fig. B.2-B.5). Mice that died were included in the analysis of clearance and replication rate, where possible.

To ease interpretation, and to explicitly represent the level under which parasites are not detectable by quantitative PCR, parasite densities are represented in the graphs in numbers per mouse. Parasite densities were converted to numbers per mouse under the assumption that mice have 58.5ml blood/kg weight.

### 3.6 Notes

This work was conducted in collaboration with Troy Day and Andrew F. Read and with technical assistance from Derek G. Sim, Matthew J. Jones and Rahel M. Salathé. It will be submitted for publication with these persons as coauthors.
3.7 Figures & Tables

Figure 3.1: Depletion of para-aminobenzoic acid (pABA) disproportionately impacts the growth of resistant parasites. Infection dynamics (A,B) and initial replication curves (C,D) of mice infected with pyrimethamine resistant (A,C; dashed lines) and susceptible parasites (B,D; solid lines) in pABA abundant (orange) and pABA depleted (turquoise) treatments. Initial replication curves were calculated by linear regression using the first two or three consecutive positive parasite counts. Stars represent the number of parasites inoculated and the time at which they were administered. n specifies the number of mice plotted and included in the analysis of total and peak parasite density (A,B) and replication rate (C,D).
Figure 3.2: Depletion of para-aminobenzoic acid (pABA) prevents the emergence of pyrimethamine resistant parasites, promotes host health and prevents the transmission of resistant parasites. Dynamics of susceptible (black, solid lines) and pyrimethamine resistant (red, dashed lines) parasites in mixed infections (A,B) and single resistant infections (C) of individual mice from Blocks 1 and 2 of Experiment 1. Stars represent the number of parasites inoculated and the time at which they were administered. Dots indicate the density of parasites detected on a particular day in instances where parasites were not detected the day before or after. Red blood cell dynamics of individual mice in Blocks 1 and 2 (D-F) and the probability of resistant parasites transmitting to mosquitoes in Block 1 (G-I), also shown. Mice received as drinking water a solution of 0.05% pABA (pABA abundant treatment, grey background) or distilled water (pABA depleted treatment, white background). Purple bar indicates the duration and timing of pyrimethamine treatment. n specifies the number of mice plotted and included in the analysis.
Figure 3.3: Depletion of para-aminobenzoic (pABA) does not prevent the emergence of artesunate resistant parasites. Dynamics of susceptible (black, solid lines) and resistant (red, dashed lines) parasites in mixed infections (A,B) and single resistant infections (C) in individual mice. Mice received as drinking water a solution of 0.05% pABA (pABA abundant treatment, grey background) or distilled water (pABA depleted treatment, white background). Green bar indicates duration and timing of artesunate treatment. Stars represent the number of parasites inoculated and the time at which they were administered. Dots indicate the density of parasites detected on a particular day in instances where parasites were not detected the day before or after. n specifies the number of mice plotted and included in the analysis of total and peak parasite density.
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<td>PYR Treated</td>
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Table 3.1: **Number of mice per treatment in Experiment 1.** Numbers in brackets indicate the number of mice that were removed from some or all analyses because they were inadvertently treated (triangle superscript, removed from all analyses); misinoculated (circle superscript, removed from all analyses); they died (cross superscript, removed from analysis of total/peak parasite density) or because the initial growth trajectory of parasites was non-linear (square superscript, removed from analysis of growth rate).
Table 3.2: **Number of mice per treatment in Experiment 2.** Numbers in brackets indicate the number of mice that were removed from some or all analyses because they were misinoculated (circle superscript, removed from all analyses) or the initial growth trajectory of parasites was non-linear (square superscript, removed from analysis of growth rate).

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Depletion of para-aminobenzoic acid prevents the emergence of pyrimethamine resistant parasites, independent of the genetic background of resistant parasites or their competitors.

4.1 Abstract

Whether drug resistant parasites emerge from an infection depends on the ecology of the within-host environment. Drug susceptible parasites competitively suppress drug resistant parasites, until they are removed from the host by drug treatment. Depleting the within-host environment of resources that are at a premium for resistant but not susceptible parasites can intensify the competitive suppression of resistant parasites, to the extent that they go extinct before drug treatment gives them the opportunity to flourish. The intensity of competitive suppression that a resistant parasite experiences depends on its genetic background and that of its competitor. So, the efficacy of resistance management strategies that harness competitive suppression, like resource depletion, might change with the genetic background of the parasite strains in an infection. Here, we investigate this proposition using the rodent malaria model, *Plasmodium chabaudi*. Pyrimethamine-resistant strains of *P. chabaudi* require more para-aminobenzoic acid (pABA) than susceptible parasites and are prevented from emerging when pABA is depleted from the host. We found that irrespective of the genetic background of resistant parasites or their competitors, pABA depletion prevented the emergence of drug resistant parasites from mixed-strain infections. pABA depletion prevented the emergence of resistant parasites primarily by intensifying competitive suppression, as evidenced by the fact that resistant parasites emerged in two-thirds of pABA depleted infections from which a competitor was absent (single infections). Since resistant parasites failed to emerge from a third of single infections, however, pABA depletion may have an additional impact on
resistant parasites that is separate from its effect on competitive suppression. By analyzing the dynamics of parasites using limiting dilution PCR, which permits the quantification of parasites at densities lower than conventional assays, we determined that pABA depletion potentiated the activity of pyrimethamine against resistant parasites and that resistant parasites of different genetic backgrounds varied in the extent to which they were impacted by the combination of pABA depletion and pyrimethamine treatment. However, a parasite’s genetic background did not appear to predict whether it went extinct in single infections. Since the efficacy of resource depletion is unaffected by the genetic background of parasite strains in an infection, resource depletion may represent a robust strategy with which to control the emergence of drug resistance.

4.2 Introduction

Drug resistance threatens our ability to treat an array of ailments, from ‘strep throat’ to malaria. Whether a resistant pathogen reaches transmissible densities within a patient, and hence spreads to others in the community, is dependent on its environmental milieu. When a resistant parasite infects a naïve individual, it encounters a host environment replete with resources and is therefore able to multiply to high densities and transmit on to new hosts. However, when a resistant pathogen emerges de novo or via horizontal gene transfer or infects an individual that already harbors a pathogen (superinfection), it enters a host environment dominated by susceptible pathogens, which have often already consumed much of the host’s resources. In these conditions of resource scarcity, the resistant pathogen population is unable to grow - it is said to have been competitively suppressed by susceptible parasites. Drug treatment removes the susceptible competitors of resistant pathogens allowing host resources to rebound and resistant parasites to emerge, in a process known as competitive release [50].

Recently, we demonstrated that depleting a within-host resource required in excess by drug resistant pathogens can so intensify competitive suppression that resistant pathogens fail to emerge after drug treatment, using a mouse malaria model (Chapter 3). Para-aminobenzoic acid (pABA) is a folate precursor that mammals do not require but which malaria parasites use to produce
tetrahydrofolate for the manufacture of purines and the metabolism of methionine [113]. Pyrimethamine resistant *Plasmodium chabaudi* parasites require more para-aminobenzoic acid (pABA) than susceptible parasites, apparently as a direct cost of the mutation conferring pyrimethamine resistance (Chapter 3). In pyrimethamine-treated infections containing both susceptible and resistant parasites, depletion of pABA from mouse hosts prevented the competitive release of pyrimethamine resistant parasites. The growth of resistant parasites in pyrimethamine-treated infections from which susceptible parasites were absent was not prevented by pABA depletion, however. We thus concluded that pABA depletion prevented the emergence of pyrimethamine resistant parasites by intensifying competitive suppression in the short period between the appearance of resistant parasites and the clearance of susceptible parasites from the mouse by drugs. Depletion of pABA also appeared to reduce the resistance of resistant parasites to pyrimethamine: when pABA was abundant, the resistant parasite population grew throughout the period of pyrimethamine treatment; in pABA depleted hosts, the population of resistant parasites remained at a density below that detectable by quantitative PCR (qPCR) throughout the period of pyrimethamine treatment and grew only after it was withdrawn (Fig. B.8). Since drug resistance often comes at the cost of an impaired metabolism (and so may often be associated with elevated resource requirements), we proposed resource depletion as a general strategy with which to combat the emergence of drug resistant pathogens.

Since resource depletion prevents the emergence of resistant parasites by intensifying competitive suppression, its efficacy as a resistance management strategy could be affected by factors that impact the strength of competitive interactions between susceptible and resistant parasites. One such factor is the genetic background (strain identity) of the competing parasites [79], the effect of which was not assessed in our original investigation of resource depletion as a resistance management strategy. The genetic background of the susceptible competitor could impact the quantity of nutrients and immune-free space available to a resistant parasite (i.e. the intensity of competitive suppression it experiences), since susceptible strains vary in their growth rate [176], the extent to which they deplete red blood cells from the host [79, 176], their immunogenicity [177] and, we expect, their pABA requirements. As the time it takes for drugs to remove
susceptible parasites from hosts can vary depending on their genetic background [178], the period of time over which a resistant parasite must endure competitive suppression might also vary with its competitor’s genetic background. Indeed, just as there is variation among susceptible strains in their sensitivity to drugs, resistant parasites of different genetic backgrounds might vary in their capacity to withstand drug treatment and/or resource depletion. This is because the level of resistance conferred by a resistance mutation [179] and the size of the costs associated with it ([180], reviewed in [54]) can vary with the genetic background on which it appears. Given the many ways that the genetic background of parasites could impact the nature of competitive suppression, the capacity for resource depletion to prevent the emergence of resistant parasites from an infection could depend greatly on the identity of parasites that make up that infection.

Here, we investigate whether the efficacy of pABA depletion, a model of resource depletion as a resistance management strategy, varies with the genetic background of the resistant parasite and its susceptible competitor. We first evolve pyrimethamine resistance onto two genetic backgrounds, distinct from that of the resistant parasite used in our previous work (Chapters 2 and 3). We then use these resistant strains, as well as that used in our previous work, to investigate the impact of pABA depletion on the emergence of resistant parasites in infections containing susceptible parasites of different genetic backgrounds. To investigate whether resistant parasites of different genetic background differ in their susceptibility to the combination of pABA treatment and pyrimethamine treatment, we employ limiting dilution PCR (ldPCR) [181,182] to quantify resistant parasites when their density lies below that quantifiable by conventional quantitative PCR (qPCR). We also use ldPCR to investigate whether pABA depletion and pyrimethamine act additively or synergistically to delay the growth of resistant parasites, using samples from a previously-conducted experiment (Chapter 3) during which the effects of pABA depletion and pyrimethamine treatment on resistant parasites, when administered alone and in combination, were investigated.
4.3 Materials and Methods

4.3.1 Experimental evolution of pyrimethamine resistance

Selection for resistance to pyrimethamine was imposed on two *P. chabaudi* strains of different genetic backgrounds, CW28 and AJ35p. These strains had never been exposed to pyrimethamine. Hosts were outbred Swiss mice, maintained on PicoLab Rodent Diet 5053 (LabDiet, USA) and 0.05% pABA solution, to facilitate parasite growth [115,116]. For each strain, ten mice were inoculated intraperitoneally with $10^6$ parasites. In order to maximize the amount of standing variation for selection to act upon, pyrimethamine treatment was not begun until parasite densities peaked, on day 6. Between days 6-12, mice were administered pyrimethamine intraperitoneally at a dose of 8mg/kg, twice a day. This regimen was chosen to mirror regimens used to treat malaria in humans, which are defined as successful only if they are capable of clearing parasites from the host [183]. In the days following the last drug treatment, blood smears were taken from all mice and the presence of parasites assessed by microscopy. As soon as parasites appeared, approximately $10^6$ parasites were passaged to each of 4 mice. To test the resistance of the passaged parasite strain, the pyrimethamine treatment regimen described above was immediately begun. Again, parasitemia was monitored via microscopy. When parasitemia reached 30% in a single mouse, the mouse was sacrificed and the parasites frozen down for storage in liquid nitrogen as strains CW29 and AJ36p (hereafter $R_{CW}$ and $R_{AJ}$, respectively). Prior to their use in experiments, the presence of the S106N mutation in the dihydrofolate reductase (DHFR) gene, which confers resistance to pyrimethamine in *P. chabaudi* [151], was confirmed by sequencing (Fig. C.1), following the protocol described in Chapter 3.

4.3.2 Exploration of the impact of pABA depletion on the competitive release of resistant parasites in infections composed of different strains

Hosts were inbred C57BL/6J mice, maintained on Laboratory Rodent Diet 5001 (LabDiet, USA), as in Chapters 2 and 3. Mice in pABA abundant treatments received a solution of 0.05% pABA as drinking water, while those in pABA depleted
treatments received distilled water.

