# Evolutionary ecology of parasites: life-history traits, phenotypic plasticity, and reproductive strategies

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# Abstract

Adaptive phenotypic plasticity, the ability of a genotype to give rise to different phenotypes in different environments, evolves to allow organisms to fine-tune their life-history traits according to the varying conditions they encounter during their lives. Reproductive investment - the manner in which organisms divide their resources between survival and reproduction - is well studied in evolutionary ecology because it is a key determinant of fitness. However, whilst plasticity in reproductive effort is well understood for free-living multicellular taxa (such as insects, birds, and mammals), the application of evolutionary theory for plasticity and life history strategies to unicellular parasites and pathogens is lacking. In this thesis, I use empirical and theoretical approaches to uncover how differential resource allocation to non-replicating, sexual stages (gametocytes) versus asexually replicating stages can be harnessed by the rodent malaria parasite *Plasmodium chabaudi* to maximise its fitness across the often very variable conditions it encounters during infections.Differential allocation between those stages is equivalent to the fundamental life-history trade-off between survival and reproduction because gametocytes are responsible for between-host transmission (i.e. reproduction of the infection) whereas asexual parasites mediate host exploitation and within-host survival. A suite of within-host models reveal that malaria parasites could gain considerable fitness benefits in the face of low levels of drug treatment if they reduce their investment into gametocyte production ("reproductive restraint"), thereby assuring the continuity of the infection and capitalising on opportunities for future transmission. In contrast, high levels of drug treatment typically select parasites to commit all of their resources to gametocyte production ("terminal investment"), to escape a host that does

not offer much opportunity for future transmission. My experiments reveal that *P. chabaudi* increases both its reproductive investment and its asexual replication rate in anaemic hosts (i.e. host that have a low density of red blood cells), suggesting that parasites profit from host anaemia and can afford high investment in gametocytes ("affluent investment"). I also uncover plasticity in a number of traits that underpin asexual replication rate, including invasion preference for different ages of red blood cells, but it is plasticity in the number of progeny (merozoites) per infected cell that is the main contributor to asexual replication rate. My experiments also reveal genetic variance in plasticity of the life-history traits investigated, which has profound implications for their evolution. Furthermore, plastic modification of these traits is associated with minimal costs or constraints, so that parasites can rapidly match life-history traits appropriately to the within-host environment. Severe anaemia is one of the deadliest symptoms of malaria, so observing that virulence and infectiousness increases in anaemic hosts has also fundamental clinical implications. Finally, the empirical and theoretical observations of affluent investment, reproductive restraint and terminal investment match theoretical predictions of how organisms should behave in varying environments, confirming *P. chabaudi* as a useful model system to test life-history theory.

# Lay Summary

Parasites are no different than other organisms in that they can change their behaviour when the environment around them changes. Just like animals or plants that are negatively affected by predators or a lack of food or other resources, parasites can encounter a range of stresses in their daily environment inside the host such as immune responses, drug treatment, or a lack of resources. In this thesis, I show that malaria parasites that infect mice hosts (our model system) can deal with such stresses by changing the balance between those parasites that become transmission stages and those that replicate and perpetuate the infection (replicating stages) in the host. Transmission stages, in contrast, cannot replicate and their sole role is to be taken up by the mosquito, which will eventually transmit parasites to a new host. My mathematical models show that parasites should produce less transmission stages and more replicating stages when they are under attack from drugs in drug-treated mice, unless the effect of drugs is so strong that all, or almost all, of the parasites are killed. In that case, parasites should jump ship and convert all replicating stages into transmission stages to get into a new host where conditions are likely to be better. My experiments show that malaria parasites replicate better and also produce more transmission stages in mice that are anaemic. Anaemic mice therefore appear to be an environment of superior quality for parasites (though why it is superior remains to be investigated). I also show that different genetic strains of parasites react differently to anaemic conditions, which has important implications for the evolution of parasite traits involved in replication in the host and transmission to mosquitoes. Finally, my results suggest that parasites can survive drugs even in absence of genetic resistance mutations and that anaemic human malaria patients

may provide better conditions for parasites, thus, drug treatment and anaemia could have previously unknown consequences for the severity and transmission of disease.

# Declaration

I declare that the present thesis has been written by me and is the result of my 4-year PhD project, conducted in the group of Prof Sarah Reece (Institute of Evolutionary Biology and Institute of Immunity and Infection Research, Ashworth Laboratories, University of Edinburgh). This work has not been submitted for any other degree or professional qualification. The following chapters received significant input from other people:

## Chapter 2

This chapter has been pre-published online in *Evolutionary Applications* (published version in appendix), and is the fruit of collaboration with Prof Nicole Mideo and Dr Megan Greischar from the University of Toronto. The original modelling framework was designed by Dr Megan Greischar, I modified the model to include killing by drugs, ran all optimisations, created the figures and wrote the first draft of the paper. Prof Nicole Mideo supervised the modelling, intensively contributed ideas, and especially helped with the epidemiological model at the end of Chapter 2. Her advice was also very helpful in Chapter 3.

## Chapter 4

Aidan O'Donnell helped with the experimental infections, Charlotte Repton helped with the data collection and Dr Petra Schneider guided me through the molecular work. Chapter 4 has been published as a paper in *Proceedings of the Royal Society of London B: Biological Sciences* (published version in appendix).

# Chapter 5

Lewis Steer helped with the data collection, and presented some aspects of this chapter in his honours thesis at the University of Edinburgh.

I have also included copies of three thematically related papers published during my PhD, but which are the result of work prior to my time in the Reece Lab, from my master and bachelor degrees. All papers included in this thesis are open-access. All experimental procedures described in this thesis were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

# Acknowledgements

First and foremost, I would like to thank my supervisor Sarah Reece for her brilliant, intense and rigorous supervision work. She knew how to push me into a new parameter space of work, so that what I actually achieved in my PhD is several magnitudes higher to what I thought I could achieve. I am also very indebted to the team in Toronto, Nicole Mideo and Megan Greischar, who, with great optimism, guided me through the jungle of modelling and were brilliant coauthors. None of the experimental work would have been a success without the know-how and help of Aidan O'Donnell, Petra Schneider, Ronnie Mooney, Charlotte Repton and Lewis Steer, so I am greatly indebted to them too. I also want to thank Nick Savill for guidance in my experimental work, for helping me with all computing-related issues and for letting me use his yet unpublished model to derive cycle durations. I want to particularly thank Petra Schneider for very patiently explaining parasite life-history and I am grateful for enlightening scientific conversations with all people mentioned above as well as with Kimberley Prior, Nick Colegrave, Laura Pollitt, Lucy Carter and Sam Rund. Finally, every one mentioned here, and especially my office mates Kimberley Prior, Ronnie Mooney and Aidan O'Donnell, managed to create the most positive working environment possible, making my four years spent in the Reece Lab an absolute delight. The final version of my thesis was significantly improved by the insightful comments and suggestions of my examiners Dr Luke McNally and Prof Mark Viney, so I am very thankful to them too.

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# Contents

# Chapter 1

# Introduction

## 1.1 General introduction

The environment shapes the selective survival and reproduction of organisms. These processes underpin fitness and thus evolution by natural selection. Organisms can evolve strategies to maximise fitness in the face of environmental change by selection on genes that produce the best strategies for the average, or most frequently encountered, environmental conditions (often termed "microevolution"). Organisms can also evolve plastic strategies ("phenotypic plasticity") in which phenotypes change as a function of the environment (Scheiner 1993). Phenotypic plasticity is adaptive when a phenotype (defined as a trait, behaviour, or strategy) is altered in a manner that maintains or increases fitness in the new environment. Microevolution and plasticity are not mutually exclusive but are two processes that operate in concert (Stearns 1992). Differential gene expression and epigenetic effects (e.g. in the context of maternal effects) underpin phenotypic plasticity and can delay microevolution in the face of environmental change because a population can reach the new optimal trait value through plasticity alone. Plasticity can also speed up microevolution, for example if environmental change exposes genetic variation in plasticity between individuals, leading to more variable trait values in a population (Fig.1.1), on which selection can act (Pigliucci 2001) or by allowing populations to subsist in the face of environmental change, thus preserving a

larger gene pool for opportunities of beneficial mutations (West-Eberhard 1989), which may eventually lead to fixation of a plastic trait (Waddington 1953).
Since parasites experience rapid and extensive variation in the environmental conditions in their lifecycle, between individuals hosts and within infections, they could reap significant fitness benefits from phenotypic plasticity. For example, filarial nematodes accelerate their development and proceed faster towards egg production when they are confronted with eosinophilia, an immune response that is likely to clear parasitic worms from the host (Babayan et al. 2010). A number of parasites have also been reported to react to indicators of future transmission. For example, the bird malaria parasite *Plasmodium relictum* increases its in-host replication and gametocyte conversion in response to mosquito probing, supposedly to profit from future transmission opportunities (Cornet et al. 2014). Similarly, the trematode *Coitocaecum parvum* changes between a typical 3-host to a 2-host lifecycle depending on the availability of appropriate intermediate hosts in the environment (Lagrue and Poulin 2007). Such strategic plasticity has however been overlooked because the focus in biomedical research has been on identifying the function of genes involved in infection. Also, this field suffers from the inherent assumption that host homeostasis is effective and so, parasites experience less environmental variation than free- living organisms (Sukhdeo and Sukhdeo 1994; Thomas et al. 2002). The medical relevance of phenotypic plasticity in parasites deserves increasing recognition (Mideo and Reece 2012; Stearns and Koella 2008) for a number of reasons:

1) Whereas evolution against anti-pathogen interventions (such as drugs or vaccines) is widely seen as a major obstacle to the long-term success of controlling disease (e.g., drug resistance mutations), plasticity of traits is also a serious danger since it allows pests, parasites or pathogens to respond on a much shorter timescale (Mideo and Reece 2012). Mosquitoes for example, appear to be capable of shifting their feeding strategies (e.g. biting earlier) to avoid encountering insecticide-treated bed nets when searching for hosts to bite (Birget and Koella 2015; Gatton et al. 2013). It also appears that in response to the front-line drug

artemisinin, the human malaria parasite, *Plasmodium falciparum* stalls asexual development to enter into a dormant stage while drugs are circulating, only to resurge in their absence (Hott et al. 2015; Teuscher et al. 2010). Furthermore, any plastic trait deployed against an intervention may subsequently facilitate the genetic fixation of the plastic trait (evolution of stabilising mutations that lead to constitutive expression of a plastically generated trait, Waddington (1953)), or facilitate the evolution of other resistance traits by increasing the size of the surviving population in which beneficial mutations can occur.

2) Even in absence of interventions, plasticity may allow parasites to adaptively respond to variable within-host environments. For example, viruses with a latent stage like varicella Zoster or herpes simplex often relapse when the host's immunity is depressed (Kinchington and Abendroth 2011), which may be an adaptive strategy to take advantage of a more favourable environment (Gandon 2016). Similarly, malaria parasites with latent stages have been shown to relapse in response to cues for transmission opportunities (Cornet et al. 2014; Scott and Otto 2014; White et al. 2011). Targeting the cues that parasites use for such plastic behaviour may deliver powerful new intervention methods: parasites could be "tricked" into making a suboptimal life-history decisions, reducing their fitness to a point-of no-return. For example, malaria parasites in vertebrate hosts could be tricked into committing all their asexual cells, which are responsible for virulence, towards sexual cells, which are non-virulent but responsible for transmission. If this is done in a controlled setting (i.e. where no transmission occurs), or in conjunction with drugs targeting sexual stages, this could be a straightforward way of ending infections.

While insights into phenotypic plasticity are increasing for a variety of parasites and pathogens, most progress is being made from model systems which are easy to manipulate and whose behaviour is well understood. Murine malaria as a model system has a long research history since its isolation from thicket rats in what is now the DR Congo (Killick-Kendrick 1978). The biology of these parasites is very similar to those of the human malarias *Plasmodium falciparum* and *P. vivax*, so observations from the murine system are suggestive of what

happens in human disease (Craig et al. 2012). The availability of multiple genotypes, the relative ease of manipulation of the within-host environment in mice, and the existence of multiple parasite life-history traits that are phenotypically plastic, makes murine malaria an ideal system to investigate the evolutionary ecology of gene by environments effects and how they shape parasite strategies. This thesis will focus on phenotypic plasticity of life-history traits of the murine malaria parasite *P. chabaudi*. In this chapter, I will first introduce the concept of phenotypic plasticity, its theoretical background and what is known about regulation of plasticity, including evolutionary costs. I will discuss how plasticity integrates with life-history evolution and focus on the theoretical background of the survival-reproduction trade-off. I will describe how that trade-off is relevant to my study system, and lay out the structure of the following chapters.

## 1.2 Phenotypic plasticity

### 1.2.1 Historical context

The environment integrates all selection pressures that drive genetic evolution but also affects the expression of phenotypes summarised under the concept of phenotypic plasticity (Scheiner 1993). Charles Darwin (1859) recognised this dual influence of the environment, which he calls "the conditions of life", though he chose to accord less weight to phenotypic plasticity in "On the origin of Species", probably so as not to divert his readers from the main message of his book, genetic evolution by natural selection: "*When a variation is of the slightest use of a being, we cannot tell how much of it to attribute to the accumulative action of natural selection, and how much to the conditions of life*" (p.102). Furthermore, he adds "*To judge how much, in the case of any variation, we should attribute to the direct action of moisture, light food, etc., is most difficult: my impression is such, that with animals such agencies have produced very little direct effects, though apparently more in the case of plants*" (Darwin 1859, p.11). Indeed, the accurate observation that plants, due to their immobility, heavily

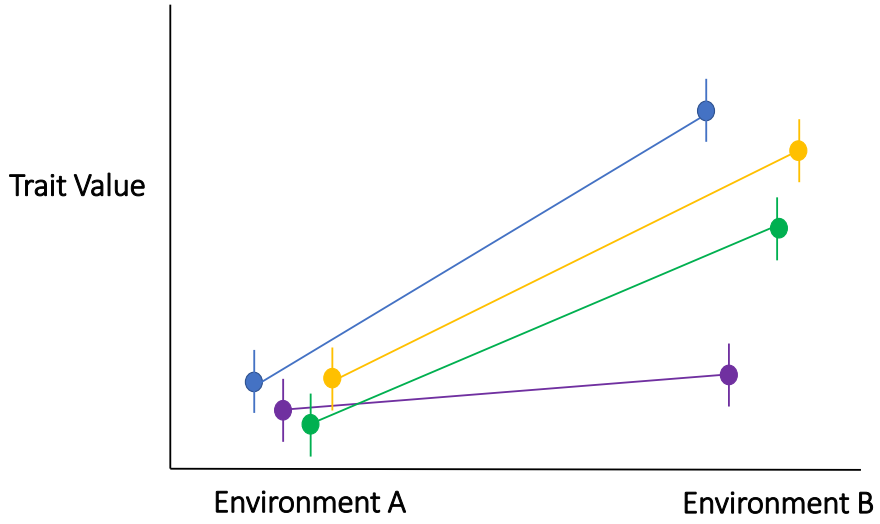


Figure 1.1. Plasticity can uncover genetic variation in a trait, thus allowing natural selection to operate and the trait to evolve. Here, the trait values of four different genotypes (different colours) are shown in two different environments. In environment A, there is little variation between the trait values of these genotypes (error bars overlap, points horizontally dodged for better visibility). Through genotype-specific plastic modification these trait values change in environment B, unravelling genetic variation (non-overlapping error bars). Since genotypes display only minor differences between trait values in environment A, evolution would be dominated by genetic drift. In contrary, genetic variation is exposed in environment B and evolution would be dominated here by natural selection (e.g. selection favours the blue over the purple genotype).

rely on phenotypic plasticity is responsible for the fact that they are the most popular organisms for the study of plasticity (Sultan 2002). Darwin was also the first to suggest that plasticity can affect genetic evolution through the fixation of plastic traits, as later demonstrated by Waddington's (1953) proof-of-principle experiments: "*Indirectly, as already remarked, they [the conditions of life] seem to play an important part in affecting the reproductive system, and in thus inducing variability; and natural selection will then accumulate all profitable variations, however ever slight, until they become plainly developed and appreciable by us*" (Darwin 1859, p.103). Darwin's initial focus on phenotypic diversity through genetic variation rather than plasticity, together with the belief that environmentally induced effects should not be heritable, may have contributed to the fact that environmental influence on phenotypic characters has until recently been considered "environmental noise" that may interfere with the measurement of the "real", heritable characters of an organism (Sultan 2002). Furthermore, there was pressure on Darwin to clearly separate his theory of evolution from that of his main contender, Jean-Baptiste Lamark, whose original theory includes a number of concepts compatible with the ideas of the theory behind phenotypic plasticity (Haig 2007). These views have changed in the last few decades with phenotypic plasticity and epigenetic effects playing an essential role, in a new, unified synthesis of evolution (Danchin et al. 2011). This new approach more explicitly recognizes that natural selection acts on phenotypic variation, underpinned by both, genetic and environmental components (i.e. through direct environmental influence and phenotypic plasticity), and that not just genes are inherited, but also the social environment, the parental environment and potential epigenetic imprinting of genes. Indeed, nowadays, one of the fundamental biological concepts in the wider public perception of evolution is that traits are influenced by both, "nature" (i.e genetics) and "nurture" (i.e environmental effects).

### 1.2.2 Theoretical Concepts

Phenotypic plasticity occurs when the expression of a given trait, coded for by a genotype, changes as the environment changes. If a trait is phenotypically plastic, it means that across a range of a given environmental variable, that trait can adopt different values. A trait may be sensitive to one environmental variable but not another one, and some genotypes may modify their traits in response to changes to that environmental variable, but others may not. Phenotypic plasticity is therefore not the property of a genotype, but the property of a *trait* (Scheiner 1993), in relation to an environmental variable to which it is responsive. Similarly, the trait may only be plastic in response to a certain range of that variable. Whereas phenotypic change through genetic evolution, e.g., through directional selection, will take, by necessity, many generations, phenotypic change through plasticity can be reached in one generation (e.g., with parental effects) or at even shorter timescales (e.g., during a lifetime via behavioural plasticity). If such phenotypic plasticity allows the organism to reach a trait value closer to the optimal for the new environment (i.e., if such phenotypic plasticity is adaptive), then it can give a significant boost to an individual's fitness and can thus be considered a powerful mechanism in evolution.

If we consider body height as an example, we can deconstruct that trait into various effects that may influence it. There may be a genetic effect that assures that an individual will be on average taller if it stems from a tall family. In contrast, there may also be an environmental effect: if the individual does not have access to sufficient nutrients, it will not be able to develop to its full genetically determined size. Finally, individuals with different genetic dispositions may react differently to nutrient availability (the gene by environment effect), if for example males increase their body height proportionally more in response to additional nutrients than females (Drummond et al. 1991). In a simple model, variation of any phenotypic trait can thus be described by the following equation:

$$\sigma(z) = \sigma(g) + \sigma(e) + \sigma(g \times e) + r.e.$$

where $\sigma$(z) is variance of the trait z, $\sigma$(g) is the additive genetic variance, $\sigma$(e) is the variance introduced by the environment (the environmental variance), (g x e) is the variance explained by the interaction between the genotype and the environment, also simply called the genotype by environment effect and *r.e.* describes random effects during trait development (developmental noise).

The effect of phenotypic plasticity is captured by the environmental variance, $\sigma(e)$, and by the genotype by environment effect $\sigma$(g x e) (from now on called G x E). The environmental variance $\sigma$(e) influences trait values of all genotypes equally and can be seen as a direct "extension" of the environment into bodily functions, that are not under control by the organism (Grether 2005). Typical examples include traits directly connected to metabolism and development e.g. the size of the adipose tissue or the muscle mass of mammals (and by extension, the weight of the animal), the growth rate of tadpoles (Berven 1982), or carotenoid deposition in the feathers of flamingos (Yim et al. 2015). Although environmental plasticity in such traits may fulfil important functions, plasticity fuelled by environmental effects alone, is not adaptive and cannot evolve, due to the absence of genetic variation. In contrast, there may be genetic variation in the exact form of the G x E effect, described by the *reaction norm*, which is the set of phenotypes produced by a single genotype across a range of an environmental variable (Stearns 1992). The reaction norm is thus the property of a *genotype* (or clone, Gotthard and Nylin 1995) or an *individual* (Nussey et al. 2007). If the trait is a continuously distributed trait, it can be described by a curve with the environmental variable plotted on the x-axis and the corresponding phenotypic value along the y-axis. That curve can take different shapes from constant (in the absence of plasticity) to linear, monotonic, quadratic and so forth (Ghalambor et al. 2007; Stearns 1992). Even non-continuously varying traits can be described by the same framework if one considers a sudden phenotypic switch in response to a threshold of a continuous environmental variable with the corresponding curve being logistic (e.g. the production of protective spines in response to predator cues in *Daphnia pulex*, Parejko and Dodson 1991). If such G x E effects lead to higher fitness of that

genotype in the new environment, it can be considered adaptive plasticity. Since different genotypes can have different reactions norms, the reaction norm itself can be considered a trait that is subject to evolution in a population.

## 1.3 Measures and evolutionary context of phenotypic plasticity

### 1.3.1 Measures of phenotypic plasticity: Reaction norms

The presence of G x E interactions imply that different genotypes have different reaction norms. If we concentrate on linear reaction norms, as we will for the remainder of this thesis, this means that the lines have different slopes, i.e., that they are not parallel. If the reaction norms cross, this is evidence of an especially strong G x E effect (Stearns 1992). Crossing reaction norms have a strong influence on positive selection for a given trait because they change the phenotype-fitness ranking of genotypes (Fig.1.2). Thus, if for example there is directional selection for an increased phenotypic value, the focal genotype may be selected in one environment but not in the other. Plasticity of a trait can be quantified by the slope of the reaction norm (Fig.1.2). In its simplest form, when there are two environments and one trait, the slope of a linear reaction norm serves as a measure of the degree of plasticity (How much does a trait change between environments?) and the pattern of plasticity (Does the trait increase or decrease between the two environments?) (Nussey et al. 2007; Pigliucci et al. 2006). A steep slope thus indicates a high degree of plasticity (Fig.1.2).

### 1.3.2 Adaptive and non-adaptive plasticity

Plasticity is not necessarily adaptive. Non-adaptive plasticity of traits can occur due to, for example, developmental constraints or trait covariation (association of two traits), keeping in mind that phenotypes may be affected by passive, environmental effects ($\sigma(e)$ in equation above) and active, plastic effects. For example, it is unclear whether there is adaptive significance to the development

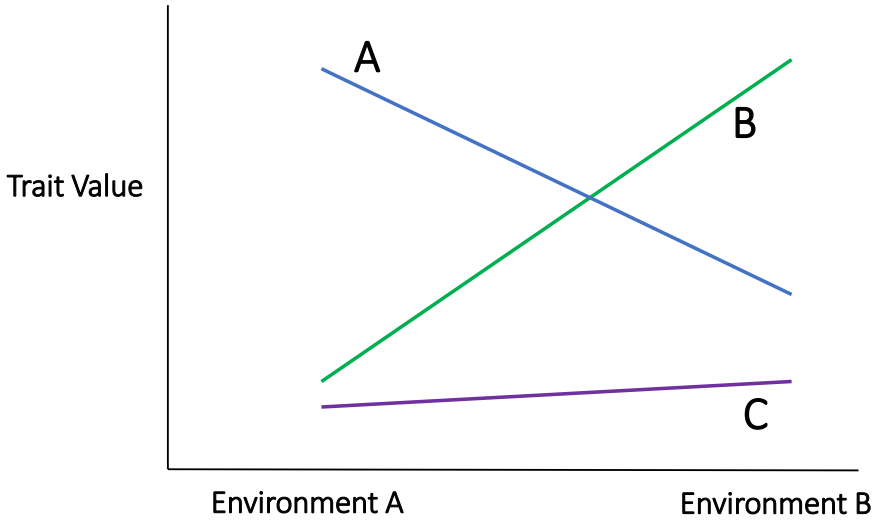


Figure 1.2. Reaction norms of three genotypes (A, B and C), across two environments. Genotype A and B have high plasticity (steep slopes) but modify their trait values in opposing directions, which is evidence of a strong genotype by environment (G x E) effect. Genotype C barely modifies its trait value between environments (shallow slope) and is therefore considered less plastic than genotype A or B.

of cross vein-less wings by *Drosophila* in response to heat-shock as a larva, the most famous example of genetic assimilation of an environmentally induced character (Waddington 1953). Non-adaptive plasticity may also be involved in the production of smaller spore sizes by the protozoan parasite *Ophryocystis elektroscirrha* if its host, the Monarch butterfly, feeds on cardenolide milkweeds, which have toxic effects on the parasite (Hoang et al. 2017).