To investigate whether the efficacy of pABA depletion as a resistance management strategy changes with the combination of strains that make up an infection, we contrasted the growth of resistant parasites in mixed, pyrimethamine treated infections in pABA abundant and pABA depleted conditions (Table 4.1). Each resistant strain was competed with a different susceptible strain - $R_{AS}$ with $S_{AJ}$, as in Chapter 3; $R_{AJ}$ with $S_{AS}$, in reversal of Chapter 3, and $R_{CW}$ with $S_{AT}$. In this system, the best predictor of the competitiveness of a strain is the minimum red blood cell density it induces [79, 110]. We chose these strains so that poorly (AS), average (CW) and highly (AJ, AT) competitive strains were represented. To investigate the possibility that pABA depletion is inherently lethal to resistant parasites in treated infections, resistant strains were also grown in pABA depleted, pyrimethamine-treated conditions in the absence of a susceptible competitor (Table 4.1, bottom row).

A detailed discussion of experimental methods can be found in Chapter 3. Briefly, mice in mixed infection treatments were inoculated with $10^6$ susceptible parasites on day 0 and $10^5$ resistant parasites five days later. Mice in single infection treatments received only the latter. Pyrimethamine treatment, at a dose of 8mg/kg was begun on day 6 and continued twice per day until day 9 and then once per day until day 12. Mice were sampled every day between days 3-33 and then again on day 35. Weight, red blood cell density and parasite density were monitored as described in Chapter 3, with the exception that the $msp-1$ qPCR assay was used in lieu of the $ama-1$ assay for the quantification of the AT and CW parasite strains following [79]. These two assays give comparable estimates of parasites density [79].

4.3.3 Exploring parasite dynamics below the qPCR limit of detection

We used ldPCR to investigate the dynamics of resistant parasites in single infections on the days, prior to their emergence, that they were undetectable by qPCR. The principal that underlies ldPCR is that, when a sample is assayed multiple times by qPCR, the number of times the assay detects that parasites
are present is proportional to the density of organisms the sample contains. For example, when samples are assayed thirty times, as they are here, ldPCR is capable of quantifying parasites at densities as small as $10^3$ per $\mu{l}$ blood, a detection level that is an order of magnitude below that of our conventional qPCR assays. All samples had previously been assayed by qPCR and only those that contained parasites at densities undetectable by conventional qPCR were included in the ldPCR assays. $R_{\text{AS}}$ was quantified by ldPCR on every even day until the day the parasites emerged. Samples of mice from which $R_{\text{AS}}$ parasites failed to emerge were similarly assayed until two consecutive zero counts were obtained or until day 12, whichever came first. $R_{\text{AJ}}$ and $R_{\text{CW}}$ were similarly quantified every odd day, after the loss of some samples from some even days. Each sample was assayed 30 times by qPCR, which was run for 45 cycles, and the number of times out of 30 that a parasite was detected was recorded. From this number the density of parasites per sample were estimated using the algorithm developed by Kennedy et al. (in prep). Samples from days 6-12 (the period of drug treatment) that contained a density of between 3-100 parasites per sample, as quantified by qPCR, were run a further two times by qPCR to get a more accurate estimate of their density. To investigate whether pABA depletion and pyrimethamine act additively or synergistically against resistant parasites, we also used ldPCR to quantify $R_{\text{AS}}$ during the even days of the drug treatment period (6-12) in the single infection treatments of Block One of Experiment 1 of Chapter 3 (Table 3.1, hereafter Experiment 2). Again, only those samples that contained a number of parasites too small to be detected by qPCR were assayed, while those containing between 3-100 parasites per sample were assayed two further times.

### 4.3.4 Statistical analysis

Statistical analysis was performed using R (version 3.2.0). Resistant parasites were considered to have emerged if they were observed growing on two or more consecutive days at any point during the infection. The probability of emergence was analyzed using binomial generalized linear models. Total parasite density was defined as the sum of the parasites produced during an infection. Total parasite density was log transformed before being analyzed using linear models or, where
heteroscedasticity was observed, generalized least squares (GLS) models. GLS models were run using the `nlme` R package, following the protocol outlined by [124]. To enable comparison between the results obtained here and those from Chapter 3, and because there is no probability of parasites transmitting when asexual densities are undetectable by qPCR (making densities below this level not meaningful in terms of emergence in the wider host population, see Chapter 3), only the parasite density data obtained by qPCR was used in the analysis of the emergence and of total resistant parasite density. To ascertain whether the different susceptible strains differed in their response to pABA depletion, we also calculated the growth rate of susceptible parasites. For this analysis we used only the data from days 3-5, since only during this period could we be sure that the growth rate of susceptible parasites was unaffected by resistant parasites (which were inoculated after blood was taken on day 5). Analysis of susceptible growth rate was performed using linear mixed effects models in the `nlme` R package following [184], with day included as a predictor variable and mouse as a random effect. Model selection was performed following [124]. This analysis also allowed us to investigate differences in the densities of the different susceptible strains on each of days 3, 4 and 5.

To analyze the dynamics of resistant parasite growth in Experiment 2, we used parasite counts obtained on the even days of drug treatment (6, 8, 10, 12). For each mouse, we calculated the growth rate of resistant parasites between each pair of days, by drawing lines between the logged parasite densities at each time interval (6-8, 8-10, 10-12) and taking their slopes. We then analyzed the impact of pABA, pyrimethamine treatment and their interaction, on the growth rate of resistant parasites at each time interval using linear models.

A number of mice received an inoculum of susceptible parasites that was lower than intended and one mouse died (Table 4.1, Fig. C.2). These mice were excluded from all analyses with the exception of the latter, which was included in the analysis of susceptible growth rate.

### 4.4 Results

**The impact of pABA depletion on resistance emergence.** In pABA abundant conditions, resistant parasites of all genetic backgrounds emerged
following drug treatment (Fig. 4.1A-C). Depletion of pABA prevented the emergence of resistant parasites from mixed infections in all but one case (Fig. 4.1D-F). The genetic background of the parasites present in infections, therefore, had no impact on the capacity for pABA depletion to prevent the emergence of pyrimethamine resistance. In pABA depleted conditions, the presence of a competitor reduced the odds of resistant parasites emerging from 0.06 to 0.66, a reduction of 97% (CI 76.5%-99.8%). This finding was consistent with our previous work. Contrary to our previous findings, however, a third of resistant parasites also failed to emerge from pABA depleted single infections (Fig. 4.1G-I).

Density & dynamics of parasites in mixed infections. The impact of pABA depletion on the density of resistant parasites in mixed infections varied with the strain composition of infections (Fig. 4.1, 4.2A; total resistant parasite density pABA*strain $\chi^2=8.29$, $p=0.016$). This finding can be attributed to differences among the resistant strains in their performance in pABA abundant conditions (Fig. 4.1, 4.2A; total resistant parasite density strain $\chi^2= 6.9$, $p=0.03$), since the different resistant strains produced the same total number of parasites in pABA depleted conditions (total resistant parasite density strain $\chi^2= 1.5$, $p=0.47$). In pABA abundant conditions, R$_{AJ}$ emerged to significantly lower densities than either R$_{AS}$ or R$_{CW}$, which produced similar numbers of parasites (Fig. 4.2A).

The effect of pABA depletion on the density of susceptible parasites was marginally impacted by the strain composition of infections (Fig. 4.1, 4.2B; total susceptible parasite density pABA*strain $\chi^2=5.21$, $p=0.07$). S$_{AS}$ appeared to be particularly sensitive to pABA depletion, producing considerably fewer parasites in the pABA depleted than in the pABA abundant treatment (Fig. 4.2B). Similarly, there was some indication that the growth rate of susceptible strains between days 3-5, during which time susceptible parasites were not faced with competition from resistant parasites, responded differentially to pABA depletion (Fig. 4.2C-E; susceptible parasite density day*pABA*strain $\chi^2=4.92$, $p=0.09$). Again, S$_{AS}$ appeared to be the most hindered of the strains. On each day of the period between days 3-5, the density of S$_{AJ}$ was lower than both S$_{AS}$ and S$_{AT}$ (which did not differ in their densities) (Fig. 4.2C-E; susceptible parasite density strain $\chi^2=11$, $p=0.004$).
Dynamics of parasites in single infections. In infections where emergence was observed (Fig. 4.3A), the resistant strains differed in their behavior during the period of pyrimethamine treatment, and these differences appeared to impact the speed with which they reached peak densities. The density of RAJ continued above the detection level of ldPCR during the period of drug treatment in all infections, as did the density of RAS in two of the three emergent infections (Fig. 4.3A). By contrast, the density of RCW dipped below that detectable by ldPCR before emerging (Fig. 4.3A), in all infections. RAJ reached its peak density first, followed by RAS and then RFW (Fig. 4.3A). Whether or not parasite densities sunk below the detection level of ldPCR thus appeared to have little to do with whether parasites emerged or not. Neither the density of parasites on day 3 (a proxy for inoculum size), mouse weight nor mouse red blood cell density appeared to predict whether a parasite would emerge (though the sample size was small).

In Experiment 2, RAS emerged in all mice (Fig. 4.4). Only in pyrimethamine-treated, pABA depleted mice did the density of RAS dip below the detection level of ldPCR, before emergence occurred (Fig. 4.4). The growth rate of RAS in pABA depleted, pyrimethamine-treated mice between days 6-8 and 10-12 was significantly slower than the growth rate expected if pABA depletion and pyrimethamine had an additive effect on RAS (*growth rate between day 6-8 paba*strain* χ_2^2*=9.7, p=0.002; *growth rate between days 10-12, paba*strain χ_2^2*=7.65, p=0.006).*

4.5 Discussion

Here, we show that pABA depletion, a model of resource depletion as a resistance management strategy, can prevent the emergence of pyrimethamine resistant parasites from infections composed of parasite strains of different genetic backgrounds. Our work demonstrates the generality of our previous findings (Chapter 3) as well as of the finding that drug treatment causes the competitive release of drug resistant parasites [16, 58, 60, 104, 143, 185], which has hitherto only been demonstrated in infections of SAJ and RAS. As well as preventing the emergence of a de novo mutant (which is likely to be highly related to its susceptible ancestor), our work suggests that resource depletion could also be deployed as a strategy with which to combat the emergence of resistant parasites, in contexts
where resistant parasites do not share the genetic background of their susceptible competitors. Such circumstances are far from rare, given that many hosts harbor co-infections [186], and may be the norm in the case of malaria. In areas where malaria transmission is high (and some where it is low), the majority of infections are composed of multiple, genetically different parasite strains [169, 187, 188]. In this epidemiological context, a spreading resistant mutant is more likely than not to share its host with a competitor, and that competitor is likely to have a different genetic background than the resistant mutant.

The presence of a competitor in the infection greatly decreased the probability of a resistant parasite emerging, in pABA depleted conditions. This suggests, as per our previous findings, that pABA depletion prevents the emergence of resistant parasites by intensifying competitive suppression of resistant parasites. This hypothesis is further evidenced by the behavior of resistant parasites in infections where we would not expect competition suppression to be intense. A number of mice received an inoculum of susceptible parasites that was smaller than was intended (Fig. C.2, subplots marked with open circles). In all of these infections, resistant parasites emerged. We hypothesize that resources in these mice were not sufficiently depleted by susceptible parasites to prevent resistant parasites taking hold. The apparent sensitivity of pABA depletion to the inoculum of the susceptible parasite warrants investigation and suggests that the relative timing of the peak in the abundance of susceptible parasites and the arrival of resistant parasites may be crucial to the efficacy of pABA depletion as a resistance management strategy. While it is reasonable to expect de novo resistant mutants to enter an infection when the susceptible parasite population is at or nearing its peak, since the probability of a mutation occurring in a population is proportional to its size, resistant parasites may not superinfect an individual in coincidence with the peak in susceptible parasite density. To compensate for the absence of susceptible parasites in such circumstances, pABA might have to be depleted to even lower concentrations than it was here, were pABA depletion to be deployed as an intervention.

Our experiment suggests that there may be an alternate, competition-independent mechanism by which the combination of pyrimethamine and pABA depletion controls resistant parasites, since resistant parasites failed to emerge
in a third of single infections. The combination of pyrimethamine and pABA depletion may simply represent the ‘straw that broke the camel’s back’, reducing the population size of resistant parasites to the extent that they became vulnerable to Allee effects and/or demographic stochasticity. It has been proposed that the innate antimalarial immune response resembles a ‘Type 2’ predation response, whereby it is able to control parasites when their numbers are small but not when they exceed a certain threshold [108]. This type of response could cause an Allee effect whereby, below a certain threshold density, the growth rate of the parasite population falls with its population size and it is doomed to disappear from the host [189]. Interestingly, our ldPCR data also suggests that positive density dependence is at work in this system - after bottoming out, the rate at which $R_{AJ}$ grows increases with its population density (Fig. 4.3A). If Allee effects are indeed at work in parasite populations, then the removal of individuals by the immune system and/or the combination of pABA depletion and pyrimethamine (see below) could have moved the parasite population below its Allee threshold. That said, our data do not suggest that there is a threshold density below which parasites are guaranteed to go extinct (or, to be more precise, it suggests that any such threshold lies below the detection threshold of ldPCR). In nine of the fifteen single resistant infections, parasite densities dipped below the detection threshold of ldPCR during the drug treatment period. Some of these parasites went on to emerge, others did not. We could not find a metric that could distinguish between the infections from which resistant parasites emerged and those infections from which resistant parasites did not emerge. So demographic stochasticity may play a more important role than Allee effects in this system present or the two forces may interact [189, 190]. That we could not predict emergence using data from a costly and sensitive technology like ldPCR (the detection limit of which is $\sim 1.5$ parasites/µl of blood as compared to the 50-100 parasites/µl of field-based microscopy [191]), illuminates the difficulty of predicting emergence in the field and the importance of understanding dynamics of resistant parasites at low densities [185].

Depletion of pABA potentiated the activity of pyrimethamine against resistant parasites, as the data from Chapter 3 revealed, and their combined administration could enhance the vulnerability of resistant parasite populations to demographic
stochasticity and/or Allee effects. We hypothesize that the synergism between pyrimethamine and pABA depletion has the same basis as the synergism between pyrimethamine and the antimalarial drug, sulfadoxine, an analog of pABA. Malaria parasites acquire tetrahydrofolate in two ways, first by producing it from pABA via the folate pathway (the endogenous route); second, by acquiring preformed folates from the host environment (the exogenous route) [147–150]. Sulfadoxine competes with pABA for the enzyme dihydropteroate synthase (DHPS) [158], in the first step of the folate pathway. Depletion of pABA also slows the production of folate via the endogenous route, by reducing the availability of the substrate of DHPS. Both sulfadoxine and pABA depletion thus force parasites to derive their folate from the extracellular environment. Pyrimethamine may prevent resistant parasites from acquiring folate via this exogenous route [192] (though it does not appear to inhibit the transporters used to import folate [150]) or prevent its conversion by its target to a usable form. Therefore, when pABA depletion and pyrimethamine are administered in combination, both folate acquisition routes are disrupted and resistant parasites (at least those carrying a single resistance mutation [125]) are prevented from growing [192]. When pABA depletion and pyrimethamine are administered separately, however, resistant parasites are able to compensate using the exogenous and endogenous routes, respectively. Resistant parasites may not be cleared from hosts when pyrimethamine and pABA depletion are given in combination, however, because pABA depletion does not actively inhibit the endogenous route like sulfadoxine does. Resistant parasites may be able to maintain some level of reproduction using latent levels of pABA in the mouse blood and so recover after the withdrawal of pyrimethamine treatment.

The combination of pABA depletion and pyrimethamine did not uniformly impact the different resistant parasite strains (Fig. 4.3A) and a strain’s capacity to withstand the treatment appeared to determine the speed with which it came to peak densities. So a strain’s susceptibility to the combination of pABA depletion-pyrimethamine could have implications for its competitiveness. One possible explanation for the variation among strains in their performance during the period of pyrimethamine treatment is that because the different strains acquired pyrimethamine resistance in three independent evolutionary events, they acquired different levels of pyrimethamine resistance or differentially acquired compensatory
mutations to offset its cost. Under this hypothesis, we would expect the different strains to carry different resistance mutations and/or different numbers of them. The nature of the evolution of pyrimethamine resistance and of our selection regimen may make these scenarios unlikely. Resistance to drugs targeting DHFR is conferred by single mutations that are typically acquired in a particular order, such that the evolutionary trajectory of resistance is of a relatively predictable and distinctly non-continuous nature [193,194]. It is therefore unlikely that the different resistant strains harbor different resistance mutations. Furthermore, each strain was subject to just one round of drug selection (for details of the experimental evolution of R_{CW} and R_{AJ} see Materials & Methods, for details of R_{AS} [122]). In this short period, it is unlikely that any of the strains could have acquired multiple mutations, so the number of resistance mutations is unlikely to vary between the strains. Previous work in our lab also suggests that there is little variation between the resistance conferred by mutations acquired in independent evolutionary events. In *P. falciparum*, artemisinin resistance can be caused by different mutations that confer different levels of resistance [179], yet when resistance to artesunate (a compound related to artemisinin) was experimentally evolved in *P. chabaudi*, the resistance phenotype did not vary among replicate lines of the same genetic background. Rather than being caused by differences in the nature or number of mutations directly related to pyrimethamine, the variable sensitivity of resistant strains to the combination of pABA depletion and pyrimethamine could be caused by differences in the strains’ genetic background. Strains of different genetic backgrounds might vary in their dependence upon exogenously acquired pABA, as the analysis of the growth of susceptible parasites in the first three days of sampling suggests (Fig. 4.2C-E), perhaps because of a differential capacity to substitute host-supplied pABA with pABA produced *de novo* or to import folates from the serum. Susceptibility to pyrimethamine might also vary among resistant strains. Variation in either the folate metabolism or pyrimethamine sensitivity of resistant parasites would cause the strength of synergy between pABA depletion and pyrimethamine to vary depending on the strain present in the infection. This could explain the observation that resistant parasites of different genetic backgrounds differ in their capacity to grow during pABA depletion-pyrimethamine treatment. To explore this hypothesis, an experiment similar in
design to Experiment 2, in which the effect of pABA and pyrimethamine treatment on resistant parasites is investigated independently and in combination, could be conducted. Alternatively, the strength of synergy between pABA depletion and pyrimethamine might remain consistent in infections composed of different strains but, because of among strain variation in growth rate, some resistant strains might be able to sustain a larger population than others during the period of pABA depletion-pyrimethamine treatment. Schneider et al. [195, 196] found that the more virulent (and hence faster growing [197]) a parasite was in untreated environments in which there were not competitors, the less it was sensitive to either pyrimethamine or artemisinin. Future work might focus on defining the virulence of these resistant parasites in untreated conditions to clarify whether, here too, virulence explains the performance of the different resistant strains.

Examining the growth of all the parasite strains in the absence of drug treatment, in single and in mixed infections, would also permit other traits, like the productivity of susceptible parasites, to be measured and the intensity of competitive suppression in infections composed of different strains to be quantified. We could then directly quantify the relationship between the intensity of competitive suppression and the efficacy of pABA depletion as a resistance management strategy. There is some indication that the intensity of competitive suppression varied among infections composed of different strains (as we would expect). In particular, $R_{AJ}$ appeared to be much suppressed by $S_{AS}$ in pABA abundant conditions (it produced the least parasites upon release). This is surprising, given that parasites with an AJ background have not previously been observed to be much competitively suppressed by parasites of the AS background [79, 110]. Furthermore, parasites of the AS background are considered to be weaker competitors than those of both the AJ or AT backgrounds but $S_{AS}$ outstripped both $S_{AJ}$ and $S_{AT}$ in terms of parasite production in pABA abundant conditions. The apparent differences in the productivity of the different susceptible parasite strains could be caused by differences in the performance of the resistant parasites with which they shared their hosts. Single infection experiments are needed to confirm that productivity varies among susceptible parasites. For now, however, we speculate that the competitive ability of parasites of the AS genetic background may have been underestimated because, when the differences
in the competitive ability of parasites of different genetic backgrounds was investigated [79], a pyrimethamine-resistant AS strain was compared to susceptible parasites of other genetic backgrounds. Due to the costs of pyrimethamine resistance, the competitive ability of \( R_{AS} \) is unlikely to have been representative of that of \( S_{AS} \) (and, again, single infection experiments could confirm this). Indeed, pyrimethamine resistance may be associated with particularly high costs in parasites with an AS genetic background, given that even \( S_{AS} \) appears to depend on pABA to grow optimally. The variability among susceptible strains in their response to pABA depletion is an important observation that could have implications for resource depletion as a management strategy. The extent to which resource depletion causes an intensification of the competitive suppression of resistant parasites by susceptible parasites, might be reduced if susceptible parasites are also impacted by resource depletion.

While the phenotypic differences between parasites of different genetic backgrounds warrants investigation, the results of this experiment suggest they will have little to no impact on the efficacy of pABA depletion as a resistance management strategy. For pABA depletion prevented the emergence of pyrimethamine resistant parasites irrespective of the genetic background or either resistant parasites or their susceptible competitors.

### 4.6 Acknowledgements

We thank Monica M. Acosta for assistance with the drug selection experiment and David A. Kennedy for useful discussions and for supplying the code with which to implement the ldPCR algorithm.

### 4.7 Notes

This work was conducted in collaboration with Troy Day and Andrew F. Read and with technical assistance from Derek G. Sim and Matthew J. Jones. It will be submitted for publication with these persons as coauthors.
4.8 Figures & Tables

Figure 4.1: Depletion of pABA prevents the emergence of pyrimethamine resistant parasites, irrespective of the genetic background of resistant parasites or their susceptible competitors. Dynamics of susceptible (black, solid lines) and pyrimethamine resistant (red, dashed lines) parasites in mixed infections (A-F) of S_{AJ} and R_{AS} (A,D); S_{AS} and R_{AJ} (B,E) and S_{AT} and R_{CW} (C,F) and single infections (G-I) of R_{AS} (G), R_{AJ} (H) and R_{CW} (I). Each line represents the dynamics of infections in an individual mouse. Stars represent the number of parasites inoculated and the time at which they were administered. Dots indicate the density of parasites detected on a particular day in instances where parasites were not detected the day before or after. Mice received as drinking water a solution of 0.05% pABA (pABA abundant treatment, grey background) or distilled water (pABA depleted treatment, white background). Purple bar indicates duration and timing of pyrimethamine treatment. n specifies the number of mice plotted and included the analysis.
Figure 4.2: The impact of pABA depletion on resistant and susceptible parasite strains in mixed infections. Density of pyrimethamine resistant (A) and susceptible (B-E) parasites of different genetic backgrounds in pABA abundant (orange) and pABA depleted (blue) conditions. In A and B, the mean total density (± standard error) is shown. In C-E, the colored lines show the mean trajectory of parasite growth between days 3-5 in each pABA treatment, the grey lines the fitted trajectory of growth in individual mice. These trajectories were estimated using a linear mixed effects model. The raw data is plotted as grey points. n specifies the number of mice plotted and included in the analysis.
Figure 4.3: **Resistant parasites vary in their performance in pABA depleted, single infections.** Dynamics of $R_{AJ}$ (brown squares), $R_{AS}$ (red circles) and $R_{CW}$ (orange triangles) in individual mice. Each line shows the dynamics in a single mouse of parasites that emerged (left panel) and did not (right panel). Densities estimated using limiting dilution PCR, or by quantitative PCR run in triplicate, are represented by a point with an error bar, which represents the 95% confidence interval around the estimate. Large points, without error bars, indicate the density of parasites detected on a particular day in instances where parasites were not detected the day before or after. Purple bar indicates duration and timing of pyrimethamine treatment. $n$ specifies the number of mice plotted.
Figure 4.4: Pyrimethamine & pABA depletion act synergistically to depress the growth of resistant parasites. Dynamics of $R_{AS}$ in Experiment 2 in individual mice treated with pyrimethamine (dashed lines, open circles) or left untreated (solid lines, solid circles), in pABA abundant (orange) and pABA depleted treatments (turquoise). The thick, grey line represents the parasite dynamics that would be expected if pyrimethamine & pABA depletion had an additive effect on parasite growth. Parasite densities estimated using limiting dilution PCR, or by quantitative PCR run in triplicate, are represented by a point with an error bar, which represents the 95% confidence interval around the estimate.
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Table 4.1: **Number of mice in each treatment.** All mice were administered pyrimethamine treatment. Numbers in brackets indicate the number of mice that were removed from some or all analyses because they were misinoculated (circle superscript, removed from all analyses) or they died (cross superscript, removed from analysis of total parasite density).
Chapter 5

A modeling framework for investigating the nature of the antimalarial immune response

5.1 Abstract

The immune response is thought to play an important role in regulating the within-host dynamics of malaria infections, particularly during the period following the peak in parasite densities. If we are to understand the ecological role of the immune response, we must first quantify the amount of parasite mortality that the immune response causes. Historically, mathematical models have defined the immune system’s shape and activity \textit{a priori}, based on intuition or information gleaned from measurements of immune cells. However, the relationship between the abundance of immune cells, of which there are many types and whose functions overlap, and immune-killing is not a direct one. We developed an exploratory modeling framework that is capable of estimating the shape of the antimalarial immune response, and the parasite traits with which it is associated, from experimental data. We first developed a model that explicitly describes the dynamics of malaria parasites and the red blood cells in which they reproduce. By estimating the deficit between the number of parasites that could be produced, given the availability of red blood cells, and the number that were observed, the model produces a ‘killing function’. With caution, this killing function can be interpreted as an index of immune-induced mortality. We fit this model to several datasets from experiments of the rodent malaria parasite, \textit{Plasmodium chabaudi}. In one of these experiments, in which the density of para-aminobenzoic (pABA) administered to hosts was varied, there was great variation in the post-peak dynamics of infections. This dataset could contain considerable information about the immune response. Preliminary analyses illuminated that the shape of the killing function depends on the genetic background of the parasites and the within-host availability of
pABA but not with the size of the parasite inoculum. Since parasite traits are explicitly described in this model, we were able to derive hypotheses about the underlying causes of the dynamics of immune activity across experiments. In the initial iteration of the model, there was no constraint on the number of red blood cells a malaria parasite can invade. The model consistently predicted that parasites invade and kill multiple red blood cells, a phenomenon that does not occur in nature. This suggests that the dynamics of red blood cell density in malaria-infected hosts cannot be explained by the activity of parasites alone and that the host may play an important role in the regulation of red blood cell abundance, as others have suggested. We demonstrate that this model framework can provide an estimate of the shape of the immune response as well as hypotheses about the parasite traits that might ultimately stimulate it.