Adaptive plasticity is defined as plasticity that allows individuals to develop a phenotype that leads to a higher fitness in a new environment than the fitness of non-plastic individuals in that new environment (Scheiner 1993). The ability of an individual to adaptively adjust the phenotype in response to changing conditions is therefore a fundamental mechanism for populations to subsist in the face of environmental change (Nussey et al. 2007). For example, trematode parasites, such as *Coitocaecum parvum*, truncate their 3-host life-cycle into a two-host life-cycle if they do not detect cues from the required intermediate host (Lagrue and Poulin 2007; Poulin 2003). This allows parasites to circumvent a situation of long waiting times for the presence of the appropriate hosts or where transmission could be precluded altogether (Lagrue and Poulin 2009).

Models predict adaptive plasticity can evolve if: i) the environment is highly variable temporally or spatially, and is frequently encountered during the lifetime of an individual ii) each environmental state is reliably associated with cues, iii) different phenotypes are favoured by selection across different environments, iv) the strength of selection is similar across environments , v) there is no phenotype that has superior fitness across all environments (Ghalambor et al. 2007; Lande 1986; Levins 1968; Scheiner 1993; Schlichting and Smith 2002), vi) the cost of plasticity is low. In contrast, when the cues are unreliable and/or the cost of plasticity is high, a single generalist phenotype tends to be favoured. If the frequency of encountering environmental states and the selection there-in are very unequal, this tends to favour phenotypes generated by genetic specialisation rather than adaptive plasticity (Scheiner 1993). Whereas there are a number of ways to test the validity of these predictions, direct evidence has been obtained from experimental evolution of plasticity in microorganisms. For example,

Leggett et al. (2013)) showed that bacteriophages evolved plastic lysis times if they experienced alternation of single and coinfections. The importance of cues, has been demonstrated in *Caenorhabditis elegans* through faster experimental evolution of the reaction norm in a plastic trait (dauer larva formation) in presence of pheromones (the cues, in this case) than in their absence (Diaz and Viney 2015). Finally, if environments are variable but poorly associated with cues, this may select either for a robust genotype or for a genotype to plastically generate diverse phenotypes in the hope that some of those phenotypes is adaptive (bet-hedging strategies, Viney and Reece 2013). For example, some isogenic lines of *C. elegans* have been shown to have higher variance of lifetime fecundity than others, which may be adaptive in unstable and unpredictable environments (Diaz and Viney 2014).

## 1.4 Regulation, limits and costs of phenotypic plasticity

### 1.4.1 Macroevolutionary constraints

The evolution of plasticity is affected by both costs and constraints. Generally, constraints that affect the evolution of any trait, including plasticity, are the presence of genetic variation, allometric connections and genetic linkage to other traits (i.e., where plasticity is not independent of other traits) as well as the influence of past selection or drift (the evolutionary history, Schlichting and Pigliucci 1998). For example, in English plantain (*Plantago lanceolata*), pollen dispersal is more efficient with higher (male) staminate flowers and pollen reception with lower (female) pistillate flowers. However, the benefits of potential plasticity in these traits (e.g. in response to different light environments) is limited by the strong positive genetic correlation between the height of male and female flowers (Young and Schmitt 1995). More generally, any phenotypic plasticity in morphological traits are developmentally constrained to fit into the general "bauplan" of the organism in question (Hall 1992). While

such constraints may be important at a "macroevolutionary" level, it is the "microevolutionary" constraints that have attracted the most interest in the research of plasticity.

### 1.4.2 Microevolutionary costs

Costs of plasticity can be defined as the reduction in fitness of more plastic genotypes compared to less plastic genotypes in the same environment and at a same trait value (Auld et al. 2010; Dewitt et al. 1998). Costs of plasticity can be conceptually divided in two categories (Callahan et al. 2008): i) First, the cost of plasticity *per se*, i.e., by carrying additional machinery for the production of plastic phenotypes e.g. maintenance costs, ii) Second, any costs directly relating the plastically produced phenotype, including production costs (Auld et al. 2010), costs related due to trade-offs with other traits and costs related to the risk of developing a suboptimal phenotype. The costs of maintenance of the machinery necessary for plasticity (e.g. organs or receptors that recognize cues, the first essential step towards a plastic response) are constitutively paid by plastic genotypes. In contrast, costs connected to the production of the plastic phenotype should be environment-dependent. These two types of costs have thus different implications for the evolution of plasticity (reviewed in Auld et al. 2010). A quantitative method to test for various costs of plasticity has been developed by Van Tienderen (1991), based on regression of plastic trait values on fitness components across different environments. Although research on maintenance costs of plasticity is scarce, studies have generally found small maintenance costs compared to the production cost of the plastic phenotype (Auld et al. 2010; Murren et al. 2015; van Kleunen and Fischer 2005). For example, DeWitt (1998) finds little evidence of any growth or fecundity costs of high-plasticity families of the snail *Physa heterostropha* compared to low-plasticity families for a same trait value in a same environment. Similarly, Scheiner and Berrigan (1998) found very little evidence of effects on fitness-related morphological traits in more plastic individuals of *Daphnia pulex*. The same accounts for plants, where maintenance cost have been found to be low and environment-dependent in *Arabidopsis*

*thaliana* (Callahan et al. 2005; Dorn et al. 2000) or only expressed in stressful environments in *Sinapsis arvensis* (Steinger et al. 2003). Costs of plasticity may also be cue-dependent. For example, while checking for the presence of predators in the context of predator-dependent plasticity, acquiring information about the environment carries an inherent risk of being attacked (also termed "information-acquisition" cost, Auld et al. 2010). Furthermore, in the case of clonal colonies of unicellular organisms (such as yeasts or malaria parasites), density-dependent plasticity (as well as a colony-wide production of the plastic phenotype) may require costly information acquisition, e.g. through quorum sensing via pheromones (Honigberg 2011) or the production of extracellular vesicles (Mantel et al. 2016, 2013; Regev-Rudzki et al. 2013).

### 1.4.3 Fixed and labile plastic traits

Constraints of plasticity are defined as all factors that prevent the genotype from producing the optimal phenotype for a given environment (Murren et al. 2015). Constraints of plasticity are strongly connected to the developmental mechanisms involved in the plastic response. One can distinguish two types of traits, those that are developmentally fixed and those that are modifiable through the lifetime of an organism (labile traits) (Scheiner 1993). Developmentally fixed traits are only plastically responsive to the environment that the organism encounters during trait development so that the phenotypic result cannot be altered during the remainder of the organism's lifetime. A typical example are parental effects such as testosterone deposition of female birds into eggs that may affect offspring traits such as body size (Schwabl 1996) or disease susceptibility (Badyaev and Uller 2009). The sensitivity of plastic traits in parasites can also be limited to a specific time window: for example, virulence of the protozoan mosquito parasite *Ascogregarina taiwanensis* depends on the environmental conditions that it experienced in the previous hosts but not in the current host (Tseng 2006).
Whereas developmentally fixed traits may carry important phenotypic costs, this may not occur for labile traits. By definition, labile traits can rapidly respond to changes in the environment and models predict that plasticity in such traits is

almost always favoured (Scheiner 1993). In contrast, developmentally fixed traits will mainly be favoured if the environmental change is predictable and slower than the generation time of the plastic organism in question (Forsman 2015; Scheiner 1993; West-Eberhard 2003). If environmental change is poorly predictable, then organisms may either rely on bet-hedging strategies (production of randomly or alternately varying phenotypes, Diaz and Viney 2014; Viney and Reece 2013) or evolve greater trait robustness (less plasticity in these traits) (Simons 2011).

## 1.5 Plasticity in life-history traits

### 1.5.1 Definition

Whereas many morphological traits tend to be developmentally fixed, behavioural traits and many life-history traits tend to be labile. Life-history traits are organismal traits that are directly linked to the fitness of an individual and thus to population growth. The quantity, quality and sex ratio of offspring, the size at birth and at maturity, investment into growth and reproduction, life span or size at time of death are commonly studied life-history traits, and all these traits are potentially connected among themselves through trade-offs (Roff 1992; Stearns 1992). The simplifying assumption is that these traits summarise the phenotype and thus ultimately, the fitness of an individual. The aim of life-history theory is understanding what determines the fitness differences between individuals or genotypes that adopt different life-history strategies (Stearns 1992).

### 1.5.2 Trade-offs

In life-history evolution, it is assumed that trade-offs can occur at three different levels: microevolutionary, macroevolutionary or physiological levels (Stearns 1992). Microevolutionary trade-offs happen at the level of a population when a trait that is under positive selection in a population is linked to another trait that causes a fitness cost. For example, bright plumage may be under positive sexual selection for bird males in many populations, may come at the cost of

increased visibility and thus increased predation (Heinsohn 2008).

Macroevolutionary trade-offs happen at the level of species, and are therefore evolutionary fixed: they can typically be identified though comparative analysis of trait variation among independent phylogenetic events. A typical example is the phylogenetic association between larger body size and longer development times and therefore older ages at maturity (Brown and Weatherhead 2004). Macroevolutionary associations between life-history traits are also relevant for parasites and pathogens. For instance, in helminths, adult size and fecundity are strongly correlated (Sorci et al. 2003). It has therefore been predicted, since anti-helminth interventions select for worms that reach maturity at smaller sizes, such interventions would select for less fecund worms, and thus reduce the overall transmission of helminthic diseases (Gemmill et al. 1999; Poulin 1998). However, Lynch et al. (2008) state that this prediction makes the strict assumptions that mortality rates are not affected by age at maturity and show that that anti-helminth -interventions could in theory drive life-history evolution for increased worm size and fecundity.

Physiological life-history trade-offs are concerned with the allocation of energy resources between two or more competing functions within a same individual. Given the central importance of life-history traits for organismal fitness, plastic allocation is expected to be widespread among all organisms. For example, *Mimulus* plants allocate more resources to the development of reproductive organs in unfavourable environments, causing them to flower earlier (Galloway 1995). This comes at the expense of a shorter lifespan compared to plants that grow in more favourable environments, that allocate more resources to vegetative growth.

Many studies have shown that physiological trade-offs are especially important when the environment imposes some sort of constraint, e.g., resource limitation or harsh environmental conditions (Albion et al. 1987; Wilson et al. 2009). Favourable environments generally allow for enough energy to be present, so that individuals can allocate sufficient resources to all competing functions- in other words, the trade-off becomes irrelevant. For example, in good

environmental conditions, a bird may lay many, large-sized eggs whereas in harsher environments, some decision would have to be made concerning the energy allocation between number and size of eggs (Lack 1966; Smith 1981). In summary, the same reaction norm concept as described above can be applied to life-history traits, with individuals plastically varying allocation to traits depending on the environment (Stearns and Koella 1986).

## 1.6 The fundamental trade-off between survival and reproduction

### 1.6.1 Age-based theoretical approaches

Selection on many life-history traits can fundamentally change depending on the age of the organisms. However, no life-history trait, subjected to age-dependent variation, has attracted more attention than reproductive effort. Determining how much energy should be to allocated to reproduction is one of the fundamental life-history problems that each organism needs to solve. The problem is how to allocate resources between survival and reproduction (Roff 1992; Stearns 1992). In most organisms, a reproductive event can be associated with significant costs while additional time and energy resources need to be committed to reproduction-related events such as finding a mate, defending a territory, producing eggs or sperm, increased predation risk and parental care. To simplify, organisms need to put at risk their somatic, non-reproductive tissues, for every reproductive event and thus may pay a cost in terms of reduced future reproduction, e.g., due to lower growth or higher risk of death (Pianka and Parker 1975). The amount of energetic resources that organisms invest into reproduction is called reproductive effort or reproductive investment.

This trade-off can be theoretically represented by tracking the "reproductive value" of an individual, which represents the age-specific expectation of all current and future offspring. However, when talking about the costs of current reproductive event, it is generally easier to talk about the "residual reproductive

value" (RRV) which solely focusses on the age-specific expectation of the future offspring. Theory predicts that young individuals, i.e., who have a high RRV, should invest less into any current reproductive event whereas older individuals with low RRV should sacrifice proportionally more of their soma for current reproductive efforts. This is because older individuals are closer to the end of their reproductive life-span, thus selecting for "terminal investment" as the prospects of future reproduction vanes (Williams 1966). In contrast, young individuals should invest less into current reproduction so as to maximise their reproductive life-span and thereby future opportunities of reproduction.

Although the theory is well developed, empirical evidence has delivered mixed messages. This is related to the fact that reproductive effort or reproductive value are notoriously difficult to measure. Reproductive value can also be considered at different scales, e.g. at the level of the reproductive season (for seasonally reproducing organism) or at the level of the whole lifetime of the organism (Clutton-Brock 1984), and these levels may interact. For example, it has been shown that, in seasonally breeding species (e.g. birds in the northern hemisphere), RRV is maximal at the start of each reproductive season but declines as the reproductive season comes to the end (Verhulst and Nilsson 2008). This "cyclical" increase and decrease of the RRV every year is overlaid upon the constraints that senescence puts on RRV (Hernandez et al. 2017; Pianka and Parker 1975). Furthermore, reproductive costs are especially hard to pin down since they are made up of many sub-costs such as mate finding, production of egg or sperm, mating itself, production of offspring, parental care, etc., all of which can trade-off or compensate each other. Finally, another major issue is that the real costs to an individual are hard to measure because individuals vary in condition, quality and environments they experience. The latter explains the positive correlations between reproductive traits that are frequently found in empirical studies even though these traits should trade-off against each other (Lindén and Møller 1989).

### 1.6.2 State-based theoretical approaches

The precise form of the trade-off between present and future reproduction may differ across species and therefore selects for different optimal age-specific reproductive tactics. In addition, the form of the trade-off is influenced by environmental conditions such as resource availability or the presence of predators or pathogens. For example, if conditions for reproduction are particularly favourable at some point (e.g., absence of predators), it would pay off for an individual to concentrate its reproductive effort into that period at the expense of future reproduction where conditions are not certain to be favourable. Similarly, if conditions for reproduction are unfavourable, it pays off an individual to invest less into current reproduction and delay reproduction-related resource expenditure to the future, when conditions are likely to improve ("reproductive restraint", Curio 1988). Finally, terminal investment is not just selected in individuals that face the end of their reproductive lifespan but also by environmental conditions that cause very high adult mortality rates, causing the cost of future reproduction to be near zero (Hirshfield and Tinkle 1975). For example, parasitic nematodes speed up their development and production of transmission stages in response to eosinophilia, a host immune response that is likely to kill adult worms (Babayan et al. 2010).

### 1.6.3 Definition of state

The complications of coherently integrating the effect of environmental and physiological conditions into age-based explanations of reproductive effort has led to the proposition of models that model an organism's reproductive decision based on its "state" (Clark and Mangel 2000; McNamara and Houston 1996, 1999). The state variable summarises the entirety of the internal (physiological) and external (environmental) conditions that an organism experiences and on which it will likely base its reproductive decision. Thus, what exactly constitutes state is highly variable for each organism. In contrast to the age-based approach, which assumes a population-wide optimal reproduction at a given age, the

state-based approach allows for inter-individual variability. For example, a bird with a larger than (population-level) optimal clutch size may still reap fitness benefits if it finds itself at a better state than other individuals in the population (e.g., due to a higher quality territory). Typical internal state variables are energy reserves, muscle mass (e.g. during male-male contests) and health. External state variables summarise all environmental effects on which organisms base their reproductive decision, for example territory quality, seasonality or their social environment (e.g. the number of conspecific competitors or helpers in the immediate vicinity) (McNamara and Houston 2008). Environmental variables and genetics both affect the internal state variables of an organism. Therefore, to simplify, I will use the term "state" only when referring to an organism's internal state, i.e., its physiological condition or quality, and use "environment" to refer to external state variables. One should keep in mind, however, that the physiological state of an organism is highly influenced by the environment (e.g., more resources or less pathogens generally increase the state of an organism).

### 1.6.4 Predictions and implications of state-based life-history theory

State-based life-history theory is a powerful method and has been proposed to be able to encompass all other fundamental life-history trade-offs as laid out by Stearns (1992). McNamara and Houston (1999) predict that all fundamental life-history trade-offs, can be summarised by four functions (assuming an annually reproducing organism): i) the probability that an organism survives until next year, ii) the state of the organism in one year's time, given that it survives until then, iii) the expected number of offspring produced which are alive in one year's time, iv) the distribution of states of the offspring. Life-history theory based on state thus leads to a few simple and intuitive predictions: i) organisms in a better state should be able to invest more into reproduction than individuals in a worse state or that experience a worse environment, ii) the costs

and benefits of a reproductive event will be dependent of an organism's state, with any reproduction-related trade-offs becoming smaller as the organisms finds itself in a better state, iii) organisms take decisions based on their state, thus organisms in a different state may respond differently to a same environmental condition, iv) an organism's state may vary over time due to environmental conditions, trade-offs or senescence. Age-based and state-based life-history, however, are not alternative ways of explaining the same phenomena, but they can effectively complement each other. Marrying the two points of views are an ongoing effort in research of life-history (e.g. Fischer et al. 2009; McNamara et al. 2009) and promises to deliver a powerful new synthesis.

Applying the concept of "age" to unicellular parasites is not straightforward. Time post-infection may be considered with eventual clearance of the infection being the equivalent of death of the individual. However for a parasite to be successful, transmission should occur before clearance, so that a parasite "individual" may simultaneously experience many hosts, especially if parasites have no specialised transmission stages (e.g. yellow-fever virus). Indeed, the very notion of what constitutes an "individual" in the context of unicellular pathogens is also a contentious one. In evolutionary terms, the selective unit, and thus the "individual", is the entirety of an infection that is made up of clonal parasite cells or organisms of a given genotype (Gardner and Grafen 2009). Due to the problem of applying the concepts of "age" and "individual" to parasites, a state-based approach has proven to be a more amenable to the life-history of parasites than an age-based approach (Thomas et al. 2002).

## 1.7 Plasticity in reproductive effort of malaria parasites

Reproductive effort at a given age and reproductive costs of a given reproductive event are notoriously difficult to measure in both, natural and experimental settings, mainly due to differences in state, environmental conditions, experience all of which generally change over an organism's lifetime (Clutton-Brock 1984;

Lindén and Møller 1989). Studying organisms with simpler reproductive lives and in an experimental setting may circumvent the above-mentioned problems. The study of apicomplexan parasites, and in particular, malaria parasites, allows a straightforward assessment of reproductive effort and state since these parasites use different stages for reproduction and survival. In malaria parasites, between host-transmission is equivalent to reproduction whereas within-host clonal replication can be seen as the equivalent of survival (Carter et al. 2013; Mideo and Reece 2012). The life-cycle of all malaria parasites consists of a vertebrate host and an insect (usually mosquito) host. If we consider the vertebrate our focal host, then mosquitoes can be considered the "vectors" responsible for between host-transmission. Once inside a vertebrate host, a parasite will increase its biomass through clonal replication inside red blood cells (see figure 1.3) for a detailed life-cycle). These clonal stages, which are asexual, are not capable of initiating an infection in the mosquito if taken up.

The only stages that can initiate mosquito infections, and therefore ensure between host-transmission, are the gametocyte stages. The latter are sexual, i.e., they can be male or female, and, once inside the mosquito, will give rise to spermatozoids or an egg-cell that mate inside the mosquito mid-gut. The progeny from this mating event will give rise to a new infection if inoculated by a mosquito to a vertebrate (Fig 1.3). It is thus the concentration of gametocytes inside the blood of a vertebrate at any timepoint that determines how infective the vertebrate host is to a mosquito and, up to a point, is directly proportional to the reproductive success of the parasite (Bell et al. 2012). Therefore, the proportion of infected cells that will give rise to gametocytes (also called the conversion rate) is the equivalent to reproductive effort (alternatively also called reproductive investment or reproductive allocation) of malaria parasites. To simplify, once in every cell cycle, a focal infected cell can either commit to producing sexually committed merozoites which will develop during the following asexual cycle, or, alternatively give rise to non-committed merozoites, which will contribute to the maintenance of the infection by asexual replication. If we consider the example of a single parasite genotype infection in a vertebrate host,

a decision needs to be taken in every cell cycle and across the whole within-host parasite population to determine how many infected cells should be committed to gametocytogenesis. Committing a cell towards gametocytogenesis comes with a clear reproductive cost: once committed, the focal cell can no longer contribute to in-host replication of the infection.

### 1.7.1 Applying life-history theory to malaria parasites

Age-based (Pianka and Parker 1975) and state-based life-history concepts (McNamara and Houston 1996, 2008; McNamara et al. 2009), which were original described with multicellular organisms in mind, are readily applicable to explain variable investment of malaria parasites during an infection (Greischar et al. 2016b). Every reproductive event should come at a cost to the "soma" of an organism (Pianka and Parker 1975). The clonal asexual parasites can be seen as the "soma of the parasite", since the entirety of the within-host population of a single parasite genotype is the unit of selection (Gardner and Grafen 2009). Asexual parasites are also related to the residual reproductive value of an infection, since every asexual parasite can give rise to gametocytes in the future, or to more asexuals which, in their turn, can give rise to gametocytes. An expanding asexual population thus also expands the residual reproductive value of the parasite. The end of the reproductive lifespan in multicellular organisms simply corresponds to the end of an infection, which can be due either to host death or immune clearance. A considerable effort has been spent over the last few years to link parasite reproductive effort to the within-host environmental and asexual density-dependent components of state and (Cameron et al. 2012; Gautret et al. 1996; Pollitt et al. 2011b; Reece et al. 2005, 2009, Schneider et al, *in prep*, reviewed in Carter et al. 2013), including which cues parasites may rely on to adjust reproductive effort in an adaptive manner (Carter et al. 2014; Greischar et al. 2014). The state of an infection is directly related to the stresses that parasites experience inside the host (e.g., resource limitation, competition with other parasites, immune killing, drug killing etc.).

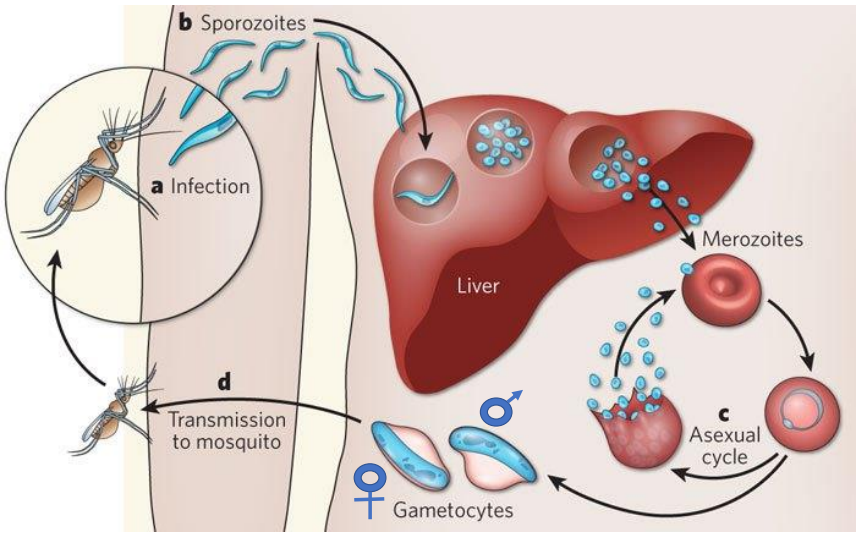


Figure 1.3. Typical life cycle of *Plasmodium* parasites, (a) a mosquito infects the host through the transfer of sporozoites, which then (b) travel into the liver where they give rise to a high number of merozoites, (c) that are released into the blood, where they initiate the asexual cycle, infecting red blood cells (RBCs), going through a number of developmental stages (ring stages, trophozoites, schizonts) until they burst out from the red blood cell, releasing merozoites into the extracellular environment. Merozoites reinvade uninfected red blood cells and initiate a new asexual cycle inside the blood. The number of merozoites produced per infected red blood cells is called the burst size. The asexual replication cycle takes around 24, 48 or 72 hours, depending on the parasites species. It is 24 hours in *P. chabaudi*. (d) During every asexual cycle a certain proportion of infected RBCs (called the conversion rate or reproductive investment) are committed towards sexual development of male or female gametocytes, which are the only stage that can be taken up a mosquito to assure transmission. Once inside the mosquito, gametocytes give rise to male and female gametes which mate and, within 10-14 days, give rise to a new generation of sporozoites, that relocate to the mosquito's salivary glands to initiate a new infection. Adapted from Michalakis and Renaud 2009.

### 1.7.2 The reaction norm of reproductive effort against within-host stresses

For malaria parasites, theory predicts that reproductive investment at a given point in time should relate to perturbation of the within-host environment in a u-shaped relationship (Carter et al. 2013, Fig.1.4). If reproductive investment is on the y-axis, and increasing stress of the within-host environment (or a decreasing state) on the x-axis, this u-shaped reaction norm can roughly be divided into three strategies according to the degree of stress that parasites experience.

#### Affluent investment

First, if parasites experience no or low within-host stress and asexual parasites are replicating well, they can afford to invest in gametocytes at a higher level without risking their in-host survival ("affluent investment", Fig.1.4, scenario A). This is a typical illustration of the "house-car" paradox: in humans, the person who invests into an expensive house may be able to afford only a small car and vice-versa, because resource (here: money) allocation is operating. However, positive associations between house and car values are much more common because individuals vary in the basic resource pool (expendable income) from which they allocate. The same applies to life-history traits that are connected by a trade-off (van Noordwijk and de Jong 1986). In the case of malaria, despite the fundamental allocation trade-off between reproduction and survival, the within-host environment provides enough energetic resources to satisfy both functions, leading to a positive correlation between state and conversion measures.

#### Reproductive restraint

Second, if the infection state worsens because within-host stresses increase, reproductive effort should decrease as parasites should prioritize within-host survival to guarantee future reproductive opportunities ("reproductive restraint",

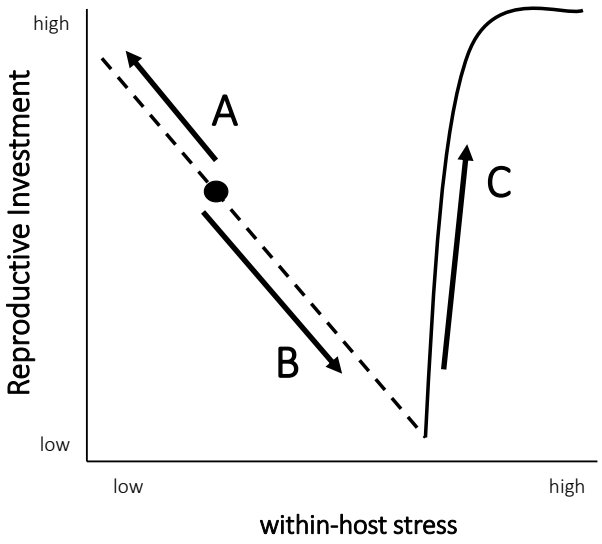


Figure 1.4. Continuous reaction norm of how much parasites should invest into transmission during an asexual cycle (reproductive investment, also called conversion rate, on the y-axis), in function of stress experienced inside the host (x-axes). The black dot indicates a hypothetical starting point. In scenario A, within-host stress decreases, allowing parasites to expand their asexual numbers, and at the same time increase their reproductive investment ("affluent investment"). In scenario B, as host stresses increase, asexual cells are killed, hence parasites decrease their reproductive investment to prioritise survival of the infection ("reproductive restraint"). In scenario C, once within-host stresses surpass a certain threshold and there is no prospect for survival of the infection in that host (e.g. due to clearance by drugs, immunity or due to host death), parasites should invest all of their resources into transmission ("terminal investment"). Whereas the switch from reproductive restraint to terminal investment is sudden (solid black line), the functional form for lower stresses is currently unknown (dotted line). Note that there is no temporal dimension to this model, i.e. parasites should react to these stresses independent of what time point they find themselves in the infection, i.e. these stresses affect parasite decisions through the intermediary of their state.

Fig.1.4, scenario B). Generally speaking, since within-host stresses increase the risk of "death" (i.e., clearance) of an infection through clearance of asexual stages, a parasite should increase its investment into "soma" to compensate for asexuals lost and thereby maximise its residual reproductive value. Thus reproductive restraint is also an adaptation that helps parasites to establish and maintain chronic infections, which is key to maximising lifetime reproductive success. Reproductive restraint has therefore a similar effect to other parasite strategies that allow the maintenance of infections such as antigenic switching, mediated by *var*-genes in *P. falciparum* (Recker et al. 2011) or the production of quiescent liver stages (hypnozoites) in *P. ovale* or *P. vixax* that allow for a resurgence of blood stage malaria (Dembele et al. 2014). Animals also frequently delay reproduction or skip breeding on a given year if current conditions are not permissive (Aebischer and Wanless 1992; Clutton-Brock 1984; Curio 1988; Reed et al. 2015). Reproductive restraint has also been described for bacteriophages, which are more likely to adopt a lysogenic strategy (hide in the genome of the infected host cell) rather than a lytic strategy (release viral progeny to burst the host cell) when the bacterial host cell's growth rate is constrained by resource limitation or if bacteriophages experience a lot of competition (Gandon 2016).

**Terminal investment**

Third, should the killing rates of asexual malaria parasites however be so high that the within-host parasite population stands little chance of recovery, then a parasite should invest all its energy into transmission. This scenario is equivalent to "terminal investment" and equates to the concept described for plants and metazoans when they experience high adult death rates (Hirshfield and Tinkle 1975) or to the inevitable end point of reproductive life (because the infection will be cleared or because the host dies), described by age-based life-history theory (Pianka and Parker 1975). Again, an equivalent can be found in bacteriophages whose lytic cycle is induced if the bacterial host cell experiences DNA damage (e.g. due to radiation) or other stress that impose a high mortality rate (Gandon 2016). The human parasite, *Schistosoma mansoni*, also seems to opt for terminal

investment when it increases its reproductive output in a drought-stressed snail, its intermediate host (Gleichsner et al. 2016).

### 1.7.3 Empirical evidence

A wide range of empirical studies suggest that malaria parasites can plastically adjust their reproductive investment in response to within-host stresses. Pollitt et al. (2011b) shows that three genotypes of *Plasmodium chabaudi*, the main model parasite for investigating life-history questions, reduce their reproductive investment if in competition with a co-infecting genotype, presumably to establish dominance over limited red blood resources. Similarly, Carter et al. (2014) show that *P. chabaudi* (genotype AJ) also reacts with reproductive restraint to cues of coinfection, but that the level of investment also depends on the timing of cue treatment, suggesting that parasites take an investment decision not just based on environmental information of the within-host environment but also on their own state.

A number of studies report increased gametocyte densities in response to red blood cell limitation (anaemia, Cameron et al. 2012; Nacher et al. 2002; Price et al. 1999) or a high proportion of immature red blood cells (reticulocytes, Gautret et al. 1996; Reece et al. 2005, a consequence of anaemia), but only two studies report that this may actually be due to increased investment (Cameron et al. 2012; Reece et al. 2005). Much of the anaemia-related data are gathered secondarily from infected patients (e.g. Price et al. 1999), from post-hoc correlations done on experiments designed to investigate hypotheses other than anaemia (Cameron et al. 2012), or only concern one of the environmental variable correlated with anaemic environments (Reece et al. 2005). Furthermore, there is little information of how the state of the infection (i.e., the dynamics of asexual stages) is affected by anaemia, since this may fundamentally affect the interpretation of reproductive effort: increased reproductive investment in anaemic environments could be due to a terminal investment response, or alternatively, to an improvement of the within-host parasite environment, and therefore the state of infection, allowing parasites to invest more into transmission without putting within-host survival

at risk (affluent investment). In this thesis, I shall present a suite of experiments that will tackle some of those unanswered questions.

There is also inconclusive evidence for how drug treatment affects reproductive effort. Subcurative doses have been shown to induce a decrease in reproductive investment in drug-sensitive *P. falciparum* parasites (Reece et al. 2010) and more virulent parasites (i.e., parasites with faster asexual growth rates) are less sensitive to clearance by pyrimethamine and arthemisinin, two antimalarial drugs (Schneider et al. 2012, 2008). But an increase in reproductive investment in response to subcurative doses has also been reported for *P. chabaudi* (Buckling et al. 1997). Although, the latter has been interpreted as terminal investment, other effects of the drug, e.g. on host physiology (e.g. oxidation status of red blood cells, Bolchoz et al. 2002) could play a role. The contradictory findings concerning the effect of drugs on reproductive investment are likely to be fuelled by inconclusive methodology on how to calculate it (Greischar et al. 2016b), variation in drug action mechanism on parasites (e.g., which stage is attacked by drugs) and the timing at which the drugs have been administered (i.e., at which state of the infection). Similar to other within-host environmental variables, making specific predictions for this particular case is not straightforward. In my thesis, I will try and fill this gap with a state-of-the art within-host model that predicts how reproductive investment of malaria parasites should vary at different intensities of drug treatment.

## 1.8 Structure of thesis

The aim of my thesis is to apply concepts from plasticity and life-history theory to the interpretation of plasticity in reproductive investment in *Plasmodium chabaudi*. The insight gained from this work should not just be relevant to this particular study system, but should first, contribute to our understanding of the trade-off between reproduction and survival and second, deliver a clinical approximation to the behaviour of human malaria parasites in human infections. Understanding the plastic investment of malaria parasites is not just a purely

academic effort to complement our knowledge of life-history evolution, but has also important applications in the fight against a disease that infects over 200 million and kills over 400'000 every year (WHO 2016). Typical applications include studying the range of behaviours that are available to parasites against intervention methods like drug treatment (White 1998) or transmission-blocking vaccines (Bousema and Drakeley 2011). The results found here will also apply to other pathogens that use distinct stages for within-host replication and between-host transmission, such as trypanosomes (Pollitt et al. 2010, Seed and Wenck 2003).

In my second chapter, I address one of the main shortcomings that has plagued the field so far, namely the absence of formal model of how reproductive investment should vary with various levels of within-host stress, but specifically focussing on drug treatment as the within-host stress of choice. I propose a state-of-the art within-host model of malaria infections (Greischar et al. 2014), modified to incorporate drug treatment that affects asexual stages, and apply optimality algorithms to obtain an optimised reproductive investment profile for various doses of drugs and timings of drug treatment. This confirms the utility of the two fundamental responses against stress, reproductive restraint and terminal investment and gives important insights into the effects of physiological and environmental state variables of an infection. Such a model also has an obvious practical application since it allows us to understand whether parasites can use variable reproductive investment as a plasticity-based resistance strategy against drugs (Reece et al. 2009).

In my third chapter, I will us the same basic within-host model as in chapter 2 but introduce a different decision making process by parasites, extending it to represent full-scale phenotypic plasticity, and thus making it more applicable to investigating state-based conversion decisions. I will also compare fitness returns between two types of plasticity (constrained and unconstrained) and the constitutive strategies of chapter 2.

In my fourth chapter, I explore the effect of an environmental state variable-resource abundance and quality, epitomised by various levels of anaemia- on

the reaction norm of reproductive investment. As stated previously, the effect of anaemia on reproductive investment is still poorly understood and has been largely devoid of interpretation in a life-history context. I also test for the existence of gene-by-environment effects by confronting various genotypes of *P. chabaudi* to experimentally created anaemia. These genotypes have been carefully selected to represent the range of life-history characteristics known so far for *P. chabaudi*, including variance in virulence (Mackinnon and Read 1999), baseline reproductive investment (Pollitt et al. 2011b) and resource use (Cameron et al. 2012). Genotypic variation in reaction norms for anaemia are a clear signal that natural selection can act on this trait, and may therefore, for example, be an integral part of evolutionary responses against antimalarial intervention methods. Furthermore, I show that these reaction norms are linked to other traits of these genotypes, which may maintain virulence in natural populations. Because anaemia is one of the main causes of death from malaria infections, especially for children under 5 years old, the results of this experiment also have an obvious clinical application. One of the additional observations is that the state of an infection (asexual growth dynamics) is also strongly affected in anaemic infections, which will form the basis of the subsequent data chapters. The fifth chapter entirely focuses on the state of infection inside anaemic environments and explores various mechanisms of how the state of asexual parasites of different genotypes is affected in anaemic environments, including parasite burst size and invasion preference in relation to red blood cell-age. Through focussing on asexual parasites, i.e., the stages that are directly or indirectly responsible for all of the virulence observed in malaria infections, the chapter is of special relevance for practical application.

In the sixth chapter, I test if there are any long-term effects of plasticity that occur when the environmental conditions (i.e., anaemia), that initially caused the plastic response, have disappeared. That chapter will therefore give much needed insight of the potential costs and limits of phenotypic plasticity, which generally are poorly understood, especially in parasite systems.

The seventh chapter is a general discussion and combines the insights obtained

from all chapters, sets them into the context of the rodent malaria study system in particular, and the field of life-history in general. The potential applications of these results to human malaria are also discussed.

# Chapter 2

# Altered life-history strategies protect malaria parasites against drugs


## Abstract

Drug resistance has been reported against all antimalarial drugs, and while parasites can evolve classical resistance mechanisms (e.g., efflux pumps), it is also possible that changes in life-history traits could help parasites evade the effects of treatment. The life-history of malaria parasites is governed by an intrinsic resource allocation problem: specialized stages are required for transmission, but producing these stages comes at the cost of producing fewer of the forms required for within-host survival. Drug treatment, by design, alters the probability of within-host survival, and so should alter the costs and benefits of investing in transmission. Here, we use a within-host model of malaria infection to predict optimal patterns of investment in transmission in the face of different drug treatment regimes and determine the extent to which alternative patterns of investment can buffer the fitness loss due to drugs. We show that over a range of drug doses, parasites are predicted to adopt "reproductive restraint" (investing more in asexual replication and less in transmission) to maximise fitness. By doing so, parasites recoup some of the fitness loss imposed by drugs, though as

may be expected, increasing dose reduces the extent to which altered patterns of transmission investment can benefit parasites. We show that adaptation to drug treated infections could result in more virulent infections in untreated hosts. This work emphasises that in addition to classical resistance mechanisms, drug treatment generates selection for altered parasite life-history. Understanding how any shifts in life-history will alter the efficacy of drugs, as well as any limitations on such shifts, is important for evaluating and predicting the consequences of drug treatment.

## 2.1 Introduction

Malaria parasites (*Plasmodium* spp.) remain one of the most severe and common causes of human disease (White et al. 2014b). Though interventions against malaria parasites have seen significant successes over the last 30 years (WHO 2015), resistance has evolved to every antimalarial drug in widespread use (Hyde 2005; White 2004; WHO 2015). In many cases, this resistance has been attributed to "classical" resistance mechanisms (*sensu* Schneider et al. 2012), including target site mutations or detoxification mechanisms (Hyde 2002, 2005). However, changes in parasite behaviour, metabolism, or life-history, i.e., "non-classical" resistance mechanisms (Schneider et al. 2012), offer additional threats to drug efficacy.

One potential mechanism for non-classical resistance is evolving traits that give rise to higher within-host parasite densities; this may offer protection against drugs by increasing the likelihood that some (genetically identical) parasites survive treatment (White 1998). Experimental rodent malaria infections confirm that more virulent parasite strains, with faster within-host replication, survive better in drug treated hosts, thus also achieving higher transmission (Schneider et al. 2012, 2008). But within-host densities are at least in part governed by a resource allocation trade-off in malaria and other sexually-reproducing microparasites: achieving higher within-host densities comes at the cost of producing fewer specialised sexual stages (gametocytes) that are required

for transmission (Carter et al. 2013; Pollitt et al. 2011b), since a parasite in a given infected host cell can follow only one of the two developmental routes. Transmission investment—by convention referred to as the conversion rate—varies plastically within artificial culture, increasing as conditions become more crowded (Bruce et al. 1990). While conversion rate can change plastically in response to changing environmental conditions, data suggest that there is parasite genetic variation for patterns of conversion across time post-infection (Pollitt et al. 2011b, Birget et al. 2017a) and that this variation can be selected upon (reviewed in Bousema and Drakeley 2011). It is well known, for example, that serial passage and culture experiments, which by their nature select for faster within-host replication, result in reduced transmission investment (Dearsly et al. 1990; Sinha et al. 2014, reviewed in Carter et al. 2013). Similarly, artificial selection for attenuation of virulence in a related parasite, *Eimeria*, resulted in indirect selection for earlier investment in transmission, which translated into a substantial reduction in total fitness (McDonald and Shirley 2009). Therefore, conversion rates represent an evolvable parasite trait essential to transmission, and the challenge is to explore if and how drug treatment might alter parasite strategies.

Malaria parasites appear to vary transmission investment in ways thought to be adaptive (Carter et al. 2013), and theory is an essential check on intuition regarding the fitness consequences of different strategies (Greischar et al. 2016c). Models have shown that reducing transmission investment—though it might appear maladaptive (Taylor and Read 1997)—can dramatically enhance parasite fitness by increasing the parasite numbers available to produce gametocytes later on and by improving persistence in the host in the face of immunity and competing strains (Greischar et al. 2016a,c; Koella and Antia 1995; McKenzie and Bossert 1998; Mideo and Day 2008). It remains challenging to show experimentally that these predicted patterns are adaptive, and actually improve parasite fitness in the face of environmental change, since techniques for forcing parasites to make alternative life-history decisions are currently not available. However, the development of improved statistical methods now allows more

accurate estimates of conversion rates *in vivo* (Greischar et al. 2016b), and theory is urgently needed to form clear expectations to compare model outcomes with natural patterns of conversion. In contrast, conversion rates are comparatively easy to integrate into mathematical models by simply varying allocation to asexual growth and gametocyte production. Mathematical models demonstrate that changing allocation patterns can have significant impacts on parasite fitness (i.e., transmission potential) and can predict the optimal pattern in different environments (Greischar et al. 2016a, 2014; Koella and Antia 1995; McKenzie and Bossert 1998; Mideo and Day 2008). Understanding how selection imposed by drugs may alter transmission investment is critical, since any changes will have both clinical and epidemiological consequences.

Here, we predict the resource allocation patterns of malaria parasites that maximise fitness in drug treated hosts. We extend a previously published mechanistic model of within-host malaria infection (Greischar et al. 2016a, 2014) and use numerical optimisation techniques to determine optimal conversion rates, i.e., the proportion of infected host cells that produce transmission stages. Into this framework, we incorporate a simple model of drug action that was parameterised for treatment of experimental rodent malaria infections with the anti-malarial drug pyrimethamine, a drug that mainly affects asexual stages (Huijben et al. 2013). By holding constant the duration and timing of drug treatment, but varying dose, this heuristic model allows us to explore the predicted impact of treatment of variable efficacy – from small to large reductions in parasite load – on parasite life-history evolution. We explore optimal investment in transmission stages, first, by assuming parasites are constrained to a constant conversion rate throughout infections and, second, by permitting parasites to employ time-varying conversion rates. Finally, we quantify the extent to which altering life-history according to these optimal patterns can buffer against the effects of drugs and we evaluate the consequences for host health and onward transmission.

## 2.2 Methods

### 2.2.1 The model

Following Greischar et al. (2016a, 2014), we use delay-differential equations to model the within-host dynamics of a malaria infection, which tracks uninfected red blood cells (R), infected red blood cells (I), extracellular malaria parasites (merozoites, M) and gametocytes (G). The change in density of uninfected red blood cells (RBCs) over time, $t$, is given by

$$\frac{dR}{dt} = \lambda\left(\frac{1-R(t)}{K}\right) - \mu R(t) - pR(t)M(t). \tag{2.1}$$

The first term represents production of new RBCs by the host. Erythropoiesis is assumed to be a logistic function of current RBC density, where $\lambda$ is the maximum realized rate of replenishing depleted RBCs and $K$ determines the homeostatic equilibrium. We assume that only uninfected RBCs count towards the homeostatic equilibrium since malaria parasites consume large amounts of haemoglobin during their development (e.g., Lew 2003) and compromise the ability of infected RBCs to carry oxygen (Schmidt et al. 1994). We have found that including infected RBCs in this term makes little qualitative difference. In the absence of infection, RBC production balances natural death (which occurs at a rate, $\mu$), so $K = \frac{\lambda R^*}{\lambda - \mu R^*}$, where $R^*$ represents the RBC density at homeostatic equilibrium. The final term represents a mass action infection process, and $p$ is the rate at which merozoites invade RBCs upon contact.

The dynamics of infected RBCs are given by

$$\frac{dI}{dt} = pR(t)M(t) - \mu I - pR\left(t-\alpha\right)M\left(t-\alpha\right)S. \tag{2.2}$$

where $S$ indicates the proportion of infected red blood cells surviving development, equal to $e^{-\mu\alpha}$ when $t > \alpha$ and in the absence of drugs. An infected cell is generated when a merozoite invades an uninfected RBC and can be lost via two different routes. First, infected RBCs can die at a background rate $\mu$. Second, infected RBCs burst to release merozoites after a period of $\alpha$ days (i.e., one day for the rodent malaria parasite, *P. chabaudi*). For simplicity,

we omit immune responses that remove infected RBCs, though simulations of this model including a saturating immune response have delivered similar optimal conversion rate profiles (results not shown).

The dynamics of merozoites and gametocytes are described as

$$\frac{dM}{dt} = (1-c(t))\,\beta pR\,(t-\alpha)\,M\,(t-\alpha)\,S - pR(t)M(t) - \mu_M M(t) \quad (2.3)$$

$$\frac{dG}{dt} = c(t)pR\,(t-\alpha)\,M\,(t-\alpha)\,S - \mu_G G(t) \quad (2.4)$$

where $c(t)$ is the proportion of parasites in a given cohort of infected RBCs that become gametocytes after successful development (i.e., the conversion rate). We allow the conversion rate to vary over the course of infection, as has been observed in experimental data (Greischar et al. 2016b; Pollitt et al. 2011b; Reece et al. 2005). The burst size, $\beta$, is the number of merozoites released from each infected RBC surviving the developmental period. Merozoites die at a rate $\mu_M$ and gametocytes die at a rate $\mu_G$.

Equations 2.2-2.4 are defined for $t > \alpha$. The dynamics of the initial inoculum of parasites, $I_0$, are governed by

$$\frac{dI}{dt} = pR(t)M(t) - \frac{I_0 S}{\alpha} - \mu I \quad (2.5)$$

$$\frac{dM}{dt} = (1-c(t))\beta\frac{I_0 S}{\alpha} - pR(t)M(t) - \mu_M M(t) \quad (2.6)$$

$$\frac{dG}{dt} = c(t)\frac{I_0 S}{\alpha} - \mu_G G(t) \quad (2.7)$$

$$S = e^{-\mu t} \quad (2.8)$$

for $t \leq \alpha$.