5.2 Introduction

The within-host dynamics of infections arise from the interactions of pathogens with different populations of host cells. Understanding these dynamics, and the interactions that underpin them, is of considerable interest to ecologists and is essential for the development of therapies and for understanding pathogen evolution. The within-host dynamics of one pathogen, the malaria mouse model *Plasmodium chabaudi*, have long been a focus of study, by dint of human malaria’s importance as a public health threat [62, 66], *P. chabaudi*’s role as a model of pathogen evolution (e.g. [59, 110, 198–200]) and its highly stereotypical dynamics. These dynamics are characterized by a period of exponential growth, followed by a ‘crisis phase’, during which parasite numbers sharply decline; a ‘plateau phase’, during which parasite numbers temporarily plateau or increase, and which gives rise to a hump in the infection dynamics, which we call ‘the shoulder’; a second clearance phase, in which parasite numbers fall to below detection levels and, lastly, a recrudescence phase (Fig. 5.1). Along with these changes in parasite density come changes in the density of red blood cells (RBCs), which provide shelter and nutrients for reproducing parasites and which are destroyed when parasites’ offspring (merozoites) burst out of them. Given their importance as a resource for parasites, the availability of red blood cells is likely to be an important driver of
the dynamics of malaria infections. Similarly, the abundance and activity of host immune cells, which suppress the growth of parasites, could determine infection dynamics.

The immune system’s role as a determinant of the dynamics of *P. chabaudi* infections and its relative importance as a regulator of parasite populations, as compared to red blood cell availability, has been the focus of both experimental and mathematical investigations. Inflammatory cytokines characteristic of the ‘innate’ immune response have been implicated in controlling the growth rate of parasites and the density at which they peak, by both experimental [201–204] and modeling studies [93, 108]. This proposition has proven controversial in the mathematical modeling literature, however, as some studies suggest that red blood cell availability alone can explain parasite dynamics during the acute phase of infection [85, 106]. The role of the immune system in the post-peak phase of infection is, by comparison, uncontroversial. Experimental studies have demonstrated that the post-peak control of malaria infections is dependent on the T-cell dependent immune response and/or B cells [128, 205, 206]. Mathematical models that describe only the dynamics of the red blood cell and parasite populations have been unable to capture the dynamics of infection from about the tenth day onward [85, 106, 108]. This failure has been taken as proof of the importance of immune activity in determining the post-peak dynamics of infection [85, 106, 108]. With its importance having been established, the next step is to elucidate the nature of the antimalarial immune response - what is its shape, to what does it respond and how is it regulated?

Mathematical models have the potential to be an important tool in the characterization of the nature of the antimalarial immune response (as they have been in the characterization of immunity to HIV i.e. [207, 208]), since they allow both the delineation and testing of hypotheses. Yet mathematical models of the antimalarial immune response have proven difficult to develop. The immune response consists of a large number of interacting cell types with redundant biological functions, which complicates the measurement of immune system activity. Where counts of immune cells are available, we do not necessarily know what they mean in terms of the phenotype of interest - killing of malaria parasites. Useful data on which to base models of the immune system, and with
which to confront them, are therefore scarce. Researchers developing models of the antimalarial response have thus been forced into making assumptions about its nature. Despite being reasonable, these assumptions may not hold. Haydon et al. [105] modeled the immune response as a function of parasite density. This approach appeals to common sense but may not be correct. Hosts may be more interested in reducing harm, of which the immune system is a potential cause, than parasite densities [209]. For these reasons, Miller et al. [109] avoided making assumptions about the factors that stimulate the immune system. Instead, they modeled immunity as a variable that came on, grew, and then abruptly turned off, at a time determined by the data. The disadvantage of this approach is that it imposes limits on the shape that the immune response can take and yields little information about the drivers of its shape and magnitude.

Since it is not the immune system per se that determines the infection dynamics in which we are ultimately interested, but rather the amount of killing done by the immune system, an alternative first step is to ask ‘how much killing is being done and when?’ In so doing, one may make a stencil of the shape of the effective antimalarial immune response, into which the products of more mechanistic models must fit. Metcalf et al. [108] demonstrated the potential of this approach. Using parameters estimated from statistical models, they projected the number of parasites expected to be present at each time point of the infection, given the availability of red blood cells, and took the deficit from that number as the number of parasites killed by the immune system.

Here, we present a preliminary attempt to define the shape of the immune response, and develop hypotheses about the parasite traits to which it might respond, by building upon the approach of Metcalf et al. [108]. We first develop a mechanistic (as opposed to statistical) modeling framework in which the dynamics of, and interactions between, red blood cells and parasites are described. We fit this model to time-series infection data and estimate the ‘killing function’ from the deficit between the number of parasites that are predicted to be present, given the availability of red blood cells, and the number that were observed. This killing function represents a putative picture of immune activity and a hypothesis of how the immune system’s importance as a regulator of the parasite population changes as the infection progresses. In addition to fitting the model to one of the datasets
used by Metcalf et al. [108], we also fit the model to data from experiments in which
the within-host availability of a micronutrient, para-aminobenzoic acid (pABA),
was manipulated. These data may contain considerable information about the
immune response, since the size (and indeed the occurrence) of the shoulder, a
phenomenon which occurs during the immunologically important post-peak phase
of infection, changes with pABA concentration. Having fit the model to these
datasets, we explore how the modeled parasite traits change with experimental
treatment and examine which of these traits are correlated with the magnitude
and shape of immune activity. In so doing, we develop hypotheses about the
putative drivers of the immune system’s shape, in addition to defining it.

5.3 Methods

5.3.1 Experimental data

We used data obtained from a number of previous experiments (Table 5.1), details
of which can be found in the relevant publications ([104], Chapter 2, Chapter
3). Briefly, inbred female C57BL/6J mice were inoculated intraperitoneally with
a given number of parasites of a given strain of P. chabaudi on day 0. Blood
was sampled from the tail daily from either day 1 or day 3 of infection until at
least day 21 post-inoculation, and red blood cell density (a measure of virulence)
and parasite density were measured by flow cytometry and quantitative PCR
(qPCR), respectively. In total, these experiments yield time-series data on the
density and virulence of five different parasite strains, of two different genetic
backgrounds, inoculated at five different inoculum sizes, in mice receiving four
different concentrations of pABA as drinking water. Four of the five strains, all
of which are of the AS genetic background, are resistant to pyrimethamine. As
a result, their growth is greatly dependent on the concentration of pABA in the
host drinking water (see Chapters 2 and 3 for explanation). To enable us to
make inferences about the shoulder, and its relationship with the killing function
and parasite traits, we looked at the data on each individual infection and by
eye assigned the infection to one of three categories: ‘present’ - it displayed the
shoulder; ‘atypical’ it displayed an atypical shoulder that was either very small,
took an irregular shape or occurred later than expected; ‘absent’ - it did not display a shoulder. The data from all experiments, and the details of each infection’s shoulder categorization, can be found in Fig. D.1 - D.4.

5.3.2 The model

We formulated a model to describe the dynamics of both the parasite and host components of malaria infections. This model is based on classical models of parasitoid infection dynamics [210, 211]. Since the cycle of red blood cell invasion and bursting in *P. chabaudi* occurs every twenty-four hours [212], we formulate the model in discrete time, with each time step representing one day. In addition to describing the density of asexual parasites $P_t$ and mature red blood cells $E_t$, we describe the dynamics of reticulocytes $R_t$ (young red blood cells), which the host produces in response to anemia. We assume that reticulocytes mature into erythrocytes every twenty-four hours (i.e. in one time step). The interaction between parasites and red blood cells are given by

$$P_t = \beta e^{-\delta_t} (R_{t-1} + E_{t-1}) f(P_{t-1})$$

(5.1)

$$E_t = (R_{t-1} + E_{t-1})(1 - f(P_{t-1})) S$$

(5.2)

where $\beta$ is the number of merozoites that emerge from a red blood cell and survive long enough to be able to invade a new red blood cell (the *effective* burst size), $\delta_t$ the killing rate, $f(P)$ the probability that a red blood cell is attacked and $S$ the survival rate of an uninfected red blood cell. At this early stage, we do not incorporate the conversion of asexual parasite stages to sexual transmission stages into the model. As only a small fraction (0.1-5%) of asexual stages are ever converted to sexual transmission stages [200], this omission is unlikely to significantly impact the fit of the model. The dynamics of reticulocyte production are in turn described as a function of red blood cell density,

$$R_t = E_0 \left( \frac{1 - S}{S} \right) \left( \frac{\kappa^\alpha + 1}{\kappa^\alpha + \left( \frac{E_{t-1}}{E_0} \right)^\alpha} \right)$$

(5.3)
where $\kappa$ is the half-saturation parameter of the reticulocyte production curve and $\alpha$, the sharpness parameter of the reticulocyte production curve. Such is this function’s definition that, at different values of $\kappa$ and $\alpha$, reticulocyte numbers can increase as a linear or saturating function of red blood cell density (Fig. 5.2). Both functions that describe erythropoiesis as a linear function of red blood cell density, and as a nonlinear function of red blood cell density, have been used to explain infection dynamics in the past [87, 106, 108]. Importantly, this function is capable of taking a shape that allows the influx of reticulocytes to be delayed until erythrocyte densities reach very low levels and the infection has entered the crisis phase, as has been observed in experiments [213, 214].

Since we do not know what the shape of the effective immune response is, we want to make the minimal number of assumptions when modeling it. We thus assume that the killing rate follows a volatility model

$$\delta_t \sim \text{Normal}(\delta_{t-1}, \sigma^2), \quad \delta_t \geq 0$$  \hspace{1cm} (5.4)

such that it can take any value above zero and can move in any direction, and estimate its distribution conditional on the data. In so doing, we allow the data to determine the shape of the killing function.

We assume that parasites encounter red blood cells randomly and in proportion to their abundance and that all red blood cells are equally susceptible to infection, so that

$$f(P) = 1 - e^{-aP}$$  \hspace{1cm} (5.5)

where $a$ is the per parasite search efficiency or the area over which a single parasite can search. While these assumptions may seem simplistic, Metcalf et al. [108] found no evidence to suggest that malaria parasites’ infection of red blood cells resemble anything other than that of a density-dependent infection. Note also that we assume that parasites exhibit no preference for red blood cells of different ages, unlike others [84, 85, 215]. This assumption was based on the results of experimental and modeling studies, which found that $P. \ chabaudi$ invades red blood cells of different ages in proportion to their availability [107, 216].

In addition to modeling the infection process, we also specify a model of the process by which the infection is observed. We assume that the measurement
error around estimates of both red blood cells and parasite density is log-normally distributed.

All parameters, barring $S$ and $E_0$, were estimated from the data. We fix the value of $S$ at $e^{-\frac{1}{40}}$, since the lifespan of a red blood cell is 40 days [217], and $E_0$ at $9 \times 10^6$ which is the approximate red blood cell density per microliter of blood in a healthy C57BL/6 mouse (author’s observation). Parameter estimation was performed using iterative filtering, implemented using the mif function of the pomp package in R [218]. Each mouse was fitted separately.