### 2.2.2 Drug action

We incorporate the model of drug action presented in Huijben et al. (2013), which was parameterised to describe the consequences of pyrimethamine for *Plasmodium chabaudi* parasites (Landau 1965) in infections of female C57BL6 mice (Schneider et al. 2012). According to this model, as long as the drug is present at a sufficiently high concentration in the host, it kills a fixed proportion (94%) of asexual parasites each day. The underlying within-host model assumed in Huijben

et al. (2013) was in discrete-time and cohorts of infected cells burst synchronously. To approximate this drug action in our model, we apply an additional death rate, $\mu_d$, to infected cells. By setting $\mu_d = -\ln(1 - 0.94) = 2.81$ we ensure that ∼94% of infected cells die within the one day parasite developmental cycle. Different drug doses, $d$, modify the length of drug action, $L$, beyond the days the drug was administered (see Figure A.1 in Appendix A, for how $L$ varies with dose):

$$L = 3.557 - \frac{2.586}{1 + e^{-8.821+d}}. \tag{2.9}$$

Therefore, parasites are subject to a drug-induced mortality rate for each day that the drugs are administered, plus an additional $L$ days afterwards. To explore the consequences of different strengths of drug treatment on optimal patterns of conversion rates, we simulate several treatment regimes: drug doses of 0-15 mg/kg, each administered for two consecutive days (days 11 and 12 post-infection). Determining the survival of infected RBCs ($S$) requires integrating these mortality rates over the delay $\alpha$. For the case of drug-treated infections, that survival term is given by

$$S = \begin{cases} \exp(-\mu t), & t < \alpha \\ \exp\left(-\left(\int_{t-\alpha}^{11} \mu d\omega + \int_{11}^{t} \mu + \mu_d d\omega\right)\right), & 11 \leq t < \alpha + 11, \\ \exp\left(-\left(\int_{t-\alpha}^{t} \mu + \mu_d d\omega\right)\right), & \alpha + 11 \leq t < L + 12, \\ \exp\left(-\left(\int_{t-\alpha}^{12} \mu + \mu_d d\omega + \int_{12}^{t} \mu d\omega\right)\right), & L + 12 \leq t < L + 12 + \alpha, \\ \exp(-\mu\alpha), & \text{otherwise.} \end{cases} \tag{2.10}$$

Given our other model parameters, these treatment regimes encompass outcomes from a small, transient reduction in parasite loads, to a strong reduction in parasite load that would prevent further transmission on the timescale of our simulation. A schematic of the model of drug action is presented in Figure A.2 in Appendix A.

### 2.2.3 Optimisation

To find optimal patterns of transmission investment, we define the "cumulative transmission potential" as our measure of fitness. This metric translates daily

estimates of gametocyte density into the probability of that density resulting in an infected mosquito, assuming mosquitoes are abundant and biting hosts on a regular basis. Although $R_0$ of a focal infection would be the most complete fitness measure, cumulative transmission potential is an obvious measure for the parasite to maximise from a within-host perspective: making the host maximally infectious to mosquitoes for as long as possible should maximise the $R_0$ of a focal infection. There are other ways for parasites to influence $R_0$, such as increasing mosquito biting on vertebrate hosts (De Moraes et al. 2014) or increasing the biting rate of infectious mosquitoes (Dobson 1988), but since we are not nesting our within-model into an epidemiological model, we assume here that parasites maximise their cumulative transmission potential. Although such nested models may provide insights on trade-offs occurring in different parts of the infectious cycle, nesting is generally not necessary to derive valid insights from a model (Mideo et al. 2008a).

The relationship between gametocyte densities and transmission probability is assumed to be sigmoidal, as has been experimentally derived for *P. chabaudi* by Bell et al. (2012). Transmission probability is defined as the probability that a given density of gametocytes in the vertebrate host results in a successful establishment of an infection in a mosquito during a bite. Bell et al. (2012) measure successful infection as the probability of observing oocysts in the midgut of a mosquito 9 days after a single feed on an infected mouse. Oocysts result from the mating between male and female gametocytes and eventually give rise to sporozoites, which invade the mosquito's salivary glands. Assuming that the host is regularly bitten by uninfected mosquitos every day, we calculate the probability of these mosquitos being infected as function of the gametocyte density on that day. To assess parasite fitness accumulated over the whole infection (20 days), we add up the probabilities that a parasite successfully transmits on a given day to at least one mosquito, obtaining the cumulative transmission potential. For example, a parasite that produces enough gametocytes on each day over the first 5 days of a vertebrate host infection to infect mosquitoes on each day, gives us a cumulative transmission potential of 5 (transmission probability of 1 on the first

day + a transmission probability of 1 on the second day +...+ a transmission probability of 1 on the fifth day). Using the parametrisation of Bell et al. (2012), our fitness function is calculated as:

$$f(\eta) = \int_0^{\eta} \frac{e^{-12.69+3.6\ \log_{10}G(t)}}{1+e^{-12.69+3.6\ \log_{10}G(t)}}dt, \tag{2.11}$$

where $G(t)$ is the gametocyte density at time point $t$, and $\eta$ is the day post-infection at which our simulated infection ends. A sigmoidal relationship between gametocyte density and transmission success has also been reported for *P. falciparum* (Huijben et al. 2010) and gives similar results if used instead of the fitness function described here (see Figure A.3 in Appendix A). Our model describes early infection dynamics, before major adaptive immune responses develop, we therefore simulate a 20 day infection over which we calculate the cumulative transmission potential, as has been done previously (Greischar et al. 2016a). Thus over a simulated infection of 20 days, the maximal fitness achieved by a parasite is a transmission potential of 20 (i.e., every days results in a successful transmission event).

We find the optimal patterns of conversion using the `optim` function (R version 3.0.2), relying on the Nelder-Mead algorithm (Nelder and Mead 1965) to maximise cumulative transmission potential in our within-host model (our objective function) over the simulated time. We set a maximum of 2000 iterations (i.e. 2000 simulations of the model) for parameters to converge on their optimal value. The Nelder-Mead algorithm is well suited for multidimensional, unconstrained optimisation without derivatives and relies on the creation of simplexes, defined by the parameter values to be optimised (Bolker 2008). We only use the optimised parameter values if the algorithm also converged on that value within the 2000 iterations.

In a first set of optimisations, we define transmission investment to be a constant ($c(t) = x$, for all $t$) and determine the optimal time-invariant conversion rate. Second, following Greischar et al. (2016a), we use cubic splines for the optimisation of time-varying conversion strategies, implemented in **R** with the `splines` package. Cubic splines require only four parameters to specify but allow

considerable flexibility in the pattern of conversion over a 20-day infection, and more complicated splines yield minimal fitness gains (Greischar et al. 2016a). Time-variable conversion, $c(t)$, in the from of a cubic spline can be described by the following equation:

$$c(t) = \exp(-\exp(j\ t^3\ +\ k\ t^2\ +\ l\ t\ +\ m)), \tag{2.12}$$

where $j$, $k$,$l$ and $m$ are the parameters that define the spline and that are to be optimised, and $t$ is time. Conversion rates must be constrained to vary between zero and one, so we take the complimentary log-log of the value specified by the spline, that is $c(t) = \exp(-\exp(\text{spline value at time } t))$. The starting values of the variables and the assumed value for each of the model parameters are given in Table 2.1, and each optimisation is initiated by setting all spline parameters to an arbitrary starting guess of 0.5. Although no numerical optimisation routine can guarantee finding a globally optimal solution, we sought to substantiate our findings by testing, for a given environment (i.e., drug dose), whether the putative optimal strategy for that environment out-performed the putative optimal strategies from other environments.

## 2.3 Results

### 2.3.1 Constant conversion rates

Following previous work (Greischar et al. 2016a), we first constrained conversion rate in our within-host model to be a constant, and determined which single rate, maintained throughout the whole infection, produced the highest estimate of our parasite fitness proxy (i.e., cumulative transmission potential). In the absence of drugs, we find a similar optimal level of transmission investment as predicted previously (Greischar et al. 2016a). Drug treatment reduces the optimal level of transmission investment, with the lowest conversion rate predicted for the highest drug dose simulated (Figure 2.1A). We found little variation in the optimal transmission investment over low and moderate drug doses, as would be expected given our assumption that the drug dose changes the number of days

Table 2.1. Model parameters.

| Parameter | Description | Value or range | Reference |
|---|---|---|---|
| $R^*$ | red blood cell density of a healthy mouse | 8.5x10$^6$ cells/$\mu$L | Savill et al. (2009) |
| $\lambda$ | maximal red blood cell production rate | 3.7x10$^5$ RBCs/$\mu$L | Savill et al. (2009) |
| $\mu$ | red blood cell death rate | 0.025/day | Miller et al. (2010) |
| $p$ | maximal per merozoite invasion rate | 4x10$^-$6/day | Mideo et al. (2008b) |
| $\alpha$ | bursting delay | 1 day | Landau and Boulard (1978) |
| $\beta$ | burst size | 10 merozoites | Mideo et al. (2008b) |
| $\mu_M$ | merozoite death rate | 48/day | Mideo et al. (2008b) |
| $\mu_G$ | gametocyte death rate | 4/day | Gautret et al. (1996) |
| $\mu_d$ | drug-induced death rate of infected cells | 2.81/day | adapted from Huijben et al. (2013) |
| $I_0$ | initial dose of infected red blood cells | 43.85965/$\mu$L | $\sim 10^4$ per mouse |
| $d$ | drug dose | 1-10 mg/kg | Huijben et al. (2013) |

of drug action rather than the killing rate (Huijben et al. 2013).

For doses below 6 mg/kg, this formulation predicts little difference in the duration of drug action (see Figure A.1 in appendix A) or consequences for parasite fitness, as can be seen in Figure 2.1B. We therefore focus on 5 mg/kg, 8 mg/kg and 15 mg/kg as representative low, medium, and high drug doses, respectively, for the remainder of our analyses. The step-wise decrease in predicted conversion rates observed from a dose of 0 to 2 mg/kg and from a dose of 8 to 10 mg/kg closely follows the fitness effects that these increasing doses would have on parasites employing a non-drug adapted conversion rate (Figure 2.1B, grey bars). Interestingly, we do not see a similar decrease in the predicted optimal conversion rate when the drug dose increases from 6 to 8 mg/kg, despite a substantial decrease in expected fitness for a non-drug adapted strategy. An explanation for this may be found in the fact that a constant

conversion rate represents a compromise, balancing the need to sustain a high enough asexual source population for conversion in the face of drug killing and having a sufficiently high conversion rate to successfully translate that asexual source population into onward transmission. Up to a dose of 8 mg/kg, slight increases in conversion rates can counteract lost fitness due to slight reductions in the asexual source population from higher doses. With a dose of 10 mg/kg or more, the asexual source population and gametocytes are reduced to such an extent that no more transmission is possible after the action of drugs. Therefore, the best option for a parasite is to restrain and increase the asexual source population that will be converted before the end of drug action.

We assume that all parasites within an infection are genetically identical; consequently, our fitness proxy is the cumulative probability of transmission over the course of infection. Since our simulated infections run for 20 days, 20 represents the maximum cumulative transmission potential that would be achieved by a parasite genotype that sustained a sufficiently high gametocyte density to transmit to mosquitoes with 100% efficacy every day. Even in the absence of drugs, parasites cannot achieve 100% transmission efficacy at every point in the simulation, especially at the beginning of the infection when parasite numbers are low; hence, the maximum cumulative transmission potential is approximately 11 for the optimal level of fixed transmission investment of 0.42 in the absence of drugs (Figure 2.1B). The grey bars demonstrate the fitness achieved by parasites employing this same conversion rate (0.42) in the face of drug treatment. As expected, parasite fitness is lost as drug treatment reduces numbers. Some fitness can be recouped by adopting lower conversion rates (the drug dose-specific optima, black bars). Indeed, with low drug doses, reduced conversion rates allow parasites to maintain roughly 90% of the fitness achieved in the absence of drugs.

### 2.3.2 Time-varying conversion rates

Next, we allowed the conversion rate to vary over the course of the infection and determined what pattern of transmission investment would maximize cumulative

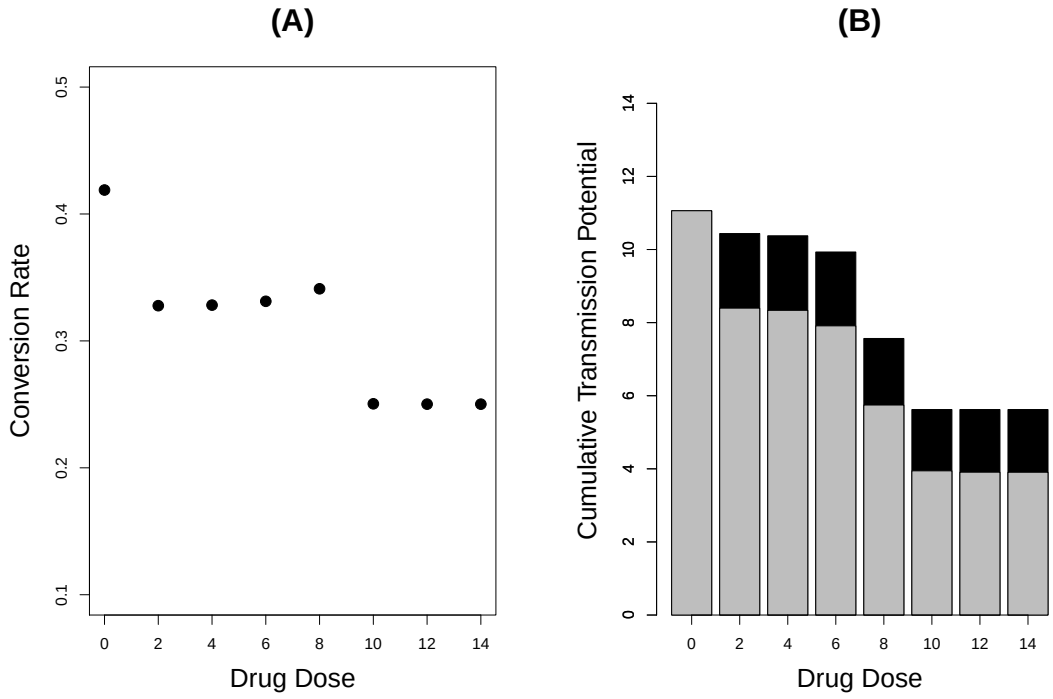


Figure 2.1. Lower conversion rates can buffer the effects of drugs. (A) Optimal constant conversion rates in the face of drug treatment (labeled as doses in mg/kg) are lower than in the absence of drugs. (B) As expected, drug treatment reduces parasite fitness (i.e., cumulative transmission potential). Grey bars indicate fitness when parasites are constrained to the drug-free optimal conversion rate ($\sim$0.42). Black bars show the fitness gains achieved by adopting the dose-specific optimal conversion rate (from A). With lower conversion rates parasites are able to recoup some of the fitness that is lost due to drugs.

transmission potential (Eqn. 2.11). The work of Greischar et al. (2016a) suggests that, in the absence of drug treatment, optimal patterns of conversion rate comprise roughly four distinguishable phases: (1) an "initial replication" phase where parasites delay gametocyte production to increase their numbers; (2) a "peak conversion" phase where parasites dramatically increase transmission investment to capitalize on their large asexual numbers; (3) a "trough" where parasites reduce transmission investment to compensate for declining numbers in the face of resource limitation; and finally, (4) "terminal investment", where

parasites invest heavily into gametocyte production before the infection ends. We find qualitatively similar strategies (with the same four phases) in drug treated infections (Figure 2.2). The corresponding dynamics of infected red blood cells and gametocytes are shown in Figure 2.3. A key difference in the predicted optimal patterns of conversion in drug treated compared to untreated infections is an earlier and faster reduction in conversion rates (i.e., greater reproductive restraint) following the initial peak conversion (compare black to coloured lines in Figure 2.2). Comparing low and medium dose treatment regimes, we find that increasing dose is accompanied by greater reproductive restraint following treatment. The best response to a high drug dose is early terminal investment, which ultimately ends the infection (see infection dynamics in Figure 2.3C).

To identify the fitness consequences of these different strategies, we plot cumulative transmission potential over the course of infections. In Appendix A, we confirm that the putative optimal strategy against a given dose outperforms the putative optimal strategies from other doses (see Figure A.4). The optimal strategies—and the corresponding cumulative transmission potential—are similar prior to drug treatment (Figures 2.2, and 2.4, respectively). After drug treatment, the transmission investment strategies diverge, and there are clear costs to parasites that employ the incorrect strategy for the drug dose they encounter within the host, especially at low doses (compare coloured to dashed grey curves in Figure 2.4). Specifically, in the absence of drug treatment and after an initial establishment phase of the infection, the optimal drug-free strategy accrues fitness at nearly the maximal rate, corresponding to almost 100% chance of transmitting to mosquitoes each day (black lines, Figure 2.4). But, this strategy performs successively worse in the face of increasing drug doses (dashed grey lines Figure 2.4; see also Figure 2.3 for corresponding infection dynamics). The optimal strategies for low, medium, and high drug doses allow parasites to recoup a substantial portion of these fitness losses (coloured lines in Figure 2.4), attributable to greater reproductive restraint immediately after drug treatment (Figure 2.2). Notice that in the face of a high drug dose, the drug-free strategy accrues no fitness following treatment (Figure 2.4C, dashed

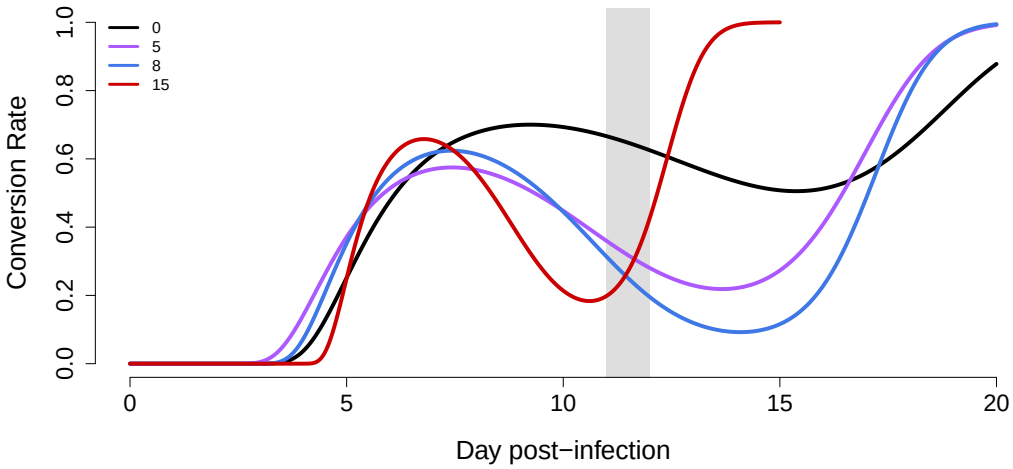


Figure 2.2. The optimal pattern of conversion over the course of infections. The black line shows the predicted best response in an untreated infection. When infections are treated (coloured lines), regardless of dose, parasites do better by reducing conversion (purple: low dose, 5 mg/kg; blue: medium dose, 8 mg/kg; red: high dose, 15 mg/kg). Drugs are administered on the days denoted by the grey bar.If drug treatment reduces the infection to a degree where parasites cannot expect any future transmission, then the best response for parasites is to terminally invest (as suggested by the red line). Note that the patterns diverge before drug treatment due to the constraints of our fitting regime; however, early differences in investment patterns contribute little to fitness differences (see text).

grey line), despite the fact that gametocytes are still circulating for days in those infections (Figure 2.3C, dashed grey line). This is because the densities are too low to achieve more than a negligible probability of transmission. In untreated infections, parasites that use reproductive restraint pay only a small fitness cost whereas parasites employing strategies against high drug doses, pay a more substantial fitness cost due to premature terminal investment (Figure 2.5A).

While reproductive restraint in response to treatment can, to some extent, buffer against the effects of drugs, our models predict that treatment still leads to reductions in parasite fitness and, importantly, reductions in transmission

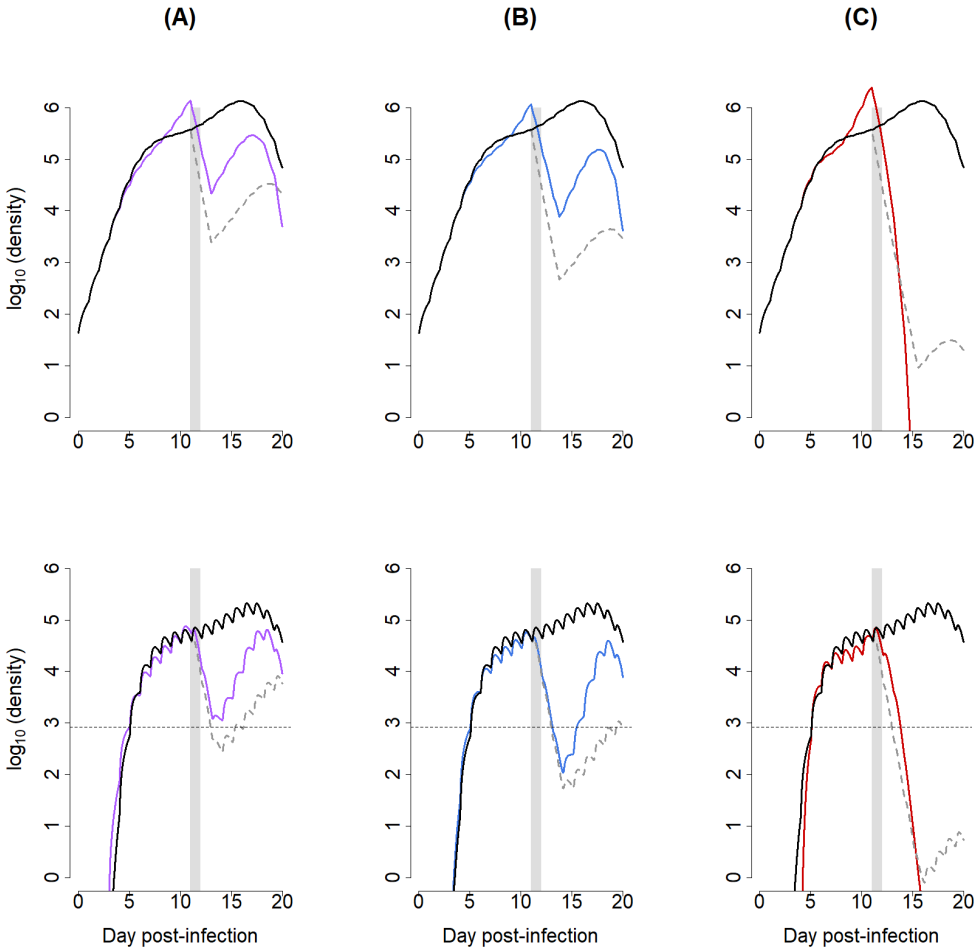


Figure 2.3. The within-host dynamics of infected red blood cells (i.e., asexual parasites; top row) and gametocytes (bottom row). Coloured lines show dynamics when parasites are using the optimal conversion profiles for a given drug treatment (A: low dose, purple; B: medium dose, blue; C: high dose, red). The black lines show dynamics in the absence of treatment, for parasites using the optimal drug-free pattern of conversion, while the dashed grey lines show how the different drug treatment regimes impact these dynamics if parasite life-history patterns are unchanged from the drug-free optimum. Grey bars denote the days of drug treatment and the horizontal lines in the bottom row indicate the gametocyte density at which there is a 10% probability of transmitting to a mosquito, according to Bell et al. (2012).

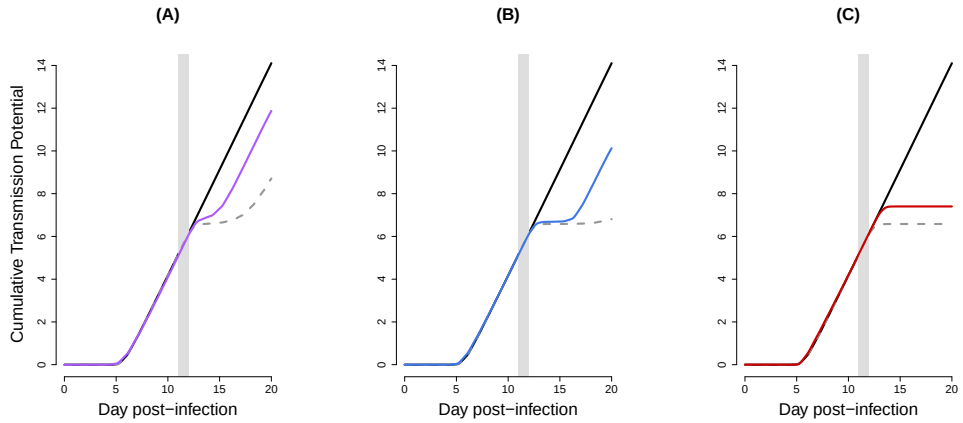


Figure 2.4. Cumulative transmission potential (fitness) over the course of infections. Given our fitness function, a parasite can maximally transmit with a probability of 1 each day, reaching a cumulative transmission potential of 20 at the end of the simulated infection. Black lines show the fitness obtained by a parasite adopting the drug-free optimal pattern of conversion over the course of an untreated infection. Dashed grey lines show the consequences of drug treatment on parasites using that same strategy in the face of drug treatment: (A) low dose, 5 mg/kg; (B) medium dose, 8 mg/kg; (C) high dose, 15 mg/kg. Coloured lines show the fitness obtained by parasites using the drug-dose specific optimal patterns of conversion (from Figure 2.2) in the face of drug treatment and indicate that parasites can recover some of the fitness lost due to drug treatment by altering patterns of conversion. Grey bars denote the days of drug treatment.

potential. Since reproductive restraint necessarily means prioritization of asexual replication and it is these parasite stages that are most responsible for the virulence (harm) of a malaria infection, there may be consequences of shifting patterns of conversion at the host (or clinical) level. Drug treatment reduces infected RBC densities, even if parasites alter their conversion rates (Figure 2.3), but what if parasites employ drug-adapted strategies in an infection that remains untreated? Figure 2.5B shows that, in an untreated host, infections composed of parasites using a drug-adapted strategy (coloured lines) are predicted to result in much more rapid declines in uninfected RBC densities, and greater anemia as measured by minimum RBC counts, compared to parasites using the best

strategy in the absence of drugs (black line).

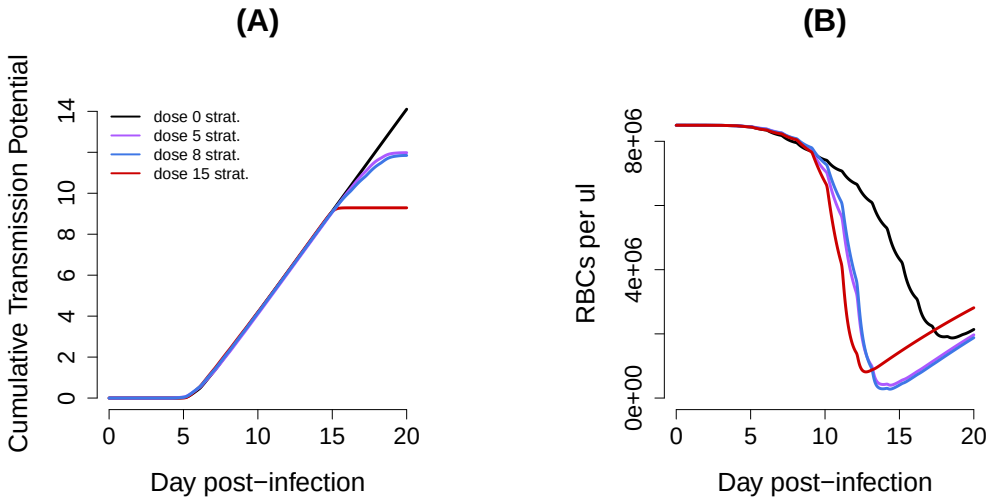


Figure 2.5. Consequences of parasite adaptation to drug-treated infections. (A) The cumulative transmission potential in untreated infections where parasites employ different conversion rate strategies. Reproductive restraint in untreated infections produces only small transmission costs (purple and blue line) compared to strategies for untreated infections (black line) whereas terminating an infection early has bigger fitness consequences (red line). (B) The dynamics of uninfected red blood cells in those infections. Simulations assume optimal strategies for untreated infections (black), infections treated with a low dose (purple), medium dose (blue), and high dose (red). The reproductive restraint predicted for drug-adapted strategies leads to earlier declines in RBCs and lower minimum values (i.e., greater anemia) when infections are not drug treated.

Of course, the likelihood of a drug-adapted strategy becoming fixed in the parasite population depends on the frequency that parasites encounter drug-treated hosts, the benefits of altered patterns of conversion in a drug-treated host, as well as the costs of that strategy in an untreated host. Using the fitness estimates for the different strategies in different environments (Table B.1 in Appendix B), we calculate the expected fitness for the drug-adapted and non-drug adapted strategies in a host population where some proportion of

hosts are treated (Figure B.1). If $b$ is the increase in fitness achieved by the drug-adapted strain in the presence of drugs (i.e., the benefit), $c$ is the reduced fitness of the drug-adapted strain in an untreated host (i.e., the cost), and $f$ is the proportion of infected hosts that are drug-treated, then it is trivial to show (see Appendix B) that the drug-adapted strategy has a higher fitness than the non-drug adapted strategy when

$$f > \frac{c}{c+b}. \tag{2.13}$$

Put another way, the drug-adapted strategy will be favoured when the ratio of the benefits to costs of the strategy is greater than the relative frequency of encountering an untreated host:

$$\frac{b}{c} > \frac{1-f}{f}. \tag{2.14}$$

Given our estimated fitnesses for the different strategies in different host environments, the drug-adapted strategy will be favoured over the non-drug adapted strategy when at least $\sim 40\%$ of infections are treated with a low or medium dose, or at least 86% of infections receive a high dose treatment. The early terminal investment strategy predicted to be optimal in the face of a high drug dose gains only a small fitness advantage in a treated host, while it suffers a large fitness cost in an untreated host (see also Table B.1), explaining why drug treatment would have to be very common to generate a sufficient selection pressure to favour that strategy.

## 2.4 Discussion

The evolution of drug resistant parasites is a serious obstacle to the control of malaria (Dondorp et al. 2009; White 2004). In addition to classical resistance mechanisms, we have shown that drug treatment can select for altered life-history of malaria parasites and, specifically, changing patterns of allocation to transmission versus asexual parasite stages. Our work predicts that reproductive restraint is adaptive in drug treated infections, allowing parasites to

compensate for the reductions in asexual densities caused by the drug. We also show that parasite adaptation to drug treatment could lead to worse outcomes for hosts that remain untreated, although as would be expected this outcome depends on the frequency with which parasites find themselves in treated hosts as well as the precise costs and benefits associated with different investment patterns in different environments.

Experimental evidence suggests that malaria parasites do alter their investment in transmission in response to drugs. Reece et al. (2010), for example, found a decrease in conversion in human malaria parasites exposed to low doses of drugs *in vitro*, as our model predicts, unless they were known to be "classically" drug-resistant parasites, which showed no change in investment (a result that highlights the multiple routes available for mitigating the effects of drugs). A similar study found no effect of drug dose on conversion rates (Peatey et al. 2009) and an *in vivo* rodent malaria experiment suggested that subcurative drug doses lead to increased conversion (Buckling et al. 1997). In contrast to the results of Reece et al. (2010), these latter two examples show parasite responses that appear maladaptive in light of our model results, raising at least two further questions. First, have parasite strategies been accurately measured? Inferring conversion rates is fraught with difficulties that have only recently been resolved, and reanalysis of past data sets could reconcile the discrepancy between theoretical predictions and empirical estimates of transmission investment (Greischar et al. 2016b). Second, are parasites capable of evolving adaptive transmission strategies to the novel selection pressure of drug treatment? Addressing this question means evaluating whether the parasites in these experiments would have achieved greater fitness than ones with different responses, which necessitates tools for manipulating parasite strategies. Advances in understanding the molecular pathways associated with commitment to gametocytogenesis (e.g., Brancucci et al. 2015) may bring such tools for experimental manipulation into reach.

Recent work has focused on dormancy as another non-classical resistance mechanism thought to be employed by malaria parasites (e.g., Codd et al.

2011; Hott et al. 2015; Paloque et al. 2016; Teuscher et al. 2010). This delayed development confers protection against the effects of fast-acting drugs that decay rapidly within a host, but whether such a strategy would be beneficial against drugs with longer half-lives is unclear. Parasites can stall their intra-erythrocytic development for many days, but only a small fraction—less than two percent—appear to successfully recover and resume development even at low drug doses (Teuscher et al. 2010). It is not clear that such a low percentage of parasites entering dormancy can explain malaria dynamics in patients (Saralamba et al. 2011). Further, the fitness consequences of dormancy are not intuitive: surviving the effects of drugs is clearly good from the parasite's perspective, but stalling development means stalling production of transmission stages and missing out on any transmission opportunities during the dormant phase. In contrast, parasites can recover substantially more than two percent of their numbers by modifying transmission investment under some treatment regimes. Indeed, Figure 2.3 suggests that parasite densities can actually increase by an order of magnitude or more within less than 4 days and this modified life-history translates to fitness gains (Figure 2.4). It is interesting to consider how these two mechanisms of non-classical resistance would affect host health. At least in the short term, dormancy should reduce pathology associated with parasite replication as well as immunopathology, while reduced investment in transmission is likely to do the opposite.

We have shown that, in principle, altered life-history can protect against the effects of drugs and while we have used a model of drug action that was parameterized for a particular drug (pyrimethamine; Huijben et al. 2013), the phenomenological description we employ should capture the effects of many different drugs. Though there will be differences among individual hosts in drug metabolism that would affect, for example, the duration of drug action, our exploration of a range of drug doses should capture much of this variation. One exception to this generality is drugs that directly target gametocytes (e.g., primaquine, White et al. 2014a). The relative susceptibilities of asexuals and gametocytes to the drug will alter the costs and benefits of producing each stage,

so different drugs may be expected to have different effects on optimal patterns of transmission investment. For example, a drug with a strong gametocytocidal effect may generate an advantage to reproductive restraint when drugs are present but promote the production of surplus gametocytes to compensate for those killed by drugs when drugs have cleared or may promote earlier production of gametocytes to compensate for lost transmission opportunities during drug treatment. Predicting evolutionary trajectories in response to such drugs will require precise calibration of the relative susceptibility of different parasite stages.

We have also ignored within-host competition and thus evolution operating at the within-host scale, but where malaria is endemic, multi-genotype infections are the rule rather than the exception (e.g. Baruah et al. 2009; Juliano et al. 2010). Previous theoretical and experimental work shows that competition favours reproductive restraint (Greischar et al. 2016a,c; McKenzie and Bossert 1998; Mideo and Day 2008; Pollitt et al. 2011b), so it is possible that our prediction of that same response in the face of drug treatment would remain unchanged. However, just as there is genetic variation for competitive ability (Bell et al. 2006; de Roode et al. 2005a,b), there is likely to be genetic variation in sensitivity to drugs (and in *P. falciparum*, differentially sensitive genotypes may often share a host; e.g.,Mideo et al. 2016). If variation in drug sensitivity is unrelated to transmission investment, then it would alter the costs and benefits to different parasite genotypes of altering that investment. Modelling the dynamic consequences of competition and the interplay between different sources of resistance on the evolution of parasite life-history would be an interesting route for future investigation. Importantly, there may also be genetic variation in the shape of the relationship between within-host gametocyte densities and the probability of transmission to mosquitos. As far as we are aware, this relationship has been quantified only a few times and only for a few distinct strains (Bell et al. 2012; Huijben et al. 2010; Paul et al. 2007). While the qualitative shapes of these relationships remain the same, there are quantitative differences in their parametrization. We found that these differences did not alter

our predictions (see figure A.3 in supplementary material), but further empirical exploration of this relationship is warranted, as is theoretical investigation of how any quantitative changes in this relationship alter evolutionary predictions. While our model allows for variation across infections treated with different drug regimes and variation over time within infections, our heuristic analysis also constrains variation at both of these scales. First, to determine when evolution should favour a drug-adapted strategy, we assumed that there were only two strategies available to parasites: the pattern of transmission investment predicted to be best in an untreated host or the one predicted to be best in the presence of a particular drug dose. In a heterogeneous host population, some intermediate parasite investment strategy may perform better than either of these two "extremes". Second, our model does not allow for parasites to directly receive and respond to cues within infections, i.e., it is not a model of plasticity. Put another way, the model implicitly assumes that parasites have perfect knowledge about the timing of drug treatment (which does not vary across treated hosts) and optimal patterns of investment may allow parasites to, in effect, prepare in advance for drug treatment. This scenario may not be too far from reality in some areas. Drug doses are standardised by WHO guidelines (WHO 2015) and hosts likely seek treatment when symptoms appear, which generally correlates with peak parasite density (Kachur et al. 2006), though there will be variation across individual hosts in the timing of early dynamics. How much fitness could be gained by allowing parasites in our model to detect and respond to drug treatment more directly is unclear, since our results suggest that differences in investment early in infections (and, in particular, before drug treatment) have little effect on parasite fitness. Consistent with this, Greischar et al. (2016a) found that investing little in transmission at the beginning of infections is adaptive in untreated hosts, regardless of other changes to the within-host environment. Thus, it seems unlikely that allowing parasites more flexibility in pre-treatment patterns of investment would result in different life-history strategies than we have predicted. On the other hand, if parasites could respond plastically to the presence of drugs in the within-host environment

(instead of through evolutionary change, as we have focused on), then this would avoid the negative consequences for host health we report.

The evolution of classical resistance is the expected result of using chemical interventions to kill parasites (or, in evolutionary terms, reduce their fitness), but, as we have shown, failing to consider the potential for non-classical resistance, like life-history evolution, can yield overly optimistic predictions about the epidemiological or clinical effects of those interventions. Similarly, Lynch et al. (2008) used models to investigate the influence of different anti-helminth interventions on nematode life-history, finding that disease control programs may frequently select for increasingly fecund worms, with ramifications for clinical outcomes and onward transmission. In an experimental system, filarial nematodes altered their reproductive schedules in the presence of specialized immune cells (eosinophils), producing transmissible stages faster and in greater numbers (Babayan et al. 2010). Since eosinophils are the same immune cells on which current experimental vaccines rely, this work suggests that nematodes could reduce the benefits of vaccination through plasticity in life-history. Finally, non-classical resistance mechanisms can not only be used by parasites but also by their vectors. For example, mosquitoes that transmit malaria and other diseases can also respond to intervention efforts with non-classical resistance, including, for example, changes in feeding behaviour or timing to avoid insecticide-treated bednets (Gatton et al. 2013; Sokhna et al. 2013).

An important question is how treatment recommendations would change in light of our predictions about optimal malaria parasite life histories. Regardless of the life-history shifts we predict here, parasites fitness and within-host densities are reduced by drug treatment. This suggests that despite the evolution of non-classical resistance, drug treatment offers epidemiological and clinical benefits. Those benefits are not as great as they would be in the absence of life-history evolution and, importantly, any hosts that remained untreated could be worse off if drug-adapted strategies became fixed in the parasite population. Further, as a result of altered patterns of transmission investment, parasites could maintain higher within-host densities in the face of drug treatment, potentially

facilitating the evolution of classical resistance. The theory developed here provides a basis for assessing the constraints and limits on parasite life-history evolution in response to human interventions.

# Chapter 3

# Phenotypic plasticity in reproductive strategies maximises the fitness of drug-treated malaria parasites

## Abstract

In response to stressful conditions (e.g. resource limitation, disease), life-history theory predicts that sexually reproducing organisms should reduce their investment into current reproduction and divert these resources into survival. Adopting reproductive restraint in such circumstances maximises fitness because it enables organisms to survive until conditions improve and reap the rewards of future reproductive opportunities. However, when future reproduction is unlikely (because of old age or environmental conditions are too harsh to survive), organisms should make a terminal investment by investing all remaining resources into reproduction. These ideas have been developed to explain variation in reproductive investment observed in insects, birds, and mammals, but should be applicable to all sexually reproducing taxa. Indeed, in chapter 2, we show that malaria parasites should evolve constitutive conversion

rate strategies of reproductive restraint if their infections are likely to be treated with low to medium doses of antimalarial drugs, or terminal investment if they expect to encounter high drug doses. A key benefit from constitutive conversion strategies is that parasites pre-empt killing by drugs by reducing conversion before treatment. This restraint enables parasites to build up the number of asexual stages so that within-host survival is maximised in the case of reproductive restraint, or the source population for transmission stage production is maximised in the case of terminal investment. Here we examine whether reproductive restraint and terminal investment also maximise fitness if parasites plastically respond to the dose of drugs they receive. Because plastic conversion strategies only enable parasites to modify conversion once drugs have been administered, they may not bring sufficient fitness benefits to evolve. However, we show that parasites should indeed react to drugs by plastically adopting reproductive restraint or terminal investment, and that plastic responses almost match the fitness returns of constitutive strategies. We also show that parasites are likely to get fitness benefits from plastic strategies only if they can drastically modify their conversion upon drug treatment. If so, plastic strategies are expected to perform better than constitutive strategies if less than 70-85% of hosts are drug-treated.

## 3.1 Introduction

Malaria parasites have a number of tools that allow them to cope with drug treatment. The best known are classical resistance mutations, for example those that allow parasites to export drug molecules in vacuoles (as in chloroquine resistance, Hyde 2005). Resistance mutations have now been reported for every antimalarial drug that is in widespread use (White 2004), including the current front-line drug artemisinin (Ashley et al. 2014). However, parasites may also use "non-classical" resistance mechanisms e.g. by plastically modifying their life-history traits. For example, *Plasmodium falciparum* has been reported to arrest its asexual development inside red blood cells in response to artemisinin

treatment, only to resurge after the drug has been cleared (Teuscher et al. 2010). Another way that parasites can protect themselves is by prioritising within-host asexual replication over between-host transmission. Asexually replicating stages are required to survive inside the host and specialised sexual stages (termed gametocytes) are essential for transmission. Because an individual parasite can either replicate asexually (i.e. producing asexually replicating progeny) or develop into a non-replicating gametocyte, investment in asexuals and gametocytes is governed by the same resource allocation trade off facing all sexually reproducing organisms. By reducing investment into gametocytes (called the conversion rate), parasites can maximise asexual replication and so, survive drugs by partially compensating for the asexual parasites killed by drugs and extending the duration of infection to benefit from future transmission opportunities. However, if drug treatment is sufficiently strong to prevent asexual numbers from recovering enough to serve as a source population for successful future transmission, then maximising the conversion rate, to escape the host, may be the best strategy. Thus, parasites are thought to adjust their conversion rates during infections in a manner that depends on how the within-host environment affects the number or replication rate of asexuals (referred to as "state", Carter et al. 2013).

Empirical data suggest that malaria parasites do react to drug treatment, and a number of other scenarios encountered within the host, by adopting reproductive restraint or terminal investment according to their state (Buckling et al. 1999; Reece et al. 2010). Whilst data and life-history theories suggest parasites have evolved adaptive phenotypic plasticity of their conversion rate, the fitness consequences of reproductive restraint or terminal investment are yet to be demonstrated. Chapter 2 takes the first step in this direction by mathematically demonstrating that fitness (defined as lifetime transmission potential) is maximised if parasites adopt drug-dose-specific patterns of conversion throughout infections (Figure 3.1). The strategies modelled in chapter 2 are phenotypically plastic: conversion rates vary during infections

and are therefore reaction norms with respect to days post-infection. However, the patterns obtained in chapter 2 are fixed by microevolution, i.e. parasites are assumed to have perfect knowledge about when the host will receive drugs and at what dose, because the best strategies require the parasites to "prepare in advance" for the appearance of drugs (Fig.3.1). This restraint enables parasites to build up the number of asexual stages so that within-host survival is maximised in the case of reproductive restraint, or the source population for transmission stage production is maximised in the case of terminal investment.

Constitutive (i.e. genetically "hard-wired") responses to drugs may indeed evolve in regions where patients generally seek treatment around the same time in the infection (e.g. when symptoms appear) and follow the same treatment regime (e.g. following WHO guidelines). However, in reality, treatment regimes are likely to vary between regions, seasons, and patient classes. For example, people with asymptomatic infections (e.g. those with non-sterilising immunity) rarely seek drug treatment; in regions far from health facilities, people are less likely to seek treatment despite potentially severe symptoms; and access to high quality drugs and compliance with recommended treatment regimes is variable (Chaturvedi et al. 2009; Kassam et al. 2016; Rao et al. 2013). Therefore, given that drug-adapted strategies are associated with fitness costs in the absence of drugs, the environments encountered by parasites during infections and across hosts may be too variable for constitutive responses against drugs to evolve. Instead, parasites may thus profit from phenotypically plastic strategies that are activated only when drugs are taken. Such a strategy corresponds to classical phenotypic plasticity where an organism alters a trait value upon encountering a different environment or a cue for a future environmental change.

Here, we examine what strategies parasites should adopt if they modify their conversion rates only when they have encountered drugs (termed "plastic strategy"). We ask whether, as for constitutive conversion profiles, they should adopt reproductive restraint in response to lower drug doses and early terminal

investment in response to high drug doses. Constitutive strategies allow parasites to prepare by adopting reproductive restraint days before drug treatment, which may give them significant fitness advantages compared to parasites that cannot anticipate a chance in their environment. However, a constitutive strategy is environment-specific and so, parasites will behave suboptimally in infections treated with unexpected doses, whereas plasticity could enable parasites to respond appropriately to any dose. In summary, we show that, as expected, constitutive strategies perform best in a given drug environment, but that parasites can reach similar levels of fitness though plastic strategies, especially at low drug doses. Importantly, plastic conversion strategies are also characterised by reproductive restraint or terminal investment, confirming that that these reproductive strategies are not just characteristics reserved to constitutive strategies.

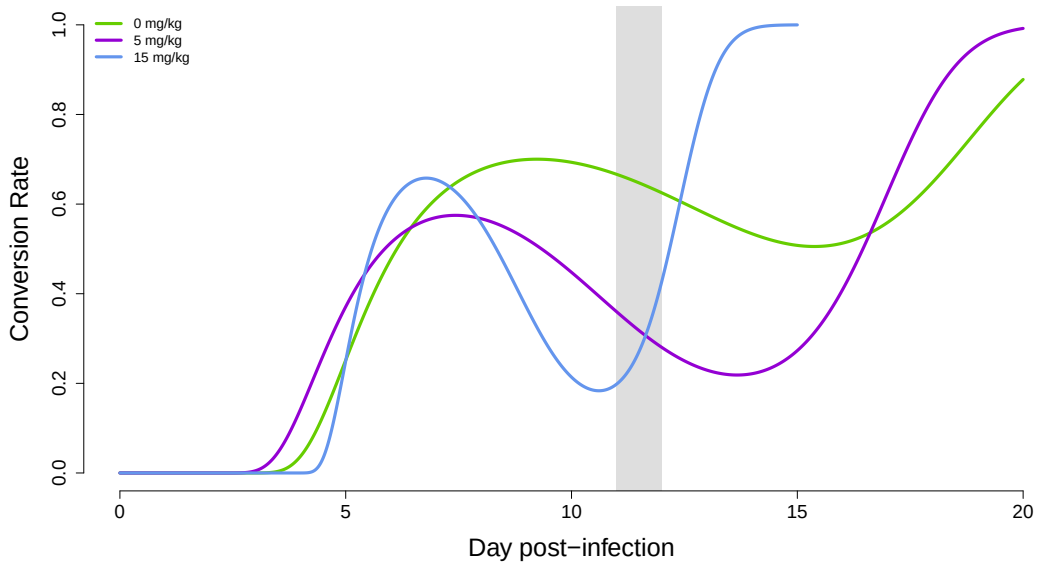


Figure 3.1. Optimal constitutive conversion rate profiles for different drug doses, with a simulated infection length of 20 days and drugs being administered at the indicated doses at day 11 and 12 (grey bar). In these conversion rate profiles, parasites pre-empt the arrival of drugs, with considerable divergence between profiles already observed from day 7 PI onwards.