5.4 Results & Discussion

5.4.1 Model fit

The model was capable of capturing the dynamics of both parasites and red blood cells, under a variety of treatment regimens (Fig. 5.3). This is somewhat unsurprising given the flexibility of both the killing function and the reticulocyte resupply function. The likelihood profiles of two infections indicated that the likelihood had not yet been maximized, so we did not consider them in our future analyses (see Fig. D.2, subplots marked with an ‘x’). We also excluded one mouse that apparently received a smaller inoculum of parasites than was intended (Fig. D.3, subplot marked with a triangle). As might be expected, given that this model is not predictive but rather something more akin to a transformation of the data, the model’s estimates of parasite density divert wildly from those one might expect when there is no data to constrain its estimates (see, for example, the predicted parasite density on days twenty onward in infections of mice inoculated with $10^5$ and administered 0.05% pABA in Fig. D.2). We thus restrict our analyses of the model output to the time period when data is available.

5.4.2 The killing function’s shape & its relation to the shoulder

We conducted a preliminary investigation of the shape of the killing function. Although the dynamics of the killing function differed among mice within and
between treatments, the killing functions of all mice shared a general shape (Fig. 5.4, D.1 - D.4). The killing rate grew in the days prior to or just after the peak in parasite density and in no case did it ever fall back to pre-peak levels. The relative consistency in the shape of the killing function is reassuring. Given that the mice are inbred and infected with the same species of parasites, we would not expect wild amounts of variation in the shape of the killing function.

We noticed two main patterns in the shape of the killing function. Firstly, the timing of the initial increase in the killing function, relative to the peak in parasite density, depended on the parasite’s genetic background (Fig. 5.4, compare rows A and B). In infections of the AS genetic background, the killing rate began to increase a couple of days prior to the peak in parasite density and peaked roughly in coincidence with it. In infections of the AJ background, by contrast, the killing rate tended to ramp up after the parasite density had peaked and begun to fall. This may suggest that red blood cell limitation has a larger role than immunity in regulating population sizes of AJ parasites, as compared to AS parasites, in the acute phase of infection. Interestingly, in mice infected with AS parasites, the killing rate and parasite density peaked roughly in coincidence with each other, irrespective of the how fast it took for parasites to reach peak densities (Fig D.1, both the killing rate and parasite density peak earlier, as inoculum size increases). We could interpret this as an indication that the time it takes for the immune response to ramp up is not preprogrammed. Interestingly, empirical studies of AS infections have found that the timing of the peak in concentration of the inflammatory cytokine interferon-gamma (IFN-γ) also changes with the timing of peak parasite density, occurring two days beforehand irrespective of how soon after inoculation parasite densities reach their peak [129]. The second pattern to emerge from our initial analysis of the killing function, was that there was a marked reduction in the killing rate following its initial increase in some mice (Fig. 5.4). This dip in the killing rate was particularly clear in mice infected with AJ22p but was also seen to a smaller extent, in some of the mice infected with parasites of the AS background. Notably, whether there was a reduction in the killing rate during the post-peak phase appeared to be related to whether the infection displayed a shoulder (Fig. 5.4, compare rows B and C), while the size of the reduction in the killing rate appeared to be related to the size of the shoulder (Fig. 5.4, compare
B1 and B2). The shoulder, therefore, appears to be an immunologically-related phenomenon, as opposed to one that arises from the dynamics of red blood cell availability. This interpretation echoes that of [108], who suggested that a post peak decline in the immune system’s efficacy could facilitate a second period of parasite growth in some mice.

To further investigate the relationship between the modality of the killing function and the shoulder. We calculated the number of parasites removed by the immune system at each time point

\[ Q = \beta (R_{t-1} + E_{t-1}) f(P_{t-1}) - P_t \]

which we take as a measure of total immune effort, and the effective reproduction rate

\[ M = \beta e^{-\delta_t} (R_{t-1} + E_{t-1}) f(P_{t-1}) / P_{t-1} \]

and plotted each infection’s progression through Q-M space in time (Fig. 5.5). The nature of an infection’s path through Q-M space was associated with whether or not it displayed a shoulder. In all of the infections, the number of parasites killed increased with time, before falling after the effective reproduction number started to fall. In most of the infections which displayed a shoulder (colored red in Fig. 5.5), the fall in the number of parasites killed was associated with an increase in the effective reproduction rate. This increase in the effective reproductive rate was in turn followed by an increase in immune effort. The paths through Q-M space of infections that displayed the shoulder were thus characterized by a kink. This kink was absent from the majority of infections that did not display a shoulder. The position of the kink in Q-M space differed among infections. In infections seeded with \(10^6\) parasites, the effective reproduction rate of AJ22p increased after a very small reduction in immune effort, whereas the effective reproductive rate of AS44p did not pick up until a much greater reduction in immune effort (Fig. 5.5 HI). Beyond the timing of the kink, whether it was even present seemed to depend on the identity of the parasite strain. Over the range of inoculum sizes, a kink in Q-M space appeared less often in infections of AS8p than in infections of other parasites strains, the majority of which share a genetic background with AS8p. Inoculum
size itself appeared to have little impact on the trajectory of an infection through Q-M space. The time it took for the number of parasites killed to be maximized increased with inoculum size. This is to be expected, given that infections seeded with smaller inocula take longer to reach peak parasite densities [123] and the size of Q is intimately linked to parasite density.

It is tempting to draw parallels between the bimodal shape of the killing function in infections that display a shoulder and the bimodal nature of the antimalarial immune response (though this must be done with caution, since the killing function represents the action of any aspect of the host environment that has a negative impact on population growth including, but not limited to, the immune system). Inflammatory TH1 associated CD4+ cells predominate in the early phase of *P. chabaudi* infection before being supplanted by TH2 associated cytokines, which have a role in parasite clearance [128]. Concentrations of IFN-γ and IL-4, the cytokines canonically associated with the TH1 and TH2 responses, respectively, reach their peak days apart [129, 206]. As a result, there is a period during which the levels of neither cytokine are maximal. Immunological studies of infections started with 10⁵ AS parasites indicate that the valley in cytokine numbers is at its deepest 10-15 days after the infection began [129, 206]. This is the period during which we observe a reduction in the number of parasites killed and an increase in the effective reproduction number in infections of 10⁵ AS123p and 10⁶ AS44p (Fig. 5.5, FH). Could this valley in immune activity give parasites an opportunity to break through and cause the shoulder? An alternative explanation is that the reduction in killing rate that we observe reflects not a reduction in immune effort but a reduction in immune efficacy, due to the emergence of a parasite mutants that the immune system cannot recognize. *P. chabaudi* parasites can express a number of different antigens and switch between them at variable rates. Both the frequency of minor antigenic variants and the expression of genes involved in antigen expression change during the exponential phase of growth [219, 220]. It is therefore possible that an antigenic escape mutant could arise early in infections and go on to cause the shoulder.
5.4.3 An investigation of the parameters that correlate with the killing function’s shape

To investigate the possible cause(s) of the reduction in immune killing that is associated with the shoulder, we next investigated whether the model parameters which describe parasite traits ($\beta$, the effective burst size; $a$, the search efficiency) varied consistently among mice that displayed a shoulder and those that did not.

We first explored the impact of the effective burst size ($\beta$), since we reasoned that the ability of a parasite to take advantage of any break in immune activity might be a function of its effective burst size. Parasites with a small burst size might still be kept in check by a down-regulated immune response, while those with large burst sizes might be able to grow. Similarly, under the hypothesis that the shoulder is caused by an antigenic variant that escapes immune killing, parasites with higher burst sizes might be expected to cause a shoulder more often than those with smaller burst sizes. All else being equal, the population size of parasites with higher effective burst sizes should be larger than those of parasites with lower burst sizes, and so they should accumulate a greater diversity of antigenic variants. We found that the estimated effective burst size of infections that displayed a shoulder was higher than those which did not display a shoulder or which displayed an atypical shoulder (Fig. 5.6A). Indeed, the effective burst size could explain why AS8p infections displayed shoulders more rarely than infections of other strains. Estimates of the burst size of AS8p tended to be lower than burst size estimates of the other strains (Fig. 5.6B), though they certainly overlapped with the burst size estimates of other strains including with AJ22p, which displays the largest shoulder. In the midst of this analysis of effective burst sizes, however, we noted that there were some infections for which the estimates of effective burst size were smaller than one. Yet the model predicted that these infections grew in size, at least at some point in the infection. How could this be?

5.4.4 Model misspecification & its impact on model interpretation

The above analysis of $\beta$ allowed us to identify an important structural problem with the model: it permits a single parasite to infect and destroy multiple red blood cells,
in deviation of what occurs in nature. In the equation that describes the number of parasites (Equation 1.1), there is no explicit constraint on the number of red blood cells that a single merozoite can parasitize. Rather, whether parasites are restricted to infecting just one red blood cell is determined indirectly by the value of $f$, which defines the probability that a red blood cell is attacked. Imagine a host is infected with a single parasite on day 0 and that it mounts no immune response. When the parasite density is small, as it is in this example, the probability that a red blood cell is attacked is solely determined by $a$, the parasite’s search efficiency. When the search efficiency is equal to the inverse of the number of red blood cells, the probability that a red blood cell is attacked multiplied by the the density of red blood cell is equal to one. So, one red blood cell is destroyed and the number of parasites produced is equal to the effective burst size. If the search efficiency is larger than the inverse of the number of red blood cells, however, the number of red blood cells multiplied by the probability that a red blood cell is infected is greater than one, so more than one red blood cell is infected by and killed by each parasite. Note that, when the search efficiency is thus inflated, the number of parasites produced (given by the effective burst size multiplied by the number of parasitized burst cells) exceeds the effective burst size. If the model, therefore, is to give an accurate estimate of the number of parasites produced, the estimate of the effective burst size must be reduced. This explains why $\beta$ and $a$ are strongly negatively correlated (Fig. 5.6C).

To examine whether the model’s misspecification had impacted the estimates of $\beta$ and $a$ in all, or just a subset, of mice we performed a simple analysis. The overestimation of the search efficiency is most problematic when red blood cell densities are large and parasite densities small (because this is when $a$’s influence on $f$ is largest), as at the beginning of the infection. We therefore investigated, for each infection, whether the estimate of the search efficiency ($a$) was greater than the inverse of the number of red blood cells at the beginning of the infection. In no case was the estimate of the search efficiency smaller than the inverse of the red blood cell density at the beginning of the infection. At least at some point during each infection, therefore, our model predicted that parasites infected multiple red blood cells. This means that we cannot correctly interpret the parameters $\beta$ and $a$ as the effective burst size and search efficiency, respectively, as originally intended.
That is not to say that $\beta$ has no relationship to the true effective burst size, however. In infections of pyrimethamine resistant parasites, an infection’s burst size is expected to be positively correlated with the concentration of pABA in the host’s drinking water, since pyrimethamine resistant parasites rely on pABA for the production of DNA and so of offspring (Chapter 2). The model’s estimates of $\beta$ are higher in infections from experimental treatments where the hosts were administered pABA (Fig. 5.6D), as would be expected. $\beta$ may thus be positively correlated with the true effective burst size. Our tentative conclusions regarding the role of burst size in determining the shape of the killing function and thus the shoulder, therefore, may still have some merit.

Since the effect of the killing rate on parasite density is mediated via its impact on $\beta$, the estimates of the killing rate might also be impacted by the model’s misspecification. We believe that though the magnitude of the killing function is likely to be impacted, its dynamics may yet represent a valid hypothesis of the shape of immune activity. The killing rate acts by reducing the size of $\beta$. Since the estimates of $\beta$ are driven down to compensate for the inflation of $a$, the killing rate may be on average underestimated. The dynamics of the killing rate may not be much impacted by the problem, however. It is important to remember that $\beta$ and $a$ are fixed parameters. When the killing rate increases, causing a reduction in the estimated production of parasites, $a$ cannot increase to compensate for the loss. The problem with the estimation of $\beta$ and $a$ does not change the fact that the only way that the model has to prevent population growth when there is none is via the killing rate.

Though its misspecification prevents meaningful interpretation of the parameters, the model’s behavior reveals an interesting fact about the biology of *P. chabaudi* infections, which must be captured by future models. That the model consistently settles on values of $a$ that permit single merozoites to infect and destroy multiple red blood cells, at least some of the time, suggests that parasite numbers alone are insufficient to explain the loss of red blood cells that we observe. This finding chimes with that of a number of previous modeling studies which have found that, in order to explain the magnitude of anemia in *P. chabaudi* infections, uninfected red blood cells must be destroyed along with infected cells. Recently, Metcalf *et al.* [22] found that the removal of uninfected red blood cells (which
they dubbed ‘bystander killing’) could counterintuitively minimize the severity of anemia that the host experiences. This lead them to suggest that the immune system may remove uninfected red blood cells as an adaptation to infection [22]. Be it adaptive or not, the immune system is thought to be responsible for severe malarial anemia in patients with *P. falciparum* infections and in semi-immune rodents infected with the *P. berghei* [221, 222]. To investigate whether, as this model suggests, bystander killing is a necessary component of a model of malarial infection dynamics, future work will focus on building a corrected version of this model that permits a parasite to infect only one red blood cell. In this new model, as in the model presented here, the killing rate will be directed at the parasites’ reproductive rate. If bystander killing is important, this new model will be unable to capture the dynamics of parasites and red blood cells simultaneously. Rather, it will overestimate parasite densities in an attempt to fit the red blood cell dynamics, or vice versa, or simply produce a poor fit to both sets of time series. It is important to recognize that bystander killing and the cessation of reticulocytosis can be confounded, in the absence of data on the age structure of the red blood cell population [105, 108]. So the validity of any conclusions we make about the importance of bystander killing from a new iteration of this model will depend on the reticulocyte supply function being correctly formulated.

Indeed, this ’subtraction’ approach, by which the failure of red blood cell availability to explain the data is used to infer the activity of immune system, is valid only in as much as the dynamics of red blood cells are adequately modeled. If the shape of the resupply function is misspecified, so that the model predicts that red blood cells are being produced when none are, there will be an inappropriate increase in the projected number of parasites produced. To bring the estimates of parasite density back in line with the data, the model might erroneously estimate an increase in the killing rate. An overestimation of the number of red blood cells being produced might also contribute to the inflation of $\alpha$, since one way that the model can remove these unwanted red blood cells is to allow parasites to infect multiple red blood cells. It is important to note that the structural problem with the model that permits this unrealistic behavior of parasites may not impact the fit of the parameters $\kappa$ and $\alpha$, which drive the shape of the reticulocyte supply function. This is because the supply of reticulocytes is a function of
the number of red blood cells destroyed, how they were destroyed matters not. Indeed, the dynamics of reticulocyte density produced by our model resemble those observed in empirical investigations of reticulocytosis, where significant densities of reticulocytes are not produced until after parasite densities have peaked (due to the suppression of the action of the hormone erythropoietin by inflammatory cytokines [223,224]). Though this is bouying, a rigorous assessment of the validity of the resupply function should be conducted.

Further iterations of the model should thus be confronted with data on the density of reticulocytes. These data may be obtained after the fact by microscopy, using the blood smears that were taken during the experiments from which we have drawn data, or by flow cytometry of qPCR during any new experiment. Even now, we anticipate that there will be discrepancies between data on reticulocyte numbers and the numbers of reticulocytes estimated from the model. Equation 2 specifies that reticulocytes become erythrocytes after one day. Reticulocyte maturation may actually take up to three days [106], depending on the immune-status and/or the anemia of the mouse. As a result, our model’s estimates of reticulocyte densities are likely to be smaller than they actually are. Since our estimates of erythrocyte numbers will be slightly larger (since two day and three day old reticulocytes are counted as erythrocytes in our model), however, the total numbers of red blood cells will be unaffected. So, as long as our assumption that red blood cells of all ages are equally vulnerable to attack stands, our disregard of the subtleties of reticulocyte maturation should not impact our model’s estimates of parasite density or killing rate. The way the model ‘records’ reticulocytes could make the fitting of the model to reticulocyte data challenging, however. To make the incorporation of reticulocyte data easier, we could define additional equations that describe the movement of reticulocytes through the three age classes (as per [106]).

Looking further into the future, to a more mechanistic model of infection dynamics, there might be value in modeling the dynamics of reticulocytosis as a function of the innate immune response, given that both the supply and maturation rate of reticulocytes are in fact variable and contingent on the quantity of cytokines associated with the innate immune response [223–225].

This preliminary work, though flawed, demonstrates that by combining the mechanistic approach to modeling infection dynamics (as used by [85, 93, 105–
with the 'subtraction' approach of Metcalf et al. [22, 108], we might identify the shape of the effective immune response as well as the parasite traits with which it is associated. This model suggests that the dynamics of parasite populations in the period following the peak in parasite density, as characterized by the shoulder, are determined more by the immune system than by the availability of red blood cells. The effective activity of the immune system during this period may be related to the effective burst size of the parasites. Before further exploring these hypotheses, we must first confirm that the anemia that occurs during malaria infections cannot be attributed to the activity of parasites alone, as the behavior of this misspecified model suggests, and build a model that accounts for the destruction of red blood cells by the host. Intriguingly, explaining the within-host dynamics of malaria infections may depend as much on understanding the interactions between populations of host cells, as on understanding their interactions with parasites.

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5.6 Notes

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5.7 Figures & Tables

Figure 5.1: **The dynamics of a typical *Plasmodium chabaudi* infection.** Schematic of the five main phases of a *P. chabaudi* infection. Each phase is delineated by a separate shade of grey.
Figure 5.2: The impact of the parameters, $\kappa$ and $\alpha$, on the shape of the reticulocyte resupply function. The shape of the reticulocyte resupply function when (left panel) the value of $\alpha$ is fixed at 5 and the value of $\kappa$ is varied and when (right panel) $\kappa$ is fixed at 0.5 and $\alpha$ is varied. The range of values along which the parameters are varied is representative of the range of the fitted values. The erythrocyte density per microliter of blood in a healthy, uninfected mouse is approximately $9 \times 10^6$ and can be reduced to $2 \times 10^6$ by *P. chabaudi* infection.
Figure 5.3: Data on & model estimates of infection dynamics in different experimental treatments. Observed (black circles) and estimated (solid lines) dynamics of parasites, erythrocytes and reticulocytes in mice infected with different numbers of parasites of different strains of *P. chabaudi*, as indicated by the label at the top of each column. In columns E and H, the dynamics of two infections are shown and in column G the dynamics of four infections are shown - each line represents an infection of a mouse administered a different concentration of pABA solution as drinking water (indicated by the line color). For ease of interpretation, each experimental treatment is represented only once in this figure. The dynamics of replicate infections can be found in Appendix D.
Figure 5.4: The dynamics of the killing function & its relationship to parasite density and the shoulder. The estimated dynamics of the killing function (thick line) and parasite density (thin line) in individual infections (separate subplots) of $10^6$ AJ22p (A) and AS44p (B,C) parasites, where the shoulder is present (A,B) and absent (C). Grey vertical lines indicate the day on which the peak in parasite density was observed. Mice were administered either a 0% (A,C) or 0.05% (B) solution of pABA as drinking water. Note that because pABA has little to no impact on the dynamics of AJ infections, a comparison of rows A and B can be treated as a comparison of the impact of different strains, rather than of the impact of pABA treatment. For ease of interpretation, the dynamics of three experimental treatments are shown here. The dynamics of the killing function in each individual infection of each treatment can be found in Fig. D.1 - D.4.
Figure 5.5: The relationship between the effective reproductive number (M) & the number of parasites killed (Q), as estimated by the model, changes through time and appears to be associated with the presence of the shoulder. Each subplot depicts an individual infection's journey through Q-M space. Stars represent where in Q-M space the infection began. Arrows indicate the infection's direction of travel at days 7, 12 and 17. The infection's place in Q-M space at days 10, 15 and 20 post-inoculation is also indicated by numbers. Labels to the right of each row indicate the number of parasites, of which strain, that the mice were infected with. To both maximize the number of inoculum size treatments that could be depicted and to ease interpretation, only infections of mice administered 0.05% pABA are shown. The shoulder and recrudescence both give rise to a kink in the trajectory through Q-M space. To prevent confusion, therefore, the dynamics of infections in which recrudescence occurred are shown from days 0-20 only.
Figure 5.6: The magnitude of $\beta$ is correlated with experimental treatment & $a$. Points indicate the values of individual infections. Data from all treatments were used for plots A and C. To facilitate comparison with Fig. 5.5 only infections from mice administered 0.05% pABA are included in B. For clarity, only data from experiments that used pyrimethamine resistant parasite strains and where pABA concentration was manipulated, were used for subplot D.
Table 5.1: Details of experiments from which we took data to fit to our model. All infections were left untreated and consisted of just the strain specified. Since all AS strains are resistant to pyrimethamine, their growth dynamics are expected to change with the pABA concentration administered to mice. Hosts in all experiments were female C57BL/6J mice.

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Chapter 6

General Discussion

Malaria parasites impact the health and wealth of 198 million people a year [62] and, with the emergence of drug resistant parasites, their impact is set to grow. Here, we examined what elements of the within-host environment limit the proliferation of malaria parasites and whether the within-host environment can be manipulated to control the emergence of drug resistant parasites. Below, we discuss our basic and applied findings, in turn.

6.1 Limiting factors of the within-host environment

In Chapters 2 and 3, we demonstrated experimentally that pABA is a limiting resource for pyrimethamine resistant *Plasmodium chabaudi* parasites and, to a lesser extent, susceptible parasites. These findings corroborate those of earlier studies, conducted in the initial development of rodent malaria as an experimental model, which found that pABA was a limiting resource for *Plasmodium chabaudi* [114] as well for a suite of other parasite species [115–117]. In contrast with studies of *P. berghei* [116] and *P. yoelii* [118], we did not find that pABA supplementation was essential for parasite growth. This suggests that trace amounts of pABA present in the bloodstream of mice, as acquired from their food or produced by gut microbiota, or parasite production of pABA [226] are sufficient to sustain the growth of *P. chabaudi*. To establish the importance of the microbiota and food as sources of pABA for malaria parasites, one could use antibiotics or design synthetic diets free of pABA to deplete the host of pABA originated from the microbiota and food, respectively. A very minimal amount of pABA might be all that is required to sustain (drug susceptible) *P. chabaudi*, if it is anything like *P. falciparum*. In culture, some strains of *P. falciparum* can undergo at
least one round of replication in medium devoid of any source of pABA or folic acid [149] and grow in a sustained manner in medium containing pABA at a concentration of just 0.5 μg per liter (a concentration between one to three orders of magnitude smaller than that found in human serum [227]). An alternative explanation for the observation that *P. chabaudi* populations grow in the absence of pABA supplementation is that pABA is a substitutable resource for *P. chabaudi* i.e. parasites are able to sustain themselves on other types of folate (e.g. 5-methyltetrahydrofolate, folic acid, pABA monoglutamate), salvaged from the host serum. Wang *et al.* [228] found that even parasites resistant to pyrimethamine (which are ostensibly very poor at salvaging folate) could be sustained for several months in medium supplemented with just 100ng/ml of folic acid and no pABA, suggesting that at least at some concentration of folate, pABA is not an essential resource for malaria parasites. Whether pABA is a fully substitutable resource for pyrimethamine resistant parasites in the field is an open question but, given that the concentration of folic acid in human serum is far lower than that which sustains parasite growth *in vitro* (6-20ng/ml) [149], it seems unlikely. Indeed, our experiments suggest that, irrespective of the way that pyrimethamine resistant parasites proliferate in the absence of pABA supplementation, their competitive ability is so poor as to make them practically inviable in the context of a harsh within-host environment in the field. The fact that pyrimethamine resistance emerged so infrequently in the field, also supports this view [70].

Our experiments represent the first time a resource has consistently been demonstrated to mediate within-host competition between *P. chabaudi* parasites and offers lessons for the study of parasite competition within-hosts. In Chapter 2, we explored how competition changes over a range of resource levels. It was important that we did so. Had we limited our experiment to the medium and high concentrations of pABA, we would have concluded that pABA did not mediate competition between resistant parasites. Our experiments also illuminate the value of using two different competition assays in conjunction. In Chapter 2, we assessed how competition changed over a resource gradient by adding a competitor to the within-host environment. We found that the intensity of competitive suppression in the unsupplemented treatment was greater than that in the high pABA treatment. These two treatments were denoted the pABA depleted and pABA abundant
treatments, respectively, in Chapters 3 and 4, wherein we used drug treatment to remove the susceptible competitor. In competitor removal experiments, we typically conclude that an organism is experiencing competition if its population size increases upon the removal of its putative competitor i.e. if competitive release is observed. Since we did not observe competitive release in the pABA depleted treatment, we might have concluded that susceptible and resistant parasites did not compete in pABA depleted environments. Only because we had grown resistant parasites in single infections and had the results of Chapter 2 to draw on, did we avoid making this erroneous conclusion. In the context of field experiments, where singly infected hosts are uncommon and addition experiments are difficult and/or non-permissible, caution should be taken in interpreting experiments in which competitive release is not observed. Lastly, our work well demonstrates Keddy’s [2] statement that manipulating resources ‘may not...yield unambiguous conclusions about mechanisms’ of competition (page 319). As discussed in Chapter 2, though we see that the intensity of competition changes over a resource gradient, it is quite possible that immune-mediated apparent competition as well as, or instead of, exploitation competition is intensified in pABA depleted conditions.