## 3.2 Methods

### 3.2.1 The within-host model

We use the same malaria within-host model as presented in chapter 2, with delay-differential equations that describe the density of uninfected red blood cells (RBCs), infected RBCs (iRBCs or asexuals), merozoites and gametocytes (Birget et al. 2017b; Greischar et al. 2016a, 2014), simulated for an infection of 20 days. The model is parameterised to reflect a *Plasmodium chabaudi* infection in lab mice, *Mus musculus*. As for chapter 2, we simulate drug treatment on day 11 and 12 post-infection (PI) with various doses of pyrimethamine, a drug that kills asexual parasites, and derived the optimal conversion rate profiles for various drug doses.The model of drug action was initially presented in Huijben et al. (2013) and was parameterised for *P. chabaudi* parasites in infections of female C57BL/6 mice (Schneider et al. 2012). We simulate treatment with a range of drug doses between 0 and 15mg/kg and select doses 0, 5 and 15mg/kg (and their corresponding strategies) as representative examples for untreated, low and high doses of drug treatment. The duration of the asexual cycle of *P. chabaudi* is one day, and during each cycle parasites decide what proportion of asexuals should be committed towards gametocyogenesis. Finally, as in chapter 2, we use the function `optim` or `optimise` in the program R (version 3.2.3) with three different modelling approaches to find the optimal plastic conversion rate strategy (defined by the parameter $c$ in equation 2.4 of chapter 2) that parasites should use in those drug treatments (see below).

In constitutive responses (described in chapter 2), the conversion decision is pre-defined by a given conversion profile (i.e. the conversion rate adopted each day during infection), which parasites follow regardless of the within-host environment in which they find themselves in. Figure 3.1 shows the optimal constitutive conversion rate profiles for three drug doses (0, 5 and 15 mg/kg), obtained in chapter 2. In all drug treated infections, conversion rates (purple and blue line) lie below the profile of an untreated infection during much of the

infection, which is evidence of reproductive restraint. However, in the case of high drug treatment, parasites adopt reproductive restraint purely to build up a large pool of asexual stages from which to make an early terminal investment. Note, that parasites in untreated and low-dose infections also make a terminal investment but this is simply a product of the simulated infections ending on day 20 PI. All parasites in drug treated infections diverge from the conversion profile of untreated hosts several days before drug treatment begins and this preparation is key to recouping the fitness lost through drug treatment.

For plastic strategies, parasites react only when drugs act, i.e. any potential fitness benefits they could gain from preparation before drug-treatment are not available to them. Here we model three types of plasticity that we call "constrained" and "unconstrained" and "constant". Constrained strategies require post drug conversion rates to be directly related to conversion rates earlier in the infection, thus making the biological assumption that parasites cannot dramatically shift their conversion rate in time (i.e. the resulting conversion profiles are smooth and without step-changes). In contrast, unconstrained strategies allow parasites to settle on a completely new conversion profile after drug treatment, thus allowing for step-changes and not requiring the conversion rate after drug treatment to be dependent on conversion before drug treatment. Finally, constant strategies allow parasites to settle on a global, constant (i.e. not variable with time) conversion rate after drug treatment, and as in unconstrained plasticity, independently of the conversion rate achieved before drug treatment.

### 3.2.2 Constrained strategy: Cubic spline with a knot

Assuming an infection length of 20 days and drug-treatment to occur on day 11 and 12 PI, we first use one of the in-built features of splines, the possibility of defining a "knot" at a given time point post-infection, which allows the spline to change its behaviour from that point onwards. If the time-variable conversion rate $c(t)$ is defined by a a cubic spline with a knot, it takes the following form:

$$c(t) = \exp(-\exp(j\ t^3\ +\ k\ t^2\ +\ l\ t\ +\ m +\ n\ (t-11)^3)), \tag{3.1}$$

where $t$ is time post-infection, $j$, $k$, $l$ and $m$ are the parameters associated with a standard cubic spline and determine the behaviour of the spline before the knot, $n$ is the parameter associated with the knot, defined at day 11 post-infection, and determines spline behaviour after the knot. We therefore first find the optimal values of $j$, $k$, $l$, $m$ and $n$ that define the optimal conversion profile along the whole infection in the absence of drugs, giving us the green line in in figure 3.2A. To reflect the fact that conversion should change only after drug treatment on day 11, we keep $j$, $k$, $l$ and $m$ fixed to their value in absence of drugs (thus assuring that the conversion profile stays the same for all drug doses before treatment) and run further optimisation, in the presence of drugs, to find the optimal value of the knot parameter $n$ for a given drug dose. All parameters (in the absence of drugs) or the knot-parameter (in presence of drugs) were set to an arbitrary starting value of 0.5 before each optimisation. Conversion rates must be constrained to vary between zero and one, so we take the complimentary log-log of the value specified by the spline. We also estimated the total fitness (see below) obtained for each of these strategies. Because the resulting conversion strategy will be constrained by the mathematical requirement for smoothness (each point of the curve must be C2-differentiable, i.e. a second-order derivative must exist at each domain of the function), we call it the "constrained strategy".

### 3.2.3 Unconstrained strategy: Disjointed splines and time-constant conversion

One issue with the knot-approach used for constrained plasticity is that the behaviour of the spline after drug treatment is constrained to be a smooth function. This likely limits the power of identifying alternative conversion strategies that parasites could use in response to drugs, for example when a sudden switch in conversion would provide greater fitness returns. Assuming that the level of conversion rate adjustment is not strongly constrained by previous conversion rates, we allow parasites to choose their conversion rate profile more freely (thus calling it the "unconstrained strategy"). To do so, we

subdivide the infection into two periods, for pre- and post-drug treatment, and use two separate splines to optimise the conversion rate profile in each of those periods. For pre- and post drug treatment, we define time-variable conversion, $c(t)$, as a cubic spline, taking the following form:

$$c(t) = \exp(-\exp(j\ t^3\ +\ k\ t^2\ +\ l\ t\ +\ m)), \tag{3.2}$$

where $t$ is time post-infection, and $j$, $k$, $l$ and $m$ are the parameters associated with a cubic spline. For both, pre- and post-drug treatment splines, these parameters are optimised separately. However, these two disjointed splines are not completely independent since the initial conditions for the post-drug spline (i.e. the starting densities of red blood cells, infected red blood cells, merozoites and gametocytes) are equal to the values of the end-point of the first spline (i.e. shortly before drug treatment). We define the pre-drug spline to be the optimal cubic spline for an untreated infection (i.e. equivalent to the green line in figure 3.1), optimised for the whole infection length (of 20 days) but cut off shortly before day 11 (i.e. corresponding to the intersection of the green line with the grey bar in figure 3.1). The post-drug treatment is a cubic spline, optimised for the last 9 days of the infection only, with simulated drug treatment (various doses between 0 and 15mg/kg) on the first two days of the post-drug period (i.e. corresponding to days 11 and 12 for the full infection). As before, all parameters were set to an arbitrary starting value of 0.5 before each optimisation. For each strategy, the total fitness (see below) was calculated.
Finally, we also derive a spline-independent and time-constant conversion rate post-drug treatment to test whether the same general tendencies can be confirmed. As for the method described in this section we assume that the pre-drug conversion is variable and identical to the optimal cubic spline for an untreated infection (i.e. equivalent to the green line in figure 3.1), optimised for the whole infection length (of 20 days) but cut off shortly before day 11. We then chose the function `optimise` in R to derive the best global conversion rate post-treatment for all drug doses between 0 and 15mg/kg of drugs, setting the starting densities of red blood cells, infected red blood cells, merozoites and

gametocytes to the values before drug-treatment.

### 3.2.4 Fitness comparisons and costs

For each conversion rate strategy, we plot the resulting dynamics of infected red blood cells (asexual parasites) and gametocytes. As a fitness measure of a parasite in an infection, we calculate the "cumulative transmission potential" across the whole infection. This metric translates daily estimates of gametocyte density into the probability of infecting mosquito, assuming mosquitoes are abundant and biting hosts on a regular basis. The relationship between gametocyte densities and transmission probability is assumed to be sigmoidal, as has been experimentally derived for *P. chabaudi* by Bell et al. (2012) and is described in detail in chapter 2. For example, a parasite that transmits every day (i.e. which has a daily transmission probability of 1) reaches a cumulative transmission potential of 20 after 20 days of simulated infection. Thus the cumulative transmission potential reached on day 20 by a parasite using a given strategy in the face of a certain drug treatment is a measure of the final fitness reached (thus also referred to as "total fitness" in this chapter). To confirm that the obtained optimal conversion profiles perform better in their given drug environment than other strategies, we simulate the performance of all drug-strategies in all drug-environments (0, 5 and 15 mg/kg). This also allows us to estimate the relative benefits of using the optimal strategy for a given drug environment compared to using a non-optimal strategy.

Because different modelling approaches imply different assumptions about the biological mechanism of conversion rate variation (constitutive, constrained, unconstrained and time-constant), a key question is which approach returns the highest fitness in a given drug environment. We therefore plot the fitness obtained through four different modelling approaches: a time-variable constitutive conversion profile (from chapter 2), the constrained plastic strategy (spline with a knot, in this chapter) and an unconstrained plastic strategy (disjointed splines, in this chapter) and optimal constant conversion (post-treatment, in this chapter).

We also construct a model to explore under which conditions plastic strategies should prevail in a population of hosts and under which conditions constitutive strategies should take over. Focusing on unconstrained plasticity only, we assume that parasites using plastic strategies can always adopt the corresponding optimal strategy whereas constitutive strategies cannot be adjusted to the drug environment. We then derive the expected ("E") fitness of a constitutive or plastic strategy in function of the frequency of a given drug treatment, assuming that all hosts in a population receive either 5 or 15 mg/kg, with the following equation (see appendix B):

$$E(\text{fitness of constitutive strategy}) = a \times f + b \times (1 - f)$$

$$E(\text{fitness of plastic strategy}) = c \times f + d \times (1 - f)$$

Where $a$ is the fitness obtained by the optimal constitutive strategy in a drug-treated host (with either 5 or 15 mg/kg), $b$ is the fitness obtained by that same drug-adapted strategy in an untreated host, $c$ is the fitness of the optimal plastic strategy in a drug treated host, $d$ is the optimal plastic strategy in a untreated host, and $f$ is the frequency of drug treatment. As fitness values we use the final cumulative transmission potential for a given strategy in a given drug environment. Finally, we calculate the expected fitness for each strategy across a range of $f$ values to derive the conditions under which a given constitutive or plastic strategies should be favoured.

## 3.3 Results

### 3.3.1 Plastic optimal conversion profiles

#### Conversion profiles

The optimal conversion profiles obtained by constrained and unconstrained plasticity for doses 0, 5 and 15mg/kg are shown in figure 3.2. For a dose of 5 mg/kg (purple), parasites adopt reproductive restraint soon after drug treatment in both modelling approaches, with conversion rates being up to 25% lower in

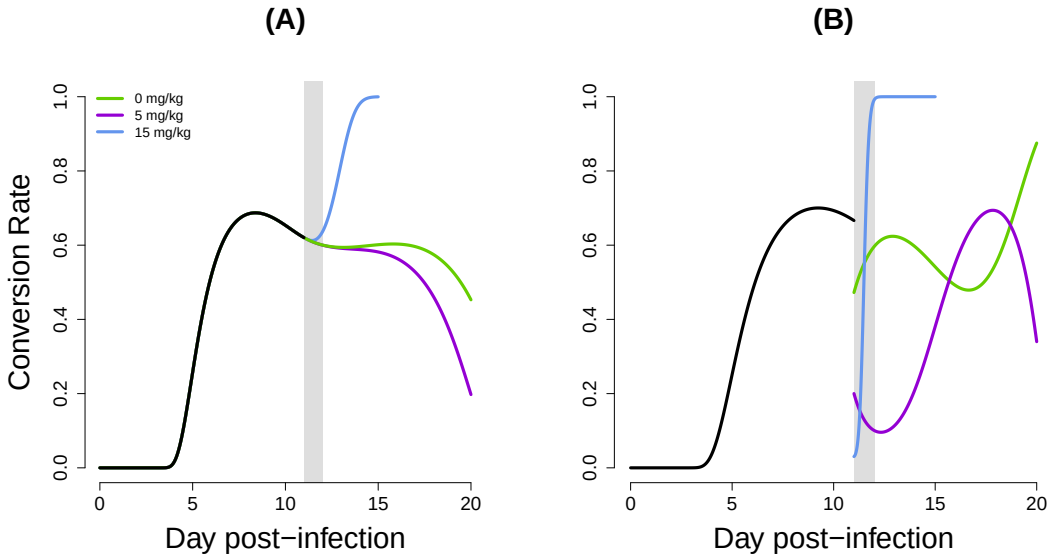


Figure 3.2. Optimal plastic conversion profiles for (A) constrained plasticity and (B) unconstrained plasticity. The grey bar indicates the period of drug treatment.

the constrained strategy and 50 % lower in the unconstrained strategy, compared to the best strategies for an untreated infection. Note, that in unconstrained strategies, the conversion rate in untreated infections (green line in Fig.3.2B) also falls (by about 20%). For both constrained and unconstrained plasticity, high drug doses select for terminal investment immediately after drug treatment (Fig.3.2 blue line). For optimal constant conversion rates, we find that low drug doses select for reproductive restraint, whereas drug doses above 9 mg/kg select for higher investment (Fig.3.3A). More specifically, conversion in the presence of 5 mg/kg of drugs is 33% lower than in untreated infections whereas the optimal conversion with 15 mg/kg of drugs is about 13% higher.

### Fitness consequences

Plots of the cumulative transmission potential against day post-infection (Fig.3.4 and Fig.3.6A) reveal that parasites benefit from plastically responding to drug treatment, and that these benefits are larger for unconstrained plasticity (Fig.3.4B). Specifically, we compare the fitness gained from the optimal strategy for each dose (solid lines) to the fitness returns from adopting the best strategy for untreated infections (i.e. the fitness of parasites that do not respond to

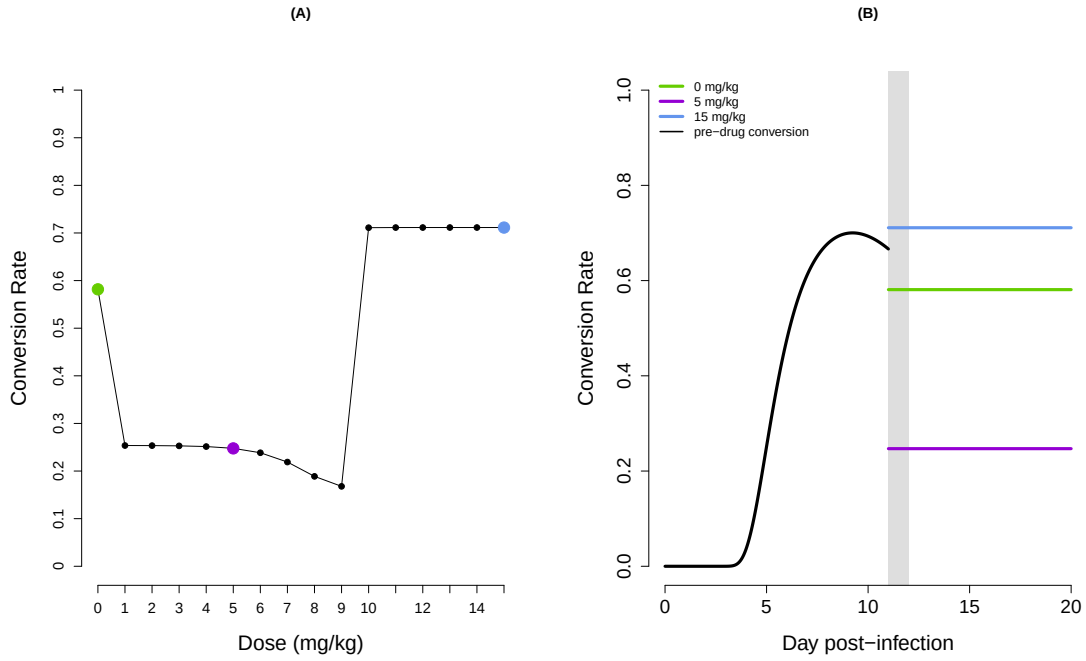


Figure 3.3. (A) Optimal global, time-constant conversion rates decrease for low drug doses but increase for drug doses over 9 mg/kg. The coloured dots highlight conversion rates associated with three representative doses (0, 5 and 15 mg/kg) used in the remainder of the analysis. (B) The reaction norm along time post-infection of the chosen doses. After drug-treatment, parasites settle on different optimal time-constant conversion rates for the remainder of the infection. The grey area indicates the days of drug treatment.

drugs, coloured dashed lines). Responding to drugs with a dose-specific reaction norm enhances fitness for unconstrained plasticity whereas the fitness benefits of constrained plasticity are minor (the cumulative transmission potential for both low and high drug doses largely overlap with the fitness returns of not responding (Fig.3.4A). A global, time-constant conversion provides significant fitness benefits only in the case of low doses of drugs (Fig.3.6B).

These differences are confirmed by comparing the total fitness gained by adopting each strategy in response to each drug dose (Fig.3.5 A and B and Fig.3.6B). For both, constrained and unconstrained plasticity as well as constant conversion (post-treatment), terminal investment brings only minor fitness returns ($< 0.5$ additional days of transmission) compared to the optimal strategy for untreated infections. Terminal investment in untreated or low drug environments generates substantial fitness costs, due to early termination of the infection (between 6 and 7 days of transmission lost compared to the optimal strategy). Reproductive restraint via unconstrained plasticity brings substantial fitness returns ($> 2.5$ days of transmission) but constrained plasticity only returns $< 0.5$ days of transmission. Adopting reproductive restraint in an untreated infection is costlier for unconstrained plasticity (a loss of 1.9 days of transmission) than for constrained plasticity (a loss of $< 0.1$ days of transmission) (Fig.3.5A and B, 5mg/kg). Adopting reproductive restraint against a high drug dose does not return any significant fitness benefits compared to using a strategy for untreated infections. In summary, in drug-treated infections, constrained plasticity brings barely any fitness benefits compared to using strategies for untreated infections, but unconstrained plasticity may return significant fitness benefits in low-dose drug treatment. The same accounts for constant conversion (post-treatment), where reproductive restraint returns significant fitness benefits compared to a non-drug strategy during treatment with low doses but only minor benefits in the case of high drug doses (Fig.3.6B).

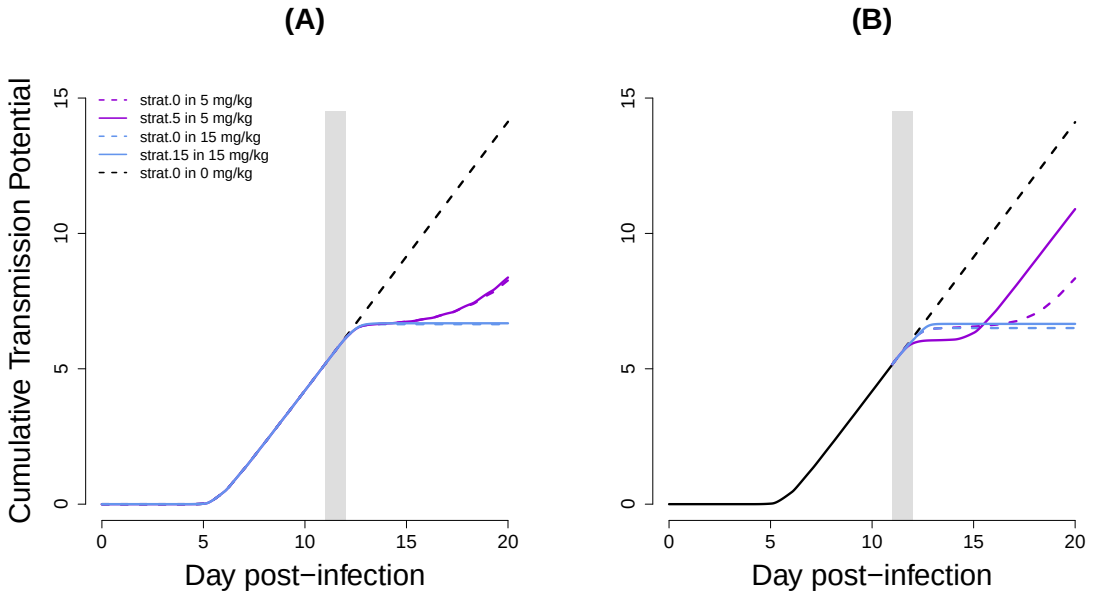


Figure 3.4. Cumulative transmission potential during the infection for (A) constrained plasticity and (B) unconstrained plasticity. The best plastic strategies for each dose are shown in solid lines whereas parasites that do not adjust their conversion in response to drugs are illustrated with a dashed line. The cumulative transmission potential of the best strategy in an untreated infection is shown by a black dashed line. The grey bar indicates the period of drug treatment.

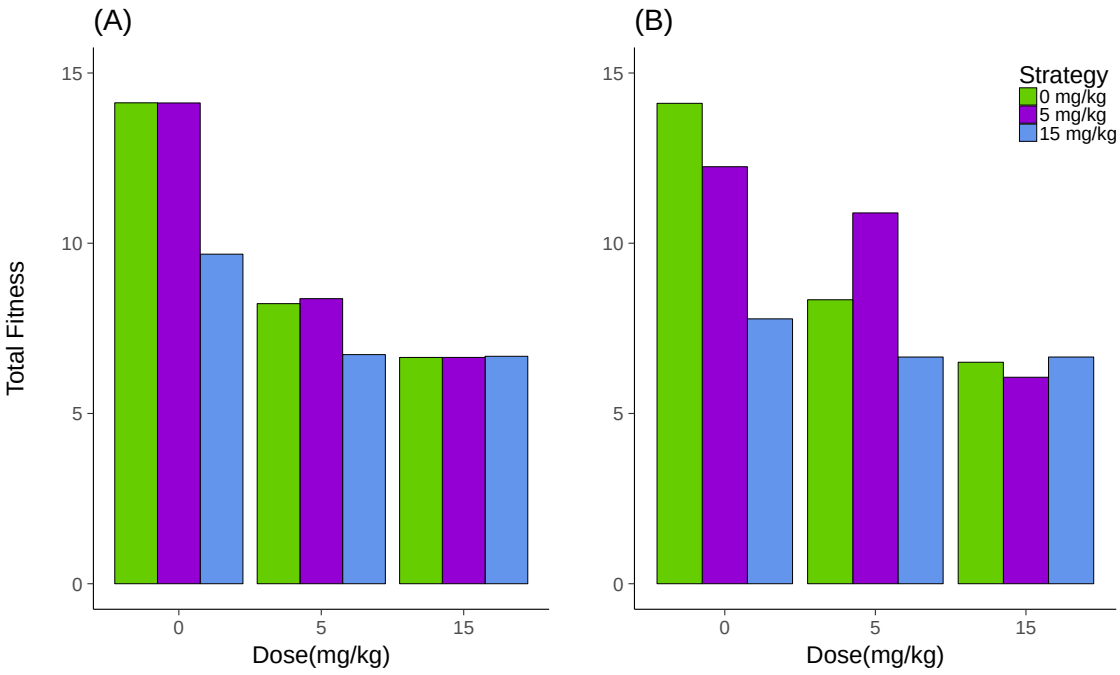


Figure 3.5. Total fitness (i.e. the cumulative transmission potential at day 20 PI) gained by dose-specific optimal strategies and non-optimal strategies for each drug dose for (A) constrained plasticity and (B) unconstrained plasticity.

### 3.3.2 Comparing plastic strategies with constitutive conversion profiles

#### Conversion profiles

For clarity, we mainly focus on constrained and unconstrained time-variable plastic strategies and how they compare to constitutive conversion profiles. The dynamics for time-constant conversion are given in appendix C (Fig.C.7). Figure 3.7 shows the conversion profiles for untreated (Fig.3.7 A) and treated infections (5mg/kg, Fig.3.7B; and 15mg/kg, Fig.3.7C) for constrained and unconstrained plasticity and the constitutive strategies obtained in chapter 2. All show reproductive restraint in a low-dose drug environment and early terminal investment in a high-dose drug environment. Reproductive restraint via constitutive strategies involves reducing conversion by up to 60%, which is similar to unconstrained plasticity (reduction of 50%), whereas in plastic strategies conversion is reduced by 25% only. All strategies reach conversion

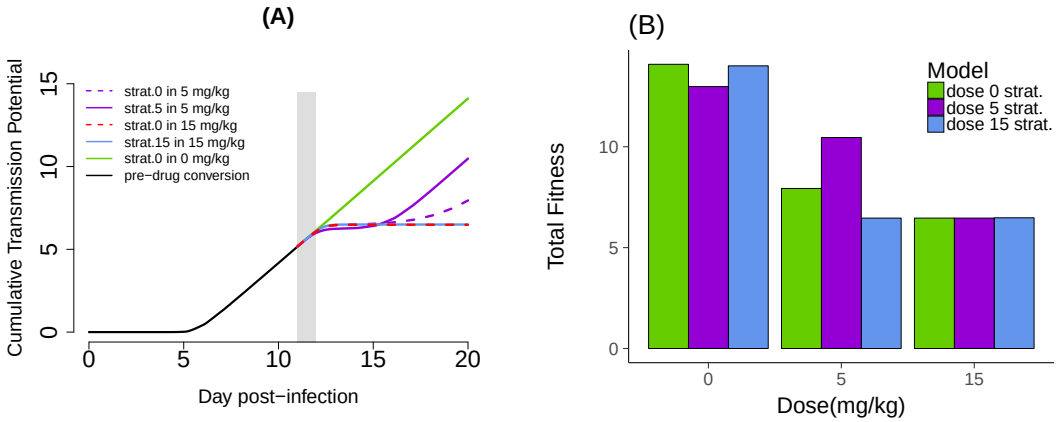


Figure 3.6. (A) Cumulative transmission potential with constant conversion post-drug treatment. Even in the constant conversion case, lowering the conversion provides significant fitness benefits in the case of low drug doses (compare solid and broken purple line) whereas the fitness benefits during treatment with higher drug doses is less obvious (compare red and blue line). (B) Total fitness (i.e. the cumulative transmission potential at day 20 PI) gained by optimal and non-optimal strategies with three different drug treatments.

rates of 99.9% shortly after drug treatment, with infections cleared by day 15, Fig.3.7C). Constitutive profiles adapted to untreated or low-dose infections and unconstrained plasticity in untreated infections end with a terminal investment. This is expected because simulated infections have a pre-determined end point and so, parasites should always proceed to terminal investment. In contrast, conversion profiles for constrained and unconstrained plasticity do not reach terminal investment in a low drug environment, which is likely a consequence of constraints related to spline-flexibility during a relatively short simulation time (9 days only, after drug treatment).

In response to low drug doses, the consequences of preemptive conversion adjustment in constitutive responses is highlighted by the 2.5x (log scale) increase in asexual numbers (red) at the time of treatment compared to plastic strategies (blue and yellow, Fig.3.7 E and F). However, the asexual dynamics of unconstrained strategies (yellow) soon recover to the same level as constitutive responses. The dynamics of gametocyte densities (Fig.3.7 G-I) behave similarly

for the constitutive profile and the unconstrained plastic strategy and gametocyte numbers quickly recover in these infections. In high-dose infections, asexual densities in constitutive strategies also lie above those of constrained and unconstrained strategies before treatment, with the aim of converting these additional asexuals into gametocytes during terminal investment. After drug treatment, both asexual and gametocyte densities, decrease rapidly in all three strategies, clearing the infection by day 15 PI.

**Fitness consequences**

Cumulative transmission potential of all optimal strategies for untreated infections are very similar (Fig.3.8A). For low drug dose infections, the optimal constitutive profile and unconstrained plastic strategy share similar transmission profiles and exceed transmission from constrained plasticity (Fig.3.8B). Specifically, preempting low-dose drug treatment (as constitutive strategies do) yields one extra day of transmission compared to a unconstrained plastic response and 3.5 days more than constrained plasticity. In contrast, for high drug doses, the optimal constitutive profile (Fig.3.8C) gains 0.7 days of transmission compared to both optimal plastic strategies which share similar transmission profiles. Total fitness (i.e. the endpoints of Fig.3.8) of the optimal strategies for their respective doses are summarised in Fig.3.9. Again, it is clear that constrained plasticity performs less well than unconstrained plasticity, especially in response to low-dose drug treatment. Interestingly, even time-constant reproductive restraint performs better than constrained plasticity in a low-dose drug environment, whereas a constant conversion strategy performs worse in all other drug environments. Parasites that use constitutive conversion profiles can prepare in advance, and so, reach higher fitness in all drug environments.
The costs of using a non-optimal strategy against a given drug treatment for all our modelling approaches are in appendix C, figure C.6 A-C. Briefly, they confirm that: (i) In untreated infections, reproductive restraint entrails a cost of around 2 days of transmission for constitutive and unconstrained plastic responses, but $< 0.1$ days for constrained plastic responses. And mistakenly adopting terminal

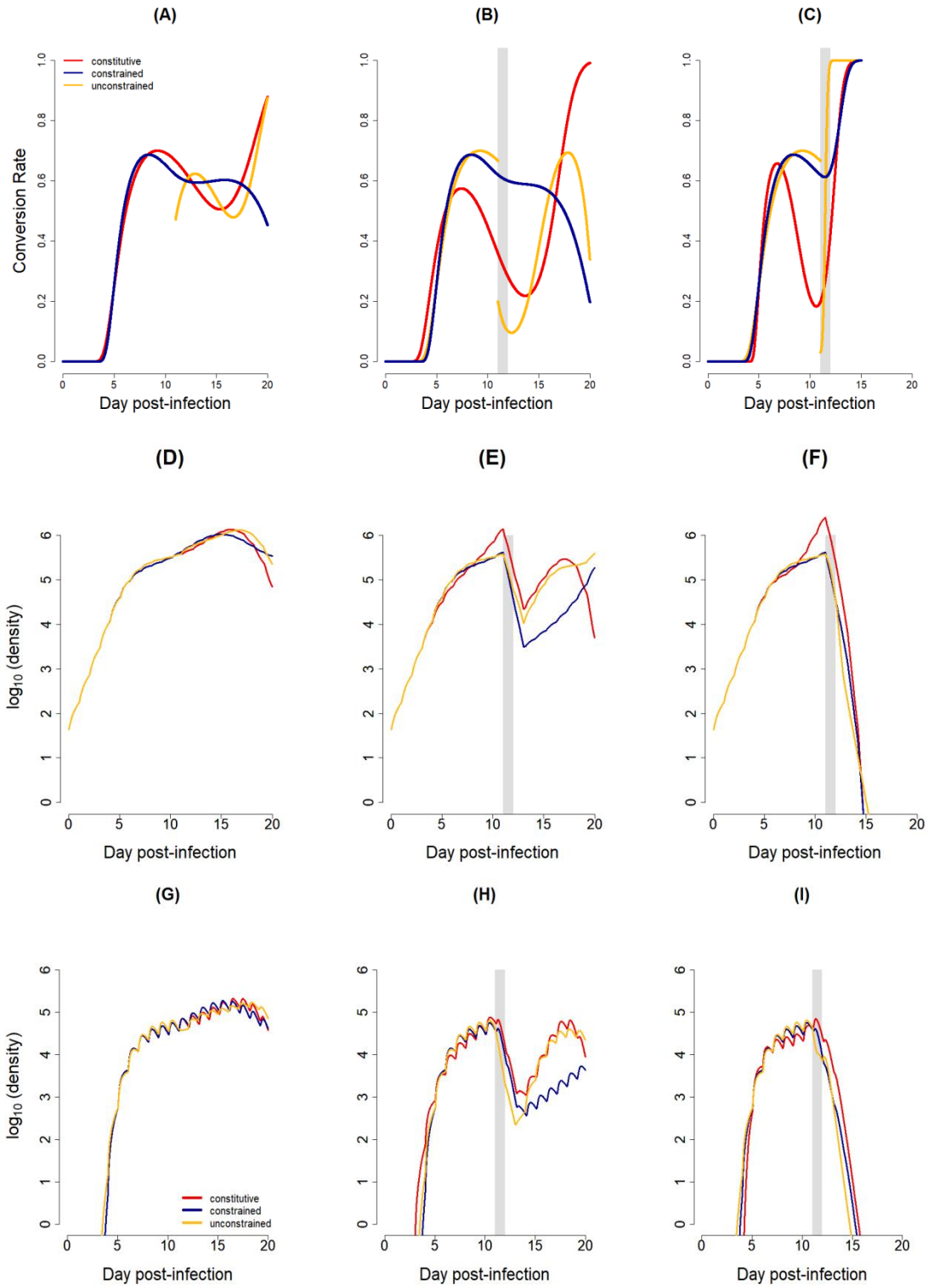


Figure 3.7. Conversion rate profiles (top row) for different strategies (constitutive: red, constrained plasticity: blue, unconstrained plasticity: orange). Columns correspond to drug dose: untreated infections (A), low dose (5 mg/kg, B) and high dose (15 mg/kg, C). The corresponding dynamics for asexual stages (D-F) and gametocytes (G-I) are given in the rows below. The grey bar indicates drug treatment.

investment is the most costly for unconstrained plasticity, causing a loss of 4.4 (constrained) and 6.3 days of transmission (unconstrained). (ii) In response to low drug doses, there is little difference in absolute fitness between adopting the strategies adapted to untreated infections but is similarly costly in constitutive and unconstrained responses (a loss of 2.5-3 days of transmission) but less costly for constrained plasticity (0.1 days). Mistakenly adopting terminal investment is similarly costly for unconstrained plasticity and constitutive conversion, causing a loss of 4.2 days of transmission compared to a cost of unconstrained terminal investment of 1.5 days. (ii) In response to high drug doses, fitness lost is minimal if parasites adopt non-optimal restrained plastic strategies during constitutive strategies and constrained plasticity. In contrast, for unconstrained plasticity, adopting reproductive restraint costs more fitness (0.6 days of transmission) than the strategy for untreated infections (0.15 days of transmission) and vice-versa for constitutive profiles.

Using some of the estimated fitnesses from above, we calculate the expected fitness of a constitutive strategy adapted to a low or high drug environment, in a host population with a particular frequency of drug treatment and compare it to the range of fitness that an unconstrained plastic strategy would obtain in the same host population (Fig.3.10). For low drug doses, we find that a plastic strategy has superior fitness if less than 70% of hosts are treated, while constitutive strategies have higher fitness for more frequent drug treatment in the population. For high drug doses, at least 85 % of hosts must be treated for constitutive high-dose strategies to spread, whereas plastic strategies reach higher expected fitness if treatment reaches less than 85% in the host population.

## 3.4 Discussion

Empirical evidence suggests that parasites respond to drug treatment with reproductive restraint and terminal investment to partially compensate for the fitness lost due to drug-killing (Buckling et al. 1999; Reece et al. 2010). Chapter 2 of this thesis has established that if parasites evolve drug-adapted

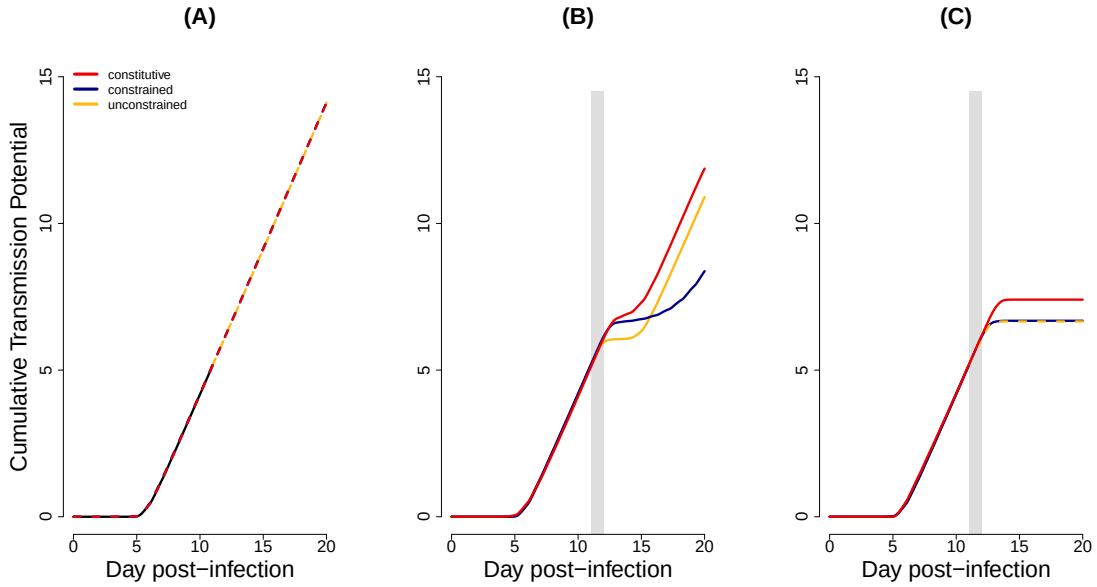


Figure 3.8. Cumulative transmission potential in (A) untreated infections, (B) infections treated with 5 mg/kg and (C) 15 mg/kg, for each conversion strategy (constitutive: red, constrained plasticity: blue, unconstrained plasticity: orange). The grey bar indicates days of drug treatment.

conversion rates (constitutive profiles), these would indeed be characterised by reproductive restraint for low drug doses or early terminal investment in the case of high drug doses. However, whilst constitutive conversion rates are predicted to vary day-to-day within each profile (so in a sense, are phenotypically plastic), the across infection patterns are pre-determined at the beginning of infections. Thus, a constitutive strategy means a parasite can only adopt the profile corresponding to the dose that it has evolved for. Notably, these profiles involve patterns of conversion diverging from untreated infections in advance of drug treatment being administered, preparing parasites for drug treatment and giving them significant fitness benefits. Here, we use three different approaches to show that reproductive restraint and terminal investment are also strategies that should evolve if parasites adjust their conversion rates in real time (i.e. classical phenotypic plasticity) in direct response to the appearance of drugs, at any dose. We either allow parasites to undergo dramatic shifts in their conversion rate at the point of drug-treatment (unconstrained plasticity and

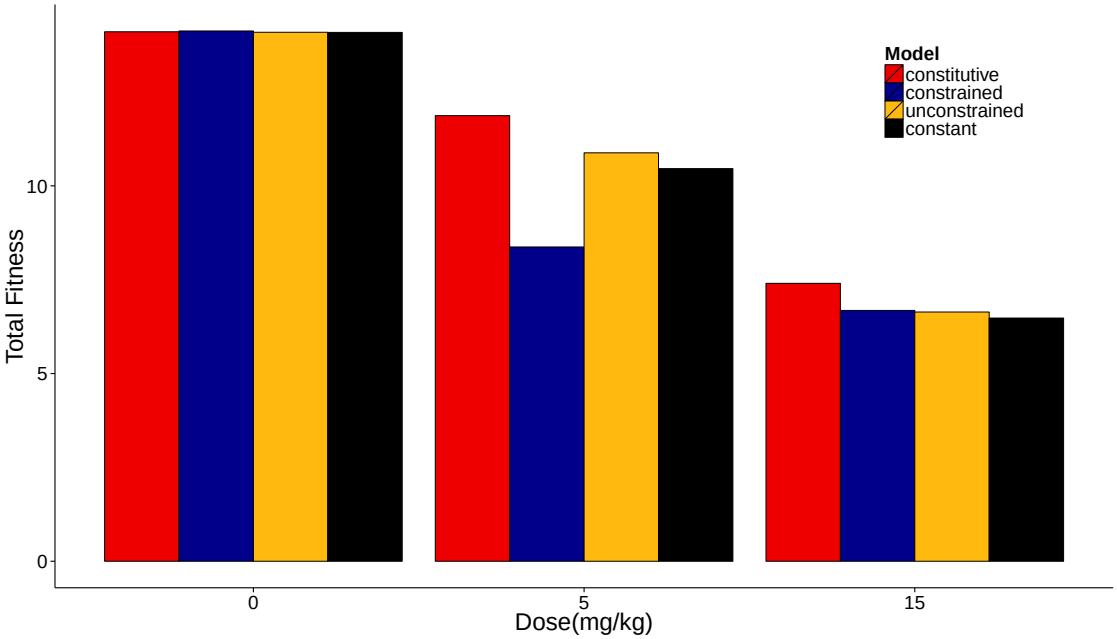


Figure 3.9. Total fitness returns (cumulative transmission potential on day 20 PI) from the dose-specific optimal reaction norms for each type of strategy in their respective drug treatments. The constitutive strategy performs best in each drug environment (red). The unconstrained plastic strategy performs better than the constrained strategy in the 5 mg/kg drug environment whereas the constrained strategy performs marginally better than the constrained strategy in a high drug dose environment. A time-constant strategy (black) provides higher fitness returns than constrained plastic strategies with low drug treatment but performs slightly worse than all other strategies in all other drug environments.

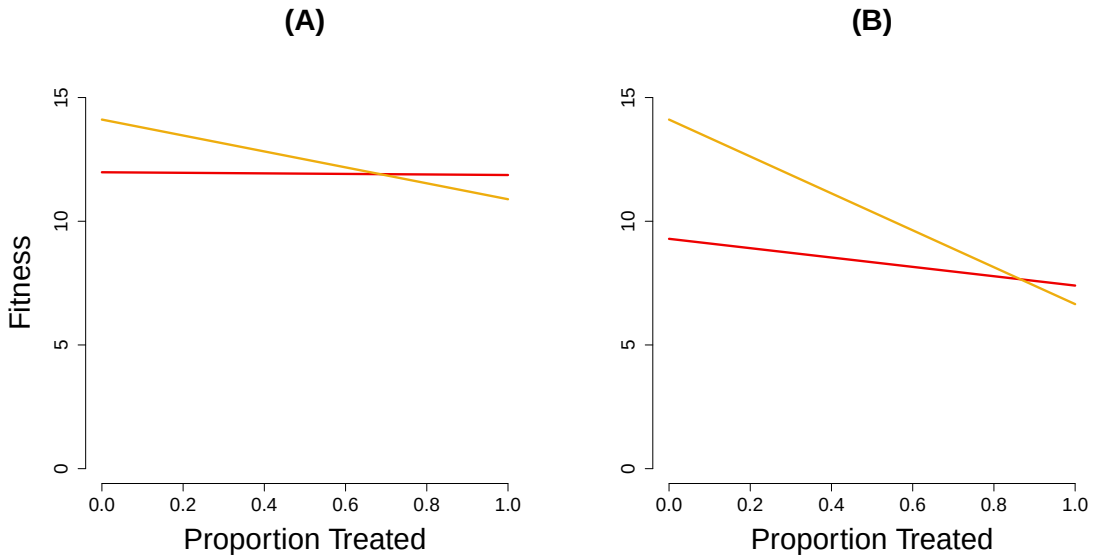


Figure 3.10. Expected fitness for constitutive (red) and unconstrained plastic strategies (yellow) for different frequencies of drug treatment in the host population, with either (A) low drug doses (5mg/kg) and (B) 15 mg/kg. At least 70% of hosts must be treated with low drug doses and at least 85% of hosts with high drug doses for constitutive strategies to reach higher expected fitnesses than plastic strategies.

constant conversion) or only allow parasites to tune conversion slightly after drug treatment (constrained plasticity). Our results reveal that parasites only receive significant fitness benefits if they can adjust their conversion dramatically i.e., adopt unconstrained plasticity. This is especially evident for reproductive restraint, where unconstrained plasticity delivers significant fitness benefits (giving an extra 2.6 days of transmission compared to the optimal reaction norm for untreated infections) but the constrained conversion profile delivers less than 0.1 days of extra fitness. We also show that even if parasites are allowed to settle on a time-constant conversion after drug treatment, their fitness could benefit in the case of low drug doses with associated reproductive restraint. This provides further evidence that plastically adjusting conversion only provides fitness benefits if it can be quickly adjusted to a level unrelated to pre-drug conversion.

These three modelling approaches have methodological advantages and disadvantages. The constrained plasticity model uses an in-built feature of a spline that allows the profile for conversion before drug treatment to be fixed, which exactly fits the need for modelling a plastic response to environmental change. However, the requirement of the function to be smooth likely reduces the possible plastic conversion rate responses that can be obtained through this method. Accordingly, we only find three distinguishable responses, one for untreated environments, one for reproductive restraint (doses below 8 mg/kg) and one for early terminal investment (doses at or above 8 mg/kg). In contrast, the unconstrained approach partially lifts the restriction by allowing parasites to choose a different spline after drug treatment. Thus, the unconstrained method yields more diverse conversion profiles post drugs (dose-specific profiles in which restraint increases with dose up to 10 mg/kg when strategies switch to terminal investment) than the knot-model. Further work is required to determine whether biological reality is best captured by our unconstrained approach but we note that in *P. chabaudi*, conversion rates can vary suddenly, and are not obviously related to conversion at a previous time-point (Greischar et al. 2016b). Finally, we note that the conclusions from the constant conversion strategies are

identical to those of other approaches, and the level of fitness obtained is similar to unconstrained plastic responses, despite the fact that chapter 2 and other work (Greischar et al. 2016a) has shown that parasites in simulated infections generally gain more fitness if they are able to vary their conversion over time. However, some behaviours like full-scale terminal investment cannot be obtained if conversion is assumed to be constant over time because the optimal value is inherently a compromise between the various selection pressures that operate on conversion during infection (discussed in more detail in chapter 2).

We now focus on comparing unconstrained plastic responses to constitutive responses (from chapter 2) because unconstrained strategies outperform constrained plastic strategies. Reproductive restraint for constitutive and unconstrained strategies returns similar fitness, suggesting that preparing in advance for drugs only delivers one additional day of transmission. For high doses, parasites using constitutive strategies are able to prepare for terminal investment by building up asexual numbers in advance. Although this option is not available to plastic parasites, they still obtain about 90% of the fitness of the constitutive strategy. Optimal constitutive strategy always outperforms unconstrained plasticity, but constitutive strategies cost fitness if deployed in a different environment than they evolved for, so which type of strategy should parasites adopt? Unconstrained plasticity delivers fitness benefits in low drug dose environments that are almost as high as the ones obtained by a constitutive strategy. Thus, the advantage of a constitutive strategy is likely to be eroded by the fitness costs paid if parasites encounter unexpected drug doses, treatment timings or durations. Indeed, we find that at least 70% of hosts must be treated with low doses or 85% treated with high doses for the corresponding constitutive strategies to spread. Thus, despite constitutive strategies having higher fitness in each treated environment to which they are adapted, they can only sustain themselves in a population in which drug treatment rates are very high. Treatment rates in most transmission settings are however generally much lower than these thresholds, with treatment rate for children under 5 alone estimated between 12-22% only (WHO 2016). Thus, there is good reason

to expect that parasites in most transmission settings would rely on plastic strategies rather than constitutive strategies.