Our modeling work (Chapter 5) suggests that, in single infections too, the availability of pABA may change the relationship between parasites and the immune system, as may the genetic background of parasites. Not only can this framework reveal the shape of the effective immune response, but it may help us to unearth what parasite traits determine the immune responses’ shape. Tantalizingly, the current iteration of the model suggests that the shape of the immune response is associated with some parameter correlated with parasite burst size, so that differences in the burst size of different parasite strains (of which growth rate is often used as a proxy) explain their differential immunogenicity.

Manipulations of pABA availability could represent a useful tool with which to investigate this hypothesis, as well as other hypotheses related to the life history traits of parasites. To investigate the extent to which a strain’s immunogenicity is merely a function of its growth rate we could experimentally evolve pyrimethamine resistance on a variety of genetic backgrounds, as we did in Chapter 4. This would make the strains (more) dependent on pABA for growth and facilitate the modification of their growth rate via alterations of the concentration of pABA in
the hosts’ drinking water. We could then experimentally derive the relationship between growth rate and immune activity in each of several strains, enabling us to ask whether there are inherent differences in the immunogenicity of different parasite strains. Manipulations of pABA availability might also be employed in the experimental investigation of the evolutionary response of parasites to resource restriction. Malaria parasites have long been used as a model system with which to investigate reproductive investment theory e.g. [229, 230]. Some of this work has focused on the question of how parasites’ investment into sexual transmission stages, and the sex ratio of those transmission stages, changes with resource availability [200, 231]. The availability of pABA can be manipulated with relative ease and economy, as compared to manipulations of red blood cell availability achieved via the administration of erythropoetin or phenylhydrazine [231], and does not cause any harm to the host. So, pABA manipulations could facilitate more experiments, and accelerate the progress of research, in this area. Whether they do so, however, will depend on the extent to which parasites are attuned to pABA as a cue for environmental quality.

In addition to revealing that pABA availability alters the character of the immune response, our model also illuminated the potential importance of the immune response in determining the availability of red blood cells. In so doing, it revealed that the extent to which the immune response is analogous to a predator is limited. After all, the impact of predators on the availability of resources of prey (here, red blood cells) is normally mediated by their effect on prey (here, malaria parasites). That said, intra-guild predators are an exception and could serve as a better analogy for the immune response, since they both predate upon and compete with their prey. As well as being important to how we think about host-parasite interactions, the choice of analogy could be of considerable importance as we look toward building a predictive rather than a descriptive model of the host immune response. Cressler et al.’s model [232] could prove to be a useful starting point for this work. They pointed out that a population of immune cells is qualitatively different from that of predators, in that the population size of immune cells is not a direct function of its prey population size (i.e. immune cells do not use parasites as resources). Rather, the population size of the immune response is constrained by the availability of host resources, which it may or may not share with parasites.
To capture these subtleties, they built a novel theoretical framework with which to model immune-parasite-resource dynamics. In their model, both the parasite and immune cells destroy the shared resource. In this sense, their framework would suit our purposes, given that both parasites and immune cells appear to destroy red blood cells. However, red blood cells do not directly promote the growth of the antimalarial immune response, and do not therefore fit Cressler et al.’s definition of a shared resource. Instead, by promoting host health, red blood cell availability indirectly promotes the growth of the immune cell population. Cressler et al.’s framework would thus need to be extended to be applied to our system or we could develop a different modeling framework altogether.

6.2 Manipulating within-host ecology for public health gain

In Chapters 3 and 4, we demonstrated that depleting a resource for which resistant parasites have greater requirements than susceptible parasites, can so intensify competitive suppression as to prevent the emergence of resistant parasites. This resistance-management intervention is the first among those that harness within-host competition (others include superinfection therapy [168] and the low dose drug treatment [58, 59]), that can be used alongside high dose drug treatment. It may thus come with less risk to host health and be more palatable to the medical community, who fear the emergence of de novo mutants. The impact of resource depletion on the emergence of de novo mutants is an obvious next step for research. However, as we explained in in Chapter 3, we do not expect de novo resistant mutants to be any less vulnerable to pABA depletion (indeed we expect the opposite).

Our work is more important as proof of the principle that resource depletion can prevent the emergence of drug resistant parasites than as a treatment, for several epidemiological and practical reasons. The use of pyrimethamine as a monotherapy ended after resistance emerged in the 1950’s (e.g. [233,234]). It has since been used in combination with sulfadoxine-pyrimethamine (S/P), to which resistance is now prevalent. The majority of resistant parasites in the field have
multiple mutations in the DHFR gene [160, 235, 236], which compensate for the fitness costs of resistance [237] (and hence pABA dependency). Such is the size and nature of the pyrimethamine resistance problem that, could we find a way to achieve pABA depletion in human hosts, it would have little impact on the rates of resistance in the field.

One of the greatest challenges in moving from proof of principle to practice will be to find ways of depleting within-host resources from human patients. Dietary manipulation is an obvious option but could be difficult to achieve outside of a medical institution. If the resources on which resistant parasites are dependent are also required by humans (unlike pABA), dietary manipulation could come at the cost of host health and might be thwarted by the body’s efforts to maintain nutrient homeostasis. That said, there are drugs that successfully reduce the serum concentrations of nutrients (i.e. statins) and a plethora of drugs are associated with nutrient deficiency as a side-effect [238]. Most suitable would be compounds similar to bacterial siderophores or transferrins: high-affinity molecules that could reversibly bind the concentration of resources available to parasites, without reducing the concentration available to patients in the long-term, and which would not cause much disruption to the day to day lives of patients. Alternatively, where hosts obtain their nutrients from the microbiota, we could use antibiotics to temporarily hinder resource supply or, where parasites take up only particular forms of a nutrient, we might interrupt the host machinery that converts the nutrient to the forms that the parasite can absorb.

Another challenge in the deployment of resource depletion as a resistance management strategy is to identify resources for which drug resistant parasites have elevated resource requirements (as discussed in Chapter 3). Where the site of the resistance mutation is known and metabolic networks mapped out, we may be able to identify resources upstream of the mutation site that resistant parasites might require in excess. Models of the flux of nutrients into and out of parasites cells (e.g. [239, 240]) could be utilized to narrow down these candidate resources. Such models could be used, first, to identify resources obtained from the host environment (i.e. those which could mediate competition between parasites) and second, to identify how an inefficiency in an enzyme impacts the flux of metabolites through the metabolic network. When pathogens are culturable, simple (but
time-consuming) experiments in which the individual ingredients of the culture medium are removed (e.g. [241]), and the response of susceptible and drug resistant parasites recorded, could be conducted. Such experiments would be useful in both confirming the predictions of metabolic analyses, and where metabolic models are unavailable, in discovering candidate resources for depletion.

Though there are obstacles that must be overcome before resource depletion can be used to prevent the emergence of drug resistant pathogens, immediate lessons regarding the role of resource supplementation in the evolution of drug resistance can be taken from our work. Our work warns that resource supplementation could accelerate the evolution of resistance by facilitating the competitive release of drug resistant parasites. Indeed, we hypothesize that folate supplementation could serve to facilitate the competitive release of S/P resistant parasites in a manner similar to way that supplementation with pABA facilitates the competitive release of pyrimethamine resistant parasites. In *P. falciparum*, sulfadoxine resistance is correlated with dihydrofolate reductase (DHFR) genotype, when folate is present in the culture medium [156], though it is also associated with dihydropteroate synthase (DHPS) mutations. Triple DHFR mutants utilize extracellular folic acid efficiently, enabling them to compensate for sulfadoxine’s inhibition of the endogenous production of folate [125]. Importantly, these S/P resistant parasites rely on salvaged folate more than wild-type parasites [148, 150]. Thus, increasing the quantity of serum folate could ameliorate the costs of S/P resistance just as pABA supplementation ameliorates the cost of pyrimethamine resistance. Reduced S/P treatment efficacy is observed significantly more regularly in women [242–245] and children [243, 245] receiving a dose of 5mg folic acid than in control patients, and blood folate levels prior to S/P treatment significantly predict treatment failure [244,246]. These observations are usually attributed to the ‘folate’ effect, by which the action of sulfadoxine is antagonized by folate (a phenomenon often observed in vitro [156,247,248]). Alternatively, treatment failure could be a consequence of the combination of high dose S/P treatment, which imposes strong selection for resistance, and high concentrations of folate, which could ameliorate the cost of S/P resistance and facilitates the competitive release of resistant parasites. Beyond its impact on the intensity of within-host competition, resource supplementation could also increase the probability of a de novo resistance mutation occurring, since
it could increase the carrying capacity of the within-host environment and permit the population size of parasites to increase beyond its normal size. The invasion of a novel mutant might also occur faster in high resource conditions, as studies of phage resistance in bacteria suggest [249]. Though resource supplementation could fuel the evolution of drug resistance (as Merkli et al. [250] found), the disadvantage of ceasing resource supplementation might be even greater.

Since there are potential difficulties with manipulating within-host resource availability, one might ask whether other aspects of the within-host environment could be manipulated to intensify the competitive suppression of drug resistant parasites. For example, could we up-regulate the immune response or other natural enemies, like phages (viruses of bacteria), that mediate apparent competition between parasites? Though it is not immediately obvious that drug resistant parasites would be differentially vulnerable to the immune response, if the costs of resistance are paid via a decrease in the growth rate of parasites, resistant parasite might well struggle more than susceptible parasites to replace themselves in the presence of an augmented immune response. Alternatively, due to the increased metabolic demands that are associated with drug resistance, resistant parasites might not be able to invest as much in immune-invasion or to deal with immune-induced oxidative stress as well as their susceptible counterparts.

Our work demonstrates that manipulations of the within-host environment can prevent the emergence of drug resistant parasites in individual hosts but it is an open question whether they could prevent the evolution of resistance in the long term. In the case of pABA depletion, we hypothesize that there is a way that parasites could evolve resistance to the combination of pyrimethamine and pABA depletion (since there is a way to evade S/P) - acquire three mutations in the DHFR. If our hypothesis is borne out, it will not support the suggestion that there is some intrinsic difference between the way that parasites evolve resistance to host-directed interventions than to drugs [135]. In fact, because of the similarities between pABA and sulfadoxine, this system gives us an opportunity to investigate this idea using experimental evolution. We can also imagine that parasites could resist resource depletion by entering a quiescent state, as they do in response to isoleucine deprivation in culture [251]. It is difficult to say whether selection would favor this phenotype, however. At least in the case of isoleucine deprivation,
parasites can survive after no longer than 24 hours in a quiescent state, so it may not be a phenotype that has much benefit in the face of long-term resource deprivation.

Understanding how parasites use, and interact within, the within-host environment is a considerable technical and theoretical challenge for ecologists. Our work shows that, with the tools of biomedical scientists and evolutionary ecologists before us, we can understand the factors that limit parasite populations within-hosts and use this understanding to develop novel, evolutionarily-robust treatment strategies. Studying the natural history of parasites could thus play an invaluable part in our efforts to resist the rise of antimicrobial drug resistance.
Appendix A  

Chapter 2 Supplemental Information

A.1 Notes on Results

A Cook’s Distance analysis revealed that the outcome of the analysis of the impact of competition on the size of $AS_{pyr}$ infections was sensitive to the exclusion of a mouse (identified with a diamond in figure A.2). This mouse’s $AS_{pyr}$ infection was approximately a quarter of the size of the others in the same treatment, presumably because the parasites peaked at a lower density and the infection was shorter in length (Fig. A.2). When this mouse was included in the analysis of total $AS_{pyr}$ density, the interaction between pABA and competition was insignificant ($total parasite density, days 3-8$ pABA*competition $\chi^2_3= 6, p= 0.11$; $total parasite density, days 3-21$ pABA*competition $\chi^2_2= 4, p= 0.13$) and competition was not deemed to have a significant impact on the size of infections in the low pABA treatment (Figure A.3). Nevertheless, that competition was significant in some pABA treatments but not in others, is consistent with the conclusion that the intensity of competition changes with pABA treatment. Moreover, this mouse was not excluded from the analysis of the impact of pABA treatment on the dynamics of competition, which was deemed to be highly significant.
A.2 Supporting Figures

Figure A.1: Dynamics of single infections in individual mice. Dynamics of AS$_{pyr}$ parasites in mice given unsupplemented water (blue lines) or low (green lines), medium (pink lines) and high (orange lines) concentrations of pABA, as drinking water. Stars represent the number of, and the time at which, parasites were administered. Dots represent the density of parasites detected on a particular day, in instances where parasites were not detected the day before or after. An open circle indicates that the mouse was inoculated with less parasites than was intended and was excluded from all analyses.
Figure A.2: **Dynamics of mixed infections in individual mice.** Dynamics of $A_{\text{pyr}}$ (solid lines) and $A_{\text{J}}$ (dashed lines) in mixed infections of mice given unsupplemented water (blue lines) or low (green lines), medium (pink lines) and high (orange lines) concentrations of pABA, as drinking water. Stars represent the number of, and the time at which, parasites were administered. Dots and squares represent the density of $A_{\text{pyr}}$ and $A_{\text{J}}$ parasites, respectively, detected on a particular day, in instances where parasites were not detected the day before or after. An open circle indicates that the mouse was inoculated with less parasites than was intended and was excluded from all analyses. A cross indicates that the mice died during the experiment. A diamond indicates that mouse was identified as an outlier and was excluded from some analyses.
Figure A.3: The impact of pABA on competition between AS\textsubscript{pyr} & AJ. Total density of AS\textsubscript{pyr} (A,C) in single (filled circles) and mixed (open circles) infections and the intensity of competitive suppression experienced by AS\textsubscript{pyr} (B, D) during days 3-8 (A,B) and days 3-21 (C,D) of infection. Model estimates of the mean and 95\% confidence intervals are shown. In A and C, the significance of the difference in the total density of AS\textsubscript{pyr} in single vs. mixed infections is indicated by the symbols, as follows - ns (not significant), one star (p<0.05), two stars (p<0.01), three stars (p<0.001). In B and D, treatments that were not significantly different from each other share the same lowercase letter annotation. n specifies the number of mice plotted and included in the analysis. These results are from an analysis where the outlier was included.
Appendix B

Chapter 3 Supplemental Information

B.1 Supporting Figures

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Figure B.1: Pyrimethamine resistant parasites, but not susceptible or artesunate resistant parasites, carry the diagnostic mutation of pyrimethamine resistance in the dihydrofolate reductase (DHFR) gene. DNA (white background) and protein (pink background) sequence alignment of the DHFR gene of the pyrimethamine-susceptible *P. chabaudi* reference strain (Accession Number: L28120.1 [172]) and the strains used in Experiment 1, Block 1 (AJ22 & AS123), Experiment 1, Block 2 (AJ35 & AS123) and Experiment 2 (AJ34 & AS117). AS123 & AS117 are resistant to pyrimethamine and artesunate, respectively. Dots indicate where the nucleotide sequences of experimental strains match the reference. The substitution of guanine for adenine (highlighted in red) confers resistance to pyrimethamine [171,172].
Figure B.2: **Dynamics of single infections of individual mice in Block 1 of Experiment 1.** Dynamics of susceptible (black, solid line) and pyrimethamine resistant (red, dashed lines) parasites in single infections of mice in pABA abundant (grey background) and pABA depleted (white background) treatments. Stars represent the number of parasites inoculated and the time at which they were administered. Dots represent the density of parasites detected on a particular day, in instances where parasites were not detected the day before or after. Purple bar indicates duration and timing of pyrimethamine treatment. Crosses indicate mice that died during the experiment, these were excluded from the analysis of total and peak parasite density. Square indicates that the mouse was removed from the analysis of growth rate.
Figure B.3: **Dynamics of mixed infections of individual mice in Block 1 of Experiment 1.** Dynamics of susceptible (black, solid line) and pyrimethamine resistant (red, dashed lines) parasites in mixed infections of mice in pABA abundant (grey background) and pABA depleted (white background) treatments. Each subplot shows the infection dynamics of an individual mouse. Stars represent the number of parasites inoculated and the time at which they were administered. Dots represent the density of parasites detected on a particular day, in instances where parasites were not detected the day before or after. Purple bar indicates duration and timing of pyrimethamine treatment. Crosses indicate mice that died during the experiment, these were excluded from the analysis of total and peak parasite density. Triangles indicate that mice were accidentally given pyrimethamine treatment, these were excluded from all analyses.
Figure B.4: **Dynamics of infections of individual mice in Block 2 of Experiment 1.** Dynamics of susceptible (black, solid line) and pyrimethamine resistant (red, dashed lines) parasites in single (top row) and mixed (middle, bottom rows) infections of mice in pABA abundant (grey background) and pABA depleted (white background) treatments. Each subplot shows the infection dynamics of an individual mouse. Stars represent the number of parasites inoculated and the time at which they were administered. Dots represent the density of parasites detected on a particular day, in instances where parasites were not detected the day before or after. Purple bar indicates duration and timing of pyrimethamine treatment. Open circle indicates that the mouse was inoculated with less susceptible parasites than was intended and was excluded from all analyses.
Figure B.5: **Dynamics of infections of individual mice in Experiment 2.** Dynamics of susceptible (black, solid line) and artesunate resistant (red, dashed lines) parasites in single (top four rows) and mixed (bottom two rows) infections of mice in pABA abundant (grey background) and pABA depleted (white background) treatments. Each subplot shows the infection dynamics of an individual mouse. Stars represent the number of parasites inoculated and the time at which they were administered. Dots represent the density of parasites detected on a particular day, in instances where parasites were not detected the day before or after. Green bar indicates duration and timing of artesunate treatment. Squares indicate mice that were removed from the analysis of growth rate. Open circle indicates that the mouse was inoculated with less susceptible parasites than was intended and was excluded from all analyses.
Resistant parasites are competitively suppressed by susceptible parasites in untreated infections. Parasite dynamics of individual mice infected with only resistant (red, dashed lines) parasites (A,C) and both susceptible (black, solid lines) and resistant parasites (B,D) in Block 1 of Experiment 1. Stars represent the number of parasites inoculated and the time at which they were administered. Dots indicate the density of parasites detected on a particular day in instances where parasites were not detected the day before or after. n specifies the number of mice plotted and included in the analysis.
Figure B.7: Depletion of para-aminobenzoic acid (pABA) has no significant impact on parasites susceptible to or resistant to artesunate in Experiment 2. Infection dynamics (A,B) and initial replication curves (C,D) of mice infected with artesunate resistant (A,C; dashed lines) and susceptible parasites (B,D; solid lines) in pABA abundant (orange) and pABA depleted treatments (turquoise). Initial replication curves were calculated by linear regression using the first three consecutive positive parasite counts. In subplot C, replication curves of resistant parasites have been overlaid, for ease of interpretation. In A & B, stars represent the number of parasites inoculated and the time at which they were administered. Dots represent the density of parasites detected on a particular day, in instances where parasites were not detected the day before or after. Green bar indicates duration and timing of artesunate treatment. In all subplots, n specifies the number of mice plotted and included in the analysis of total and peak parasite density (A,B) and replication rate (C,D).
Figure B.8: Depletion of para-aminobenzoic (pABA) appears to potentiate the activity of pyrimethamine against resistant but not susceptible parasites. Infection dynamics of mice infected with (A) pyrimethamine resistant (dashed lines) and (B) susceptible parasites (solid lines) in pABA abundant (orange) and pABA depleted treatments (turquoise). Stars represent the number of parasites inoculated and the time at which they were administered. Dots indicate the density of parasites detected on a particular day in instances where parasites were not detected the day before or after. Purple bar indicates duration and timing of pyrimethamine treatment. n specifies the number of mice plotted.
Appendix C

Chapter 4 Supplemental Information

C.1 Supplementary Figures

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Figure C.1: Resistant parasites carry the diagnostic mutation of pyrimethamine resistance in the dihydrofolate reductase (DHFR) gene. DNA (white background) and protein (pink background) sequence alignment of the DHFR gene of the pyrimethamine-susceptible *P. chabaudi* reference strain (Accession Number: L28120.1 [172]) and the six strains used here. Dots indicate where the nucleotide sequences of experimental strains match the reference. The substitution of guanine for adenine (highlighted in red) causes the substitution of serine for asparagine at amino acid position 106 and confers resistance to pyrimethamine [171,172].
Figure C.2: Dynamics of mixed infections in individual mice. Dynamics of susceptible (black, solid line) and resistant (red, dashed lines) parasites in pABA abundant (shaded background) and pABA depleted (white background) treatments. Each subplot shows the infection dynamics of an individual mouse. Stars represent the number of, and the time at which, parasites were administered. Dots represent the density of parasites detected on a particular day, in instances where parasites were not detected the day before or after. Purple bar indicates duration and timing of pyrimethamine treatment. Cross indicates that the mouse died and so was omitted from the analysis of total density. Open circle indicates that the mouse was inoculated with less susceptible parasites than was intended and was excluded from all analyses.
Appendix D

Chapter 5 Supplemental Information

D.1 Supporting Figures
Figure D.1: Experimental data & model estimates of the dynamics of individual AS8p infections. Each vertical pair of subplots show the observed (black circles) and estimated (thin lines) dynamics of parasite density (P, upper subplots) and red blood cell density (E, lower subplots) in an individual mouse infected with AS8p. Thick lines in upper subplots show the model’s estimates of the killing rate ($\delta$). In the top left corner of each upper subplot, 0 indicates that there the shoulder was absent, 1 that the shoulder was present but atypical & 2 that a typical shoulder was present, as determined by eye from the dynamics of the data. Grey vertical lines on upper subplots indicate the day on which the peak in parasite density was observed. Labels to the right of each row of plots indicate the size of the parasite inoculum and the concentration of pABA administered to each mouse.
Figure D.2: **Experimental data & model estimates of the dynamics of individual AS123p infections.** Each vertical pair of subplots show the observed (black circles) and estimated (thin lines) dynamics of parasite density (P, upper subplots) and red blood cell density (E, lower subplots) in an individual mouse infected with AS123p. Thick lines in upper subplots show the model’s estimates of the killing rate ($\delta$). In the top left corner of each upper subplot, 0 indicates that there the shoulder was absent, 1 that the shoulder was present but atypical & 2 that a typical shoulder was present, as determined by eye from the dynamics of the data. A cross in the upper right corner of the upper subplot indicates that the likelihood of the model was not maximized and the infections was removed from the analyses. Labels to the right of each row of plots indicate the size of the parasite inoculum and the concentration of pABA administered to each mouse.
Figure D.3: **Experimental data & model estimates of the dynamics of individual AS44p infections.** Each vertical pair of subplots show the observed (black circles) and estimated (thin lines) dynamics of parasite density (P, upper subplots) and red blood cell density (E, lower subplots) in an individual mouse infected with AS44p. Thick lines in upper subplots show the model’s estimates of the killing rate ($\delta$). In the top left corner of each upper subplot, 0 indicates that there the shoulder was absent, 1 that the shoulder was present but atypical & 2 that a typical shoulder was present, as determined by eye from the dynamics of the data. A triangle in the upper right corner of the upper subplot indicates that the mouse did not receive a smaller inoculum of parasites than was intended and was removed from the analyses. Labels to the right of each row of plots indicate the size of the parasite inoculum and the concentration of pABA administered to each mouse.
Figure D.4: **Experimental data & model estimates of the dynamics of individual AJ22p infections.** Each vertical pair of subplots show the observed (black circles) and estimated (thin lines) dynamics of parasite density (P, upper subplots) and red blood cell density (E, lower subplots) in an individual mouse infected with AJ22p. Thick lines in upper subplots show the model’s estimates of the killing rate ($\delta$). In the top left corner of each upper subplot, 0 indicates that there the shoulder was absent, 1 that the shoulder was present but atypical & 2 that a typical shoulder was present, as determined by eye from the dynamics of the data. Labels to the right of each row of plots indicate the size of the parasite inoculum and the concentration of pABA administered to each mouse.


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Education
The Pennsylvania State University University Park, Pennsylvania 2011-2016
Ph.D. in Biology, May 2016
University of Cambridge Cambridge, United Kingdom 2007-2010
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Research Experience
Doctoral Research The Pennsylvania State University 2011-2015
Dissertation Advisor: Prof. Andrew F. Read
Investigated the impact of within-host resource availability on competition between malaria parasites and the evolution of drug resistance.
Research Associate EcoHealth Alliance 2010-2011
Research Advisors: Dr. Peter Daszak & Dr. Tiffany L. Bogich
Investigated the processes that drive the emergence of infectious diseases.
Undergraduate Research University of Cambridge 2009-2010
Research Advisors: Prof. Leslie A. Knapp
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Teaching Experience
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Infectious Disease Evolution Across Scales Research Exchange Grant 2014
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Awarded by The Pennsylvania State University
University Graduate Fellowship 2011-2012
Awarded by The Pennsylvania State University

Publications