The evolution of plasticity in a trait is expected under certain conditions (Ghalambor et al. 2007; Lande 1986; Levins 1968; Scheiner 1993; Schlichting and Smith 2002): i) the environment is highly variable in time and/or space, ii) each environmental state is reliably associated with cues, iii) different phenotypes are favoured by selection across different environments, iv) the strength of selection is similar across environments, v) there is no phenotype that has superior fitness across all environments, and vi) the cost of plasticity is low. Plastic conversion of malaria parasites against drugs may satisfy these criteria:

i) the probability of a host receiving drugs, or the timing or dose of drugs, varies and a given parasite genotype will encounter different types of host because its vector decides which host to inject the parasite into,

ii) the effects of drugs are reliably associated with cues that parasite can detect (e.g. parasite death rates or replication rates),

iii) different conversion rates are favoured by selection across different environments (our three representative drug environments),

iv) although there is little doubt that selection is stronger in drug-treated infections, experimental evidence suggests that malaria parasites can survive drug treatment through plastic modification of other life-history traits such as cell dormancy (Teuscher et al. 2010). Furthermore, for very strong selection differentials between environments, one may expect genetic specialisation rather than plasticity to evolve (Scheiner 1993), and there is indeed abundant evidence of genetic resistance mutations against antimalarial drugs,

v) all constitutive strategies modelled suffer some fitness costs if deployed in response to a drug dose they are not adapted for (though, reproductive restraint is the least costly),

vi) we do not consider the traditional costs of plasticity (i.e. maintenance of the plastic machinery, of acquiring information about the environment, of coordinating a conversion response across the within-host parasite population Auld et al. 2010). However, the implicit cost of using a plastic strategy is that

parasites cannot pre-empt drug treatment and the best-case scenario (from the parasites' point of view), is adjusting conversion immediately after treatment. Any time lags in responding will further exacerbate the fitness differences between constitutive and plastic strategies. Time-lags should be empirically tractable thanks to the availability of superior methods to estimate conversion (Greischar et al. 2016b). We also consider the costs associated with misdirecting a plastic phenotype (local, phenotypic cost, *sensu* Murren et al. (2015)), and if parasites could be prevented from altering conversion then the costs of not adopting reproductive restraint (or terminal investment) in response to low (and high) doses infections could be quantified.

A further consideration is that, in addition to drug treatment, parasites should also adjust conversion rates to cope with other within-host stressors, such as competition between coinfecting genotypes (Greischar et al. 2016a; Pollitt et al. 2011b), and variation in red blood cell (RBC) availability (chapter 4). It seems remote that a constitutive conversion profile adapted to a particular drug dose will coincidentally maximise fitness in the face of RBC dynamics experienced during infections and the likelihood of encountering within-host competition. Moreover, given that drugs are a relatively new selection pressure, we suspect that empirical observations of parasite responses to drugs are actually the result of co-opting plastic strategies to cope with RBC dynamics and competition (Carter et al. 2013; Reece et al. 2009).

In conclusion, we show that plastic conversion rate strategies, characterised by reproductive restraint and terminal investment, can be co-opted by parasites to partially compensate for the fitness costs of drug pressure. We also find that such strategies return the largest fitness benefits if parasites can drastically modify their conversion following drugs treatment. If parasites can do so, plastic strategies are expected to be dominant across most transmission settings. Our findings support empirical observations but available data only relate to part of the reaction norm: experimental data that show that parasites adopt a range of conversion rates between restraint and terminal investment are required. Further modelling work should consider how the benefits of plasticity

in conversion interact with other life-history traits selected for by drugs, such as virulence (Schneider et al. 2012) and dormancy (Hott et al. 2015; Teuscher et al. 2010). Should parasites rely on a suite of life-history traits to cope with drugs or is plastic conversion sufficient? How does the evolution of life-histories interact with the evolution of genetically encoded drug resistance? For example, reproductive restraint may increase the size of the pool of genomes in which beneficial mutations can occur. Furthermore, can parasites evolve conversion strategies against particular types of drug? For example, regardless of dose, reproductive restraint may be the best strategy against drugs that have a gametocytocidal effect, like artemisinin. Finally, reliable methods are needed to assess the relevance of plastic conversion rates in natural human infections. For example, can the signature of reproductive restraint be detected from a drug clearance curves? If human malaria parasites adopt reproductive restraint in response to drugs, then suboptimally treated infections are even harder to clear than clearance curves derived from high-dose treatments would predict.

# Chapter 4

# Phenotypic plasticity in reproductive effort: malaria parasites respond to resource availability

## Abstract

The trade-off between survival and reproduction is fundamental in the life-history of all sexually reproducing organisms. This includes malaria parasites, which rely on asexually replicating stages for within-host survival and on sexually reproducing stages (gametocytes) for between-host transmission. The proportion of asexual stages that form gametocytes (reproductive effort) varies during infections – i.e., is phenotypically plastic – in response to changes in a number of within-host factors, including anaemia. However, how the density and age structure of red blood cell resources shape plasticity in reproductive effort and impacts upon parasite fitness, is controversial. Here, we examine how and why the rodent malaria parasite *Plasmodium chabaudi* alters its reproductive effort in response to experimental perturbations of the density and age structure of red blood cells. We show that all four of the genotypes studied increase reproductive

effort when the proportion of red blood cells that are immature is elevated during host anaemia, and that the responses of the genotypes differ. We propose that anaemia (counterintuitively) generates a resource-rich environment in which parasites can afford to allocate more energy to reproduction (i.e. transmission) and that anaemia also exposes genetic variation to selection. From an applied perspective, adaptive plasticity in parasite reproductive effort could explain the maintenance of genetic variation for virulence and why anaemia is often observed as a risk factor for transmission in human infections.

## 4.1 Introduction

Parasites are exposed to rapid and extensive variation in the environmental conditions they experience inside their hosts and vectors, both during infections and across different hosts (e.g. dynamic immune responses, competition with other parasites, fluctuations in the availability and quality of resources). Therefore, parasites, like any organism which experiences frequent environmental change, could use phenotypic plasticity – the ability to match phenotypes to different environmental conditions – to maintain their fitness in the face of changing conditions (Roff 1992; Stearns 1992). However, because parasites have traditionally been viewed as organisms with inflexible strategies, their environmental sensing mechanisms are assumed to be directed towards maintaining homeostasis (Mideo and Reece 2012). Thus, adaptive plasticity in parasites has generally been overlooked in evolutionary biology and is controversial in applied bioscience (Kochin et al. 2010a; Stearns and Koella 2007). For malaria parasites (*Plasmodium* spp.), there is mounting evidence that allocation to within-host growth versus between-host transmission is phenotypically plastic (Birget et al. 2017b; Carter et al. 2013). Malaria parasites rely on asexually replicating stages for within-host survival and on sexually reproducing stages (gametocytes) for between-host transmission. Therefore, for malaria and all other parasites that use distinct stages for transmission and within-host replication (e.g. trypanosomes) between-host transmission

is equivalent to "reproduction" of an infection and within-host replication determines the "survival" of an infection (Carter et al. 2013; Pollitt et al. 2011a; Reece et al. 2009). The proportion of asexual stages in each cycle of replication that commit to forming gametocytes represents the parasite's reproductive effort (called the "conversion rate" in parasitology). Why conversion rates are generally low but highly variable in malaria parasites are long-standing questions in parasitology (Carter et al. 2013; Taylor and Read 1997) and explaining plasticity in reproductive effort is a major aim of evolutionary biology (McNamara et al. 2009; Pigliucci 2001; Williams 1966).

The fundamental life-history trade-off between survival and reproduction has attracted much theoretical and empirical attention (Clutton-Brock 1984; McNamara and Houston 2008; Pianka and Parker 1975; Williams 1966). The consequences of diverting energetic resources from maintenance of the organism into reproduction means that organisms must balance their current reproductive effort against their prospects for survival and future reproduction. For most organisms, a reproductive event comes with several types of costs (e.g. egg production, parental care, competition for mates), many of which can affect an organism's survival (Fisher 1930). For example, breeding-associated immobility or the need for increased foraging to feed offspring can expose parents to increased predation risk (Ghalambor and Martin 2001; Schneider and Griesser 2014). For malaria parasites, allocating cells to become gametocytes comes at the instant cost of a reduced number of asexual stages, needed to perpetuate the infection. Theory predicts that, compared to older organisms, whose residual reproductive value (RRV, the age-specific expectation of future offspring) is lower, young organisms should allocate less into current reproduction ("reproductive restraint") so as to minimise risk to their survival prospects (Pianka and Parker 1975). In contrast, reproductive effort should increase as the probability of future reproductive success decreases (Williams 1966), and in the last reproductive event of their life, i.e., when the RRV is very small, organisms should allocate all of their remaining energy to reproduction ("terminal investment").

In addition to the age of the organism, the current environment and an organism's physiological characteristics are also expected to affect reproductive effort. Accounting for these effects, summarised as "state variables", has given rise to theory that complements age-based life-history theory (Clark and Mangel 2000; McNamara and Houston 1996, 1999, 2008). For example, terminal investment may not just be observed when age is the main reason for an organism's death, but also if external factors cause the probability of future reproduction to be near zero (Hirshfield and Tinkle 1975). Conversely, if current conditions are not conducive to reproduction (e.g. resources are limited), an organism may be selected to exert reproductive restraint and delay reproduction until environmental conditions improve (Curio 1983, 1988; Monaghan and Nager 1997). Finally, state-based and age-based life-history interact because physiological state varies over an organism's lifetime (McNamara et al. 2009; Nussey et al. 2013). In summary, an optimally behaving organism is expected to adjust its reproductive effort over successive bouts of reproduction according to interactions between environmental conditions, energetic reserves, and expected lifespan. The predictions of age- and state-based theories are supported by empirical data from diverse laboratory models and natural systems (Clutton-Brock 1984; Hamel et al. 2010; Kaitala 1991; Krams et al. 2015; Pianka and Parker 1975; Reed et al. 2015) confirming the fitness benefits of adjusting reproductive effort in relation to circumstances.

Life-history theory has also been applied to explain plasticity in the reproductive effort of malaria parasites, which appear to adjust conversion rates in response to information about their within-host environment and the density of clone-mates within the host (Buckling et al. 1999, 1997; Cameron et al. 2012; Carter et al. 2014; Greischar et al. 2016a; Pollitt et al. 2011b; Wargo et al. 2007b). A clonal parasite population inside a host is the selective equivalent of a single organism (Gardner and Grafen 2009) and can experience considerable variation in within-host environments during infections and in different hosts.

Characteristics of the in-host environment can affect state, which for malaria parasites, is associated with asexually replicating stages because they make up the bulk of parasite biomass and produce gametocytes. Environmental conditions thought to affect parasite state are immune attack, drug treatment, competition with con-specific parasite strains, and a variable supply of red blood cells (Carter et al. 2013). Parasites should therefore prioritize allocation to asexual stages to maintain the infection when faced with a moderate loss of state because prolonging in-host survival rewards parasites with future transmission opportunities (Birget et al. 2017b; Mideo and Day 2008; Pollitt et al. 2011b; Wargo et al. 2007a). In contrast, parasites should allocate all remaining resources to transmission when faced with a situation where in-host survival is unlikely (e.g. clearance by drugs or strong immunity) (Buckling et al. 1999, 1997; Carter et al. 2013; Peatey et al. 2009) or when the infection is likely to end due to host death. Finally, an increase in state (e.g. due to an enrichment of the environment) allows parasites to allocate more to gametocytes, since within-host survival is not at risk. These strategies can be represented in a reaction norm for conversion rate which illustrates how the circumstances that parasites find themselves in determine the allocation parasites make to gametocytes at each cycle of replication (figure 4.1).

Mounting evidence suggests that malaria parasites have evolved to adjust conversion rates according to circumstances – including changes in the supply of an essential resource – red blood cells (RBC) (Cameron et al. 2012; Nacher et al. 2002; Price et al. 1999; Reece et al. 2005). Most studies report an increase in gametocyte densities when hosts become anaemic (i.e. when the density of RBCs is low). Only two studies link elevated gametocyte densities to higher conversion in response to anaemia (Cameron et al. 2012; Reece et al. 2005), and most findings are based on post-hoc correlations of observational data in which confounding changes in asexual densities cannot be accounted for (Nacher et al. 2002; Price et al. 1999; von Seidlein et al. 2001). Moreover, it is not known whether increased conversion occurs because parasites are making a terminal investment (e.g. due

to RBC limitation or because an anaemic host has a high mortality risk), or because the in-host environment has improved and parasites can afford to reproduce. These contradictory hypotheses emerge because anaemia is complex. The population of circulating RBC within the host is characterised quantitatively by their overall density (i.e. number of RBC/ mL blood) and qualitatively by their age structure (the frequency of immature RBC, termed reticulocytes, versus mature RBC, termed normocytes). The density and/or frequency of reticulocytes and normocytes matters because different parasite species preferentially invade different age classes (Carter et al. 2013; Ott 1968). A further complication arises because parasites may not be selected to respond to anaemia per se, but simply use it as a proxy for the appearance of immune responses that have a major effect on state (Haydon et al. 2003; Kochin et al. 2010b; Miller et al. 2010).

Here, we avoid confounding changes in immune responses and variation in asexual density to ask how parasites adjust conversion rates in response to the in-host environmental characteristic anaemia. Our approach enables us to examine several different parasite genotypes of the rodent malaria parasite *Plasmodium chabaudi* to assess genetic and genotype-by-environment influences on conversion rate. We find that all genotypes increase their conversion in response to anaemic conditions and that different genotypes do so to different extents. Further analysis suggests that parasites respond to the frequency of reticulocytes versus normocytes and that parasites increase conversion because they are taking advantage of an increase in resources rather than making a terminal investment.

## 4.2 Methods

We pre-treated hosts with different doses of a drug that modifies the age structure (measured as the proportion of reticulocytes) and overall density of RBC in the blood (phenylhydrazine, PHZ), then inoculated mice with parasites of one of several genotypes. We then measured the density of asexual parasites and

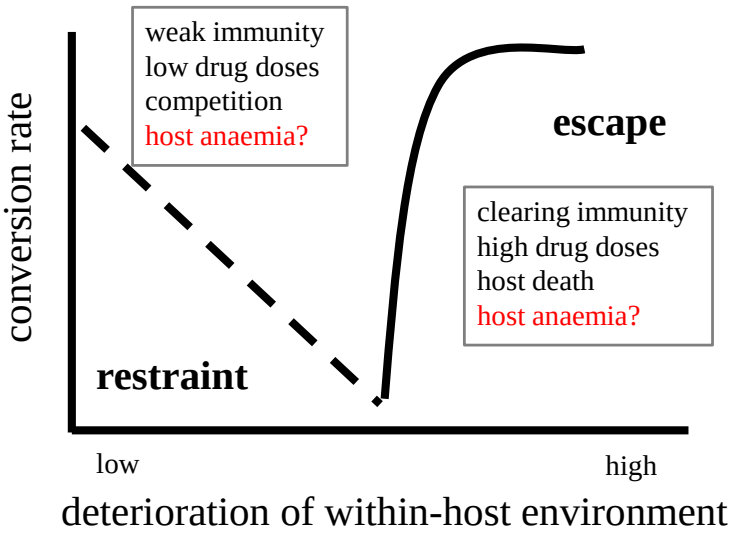


Figure 4.1. Cartoon of a reaction norm for conversion rate against conditions experienced by parasites inside the mammalian host, adapted from Carter et al. (2013). Importantly, the x-axis does not represent time since infection, but a given stress, or combination of stressors experienced at any point during the infection that reduces the condition/state of parasites. The dotted line represents a decrease in conversion rate ("restraint") as the parasites experience a loss of condition/state, but the exact functional form of this is not known. If the within-host environment has deteriorated substantially and recovery of condition/state is unlikely or impossible parasites should make a terminal investment by putting all resources into transmission ("escape"). Factors in the boxes denote circumstances thought to induce reproductive restraint or terminal investment, but the effect of variable red blood cell resources during anaemia is unclear.

gametocytes to infer the conversion rate.

### 4.2.1 Parasites and hosts

We obtained C57BL/6 female mice (aged 6–8 weeks) in-house (University of Edinburgh) and *Plasmodium chabaudi* clones AJ, AS, CR and ER from the Edinburgh Malaria reagent repository (University of Edinburgh). *P. chabaudi* was isolated between 1948 and 1974 from African thicket rats, *Thamnomys* spp.,

in Central Africa (Killick-Kendrick 1978). After cloning, the parasite genotypes have been cryopreserved and undergone regular transmission through mosquitoes to maintain their wild type phenotypes (Spence et al. 2013). The four *P. chabaudi* genotypes chosen span the diversity of conversion rates and virulence reported from previous experiments (Bell et al. 2006; Mackinnon and Read 1999; Pollitt et al. 2011b). Four days after PHZ treatment (see below), hosts were infected intravenously with $5x10^6$–$1x10^7$ parasitized RBC.

### 4.2.2 Perturbing red blood cell resources in the within-host environment

We used PHZ to generate different RBC resource environments in the blood. Phenylhydrazine causes the clearance of circulating red blood cells (Savill et al. 2009) and the resulting anaemia stimulates the release of immature RBC (reticulocytes) from the bone marrow. We found in a pilot study that injecting 120 mg/kg, 30 mg/kg and 0 mg/kg (control) of PHZ generated non-overlapping environments in terms of both total red blood cell density and reticulocyte proportion (see appendix, figures D7 and D8) two days after injection, and that these differences persisted for 5 more days (see supplementary material). The variation in total red blood cell density and reticulocyte proportion observed for these doses corresponds to environments typically encountered by parasites during infection: the control treatment emulates the initial environment encountered in a healthy host, the 120 mg/kg treatment reflects conditions after the peak of infection when hosts are most sick, whereas the 30 mg/kg treatment resembles the RBC environment in a recovering host.

### 4.2.3 Experimental design and data collection

Four days before infection (day -4 post infection, PI) we injected mice with 120 mg/kg (n=23 mice), 30 mg/kg (n=23) or 0 mg/kg (control treatment, n=22) PHZ and on day 0 PI we infected each mouse with 1 of 4 clones. This gave us a fully cross-factored design with 12 different treatment combinations (4 genotypes

x 3 PHZ treatments). From day 1 PI to day 3 PI, we monitored mice daily at 9am by taking 2$\mu$l of blood to quantify RBC density (Ferguson et al. 2003), making a thin blood smear, and collecting 10$\mu$l blood for RT-qPCR to quantify gametocytes approximately 35 hours old (since bursting from a committed schizont) (Wargo et al. 2006), measuring expression of the *CG2* gene (GenBank Accession number PC302249.00.0), which is only expressed at the gametocyte stage (Wargo et al. 2007b). We did not perform technical replicates of the PCR. RNA extraction was performed as described in (Schneider et al. 2015) with minor changes to the protocol. We extracted RNA using the Kingfisher Flex Magnetic Particle Processor (Life Technologies). Additional adjustments were made to the manufacturer's protocol AM1830DW to improve RNA recovery. To ensure full lysis of the blood, 350ul of lysis binding solution and medium mixing speeds were used. Wash steps have been lengthened to 45 seconds and elution to 7 minutes 30 seconds. Minimum temperatures were set to 20°C to prevent variability resulting from low ambient temperatures. All bead transfer steps have been lengthened and an extra bead transfer step was introduced when moving samples from the sample plate and into the elution plate.

We deliberately focussed on the early infection dynamics to maximise the likelihood of observing parasite responses to the RBC environments they encountered upon infection, rather than confounding factors that develop as infections progress (e.g. immune responses, divergence in parasite densities between treatments). Blood smears were used to estimate the density of asexual parasites and the proportion of RBC that were reticulocytes or normocytes. All host measures relating to the RBC environment were inferred from the age structure and total density of red blood cells; the density of reticulocytes was estimated as the proportion of reticulocytes multiplied by the total red blood cell density and the density of normocytes as (1−the proportion of reticulocytes), multiplied by total RBC density.

### 4.2.4 Estimation of conversion rate and statistical analysis

Data were analysed using R version 3.0.2. We used ANOVAs to assess the effect of PHZ on the within-host environment and on asexual parasite density. Since the proportional data (RBC frequency) obtained from this experiment are non-binomial, linear models were used with the pre-condition that the values satisfy linear modelling assumptions (Warton and Hui 2011). We then carried out two analyses to ask how anaemia affects conversion rates. Using ANCOVAs, we first tested the effect of genotype and PHZ treatment on conversion while also controlling for asexual density. All test statistics, degrees of freedom and p values reported are from maximum likelihood-based deletion tests (i.e., comparing a model with and without the explanatory variable of interest) (Crawley 2012). Second, we fitted four ANCOVA models that decomposed the effects of PHZ into the densities and frequencies of RBC types to identify which in-host environmental variables correlate most closely with conversion rates. These models were simplified using maximum likelihood-based deletion tests as above and the minimal models were compared by AIC. To infer conversion rate, we compared the summed gametocyte densities on days 2 and 3 PI to the summed density of their source populations of asexual parasites whose densities were recorded on days 1 and 2 PI. In absence of differences in asexual parasite density, analysing pooled gametocyte densities directly avoids the difficulties of accurately calculating conversion rate by PCR (Carter et al. 2013; Greischar et al. 2016b). Gametocyte densities on days 2 and 3 PI may include gametocytes produced in donor mice. However, since all mice were infected from the same donor mouse and the number of gametocytes or sexually committed parasites in inocula is small, this is unlikely to influence differences between treatments. For each statistical model, were examined diagnostic plots to assess whether linear modelling assumptions are satisfied (Crawley 2012; Warton and Hui 2011).

## 4.3 Results

### 4.3.1 Modifying resources in the within-host environment

For the duration of experimental infections, hosts in the different PHZ treatment groups had significantly different total RBC densities and age structures (figure 4.2, table D3 in appendix D). As expected, normocyte density was reduced by PHZ in a dose-dependent manner, whereas the density and proportion of reticulocytes varied in the reverse fashion (figure 4.2). There are no genotype or genotype by PHZ treatment effects, i.e. within a given PHZ treatment, all genotypes encountered the same RBC environment (table D3). The mean total RBC density from all genotypes combined (expressed as $10^9$ cells per ml) from day 0 PI to day 2 PI varied from 3.03($\pm$0.18) to 4.57($\pm$0.17) in the 120 mg/kg treatment, from 5.63($\pm$0.14) to 5.68($\pm$0.13) in the 30 mg/kg treatment and from 7.04($\pm$0.34) to 7.71($\pm$0.15) in the control treatment. The proportion of reticulocytes varied from 0.21($\pm$0.02) to 0.27($\pm$0.001) in the 120 mg/kg treatment, from 0.12($\pm$0.01) to 0.13($\pm$0.008) in the 30 mg/kg treatment and from 0.01($\pm$0.002) to 0.02($\pm$0.002) in the control treatment.

### 4.3.2 Conversion rate in perturbed within-host environments

Asexual density increases from day 1 to day 2 PI but there is no significant difference in their summed densities between genotypes within each PHZ treatment ($F(3, 62) = 1.62$, $p = 0.193$, figure 4.3) or between PHZ treatments ($F(2, 65) = 2.81$, $p = 0.067$, table D4). Thus, the summed gametocyte densities on days 2 and 3 PI reflect the conversion decision taken by asexuals on days 1 and 2 PI. However, we also control for asexual density in the following statistical models to account for any subtle variation between individual infections. The mean total gametocyte densities (over days 2 and 3 PI) vary significantly across PHZ treatments and genotypes. All genotypes increase their gametocyte densities (0 mg/kg $<$ 30 mg/kg $<$ 120 mg/kg) with increasing PHZ dose, but they vary in the magnitude of their responses (figure 4.4, table D5). Once

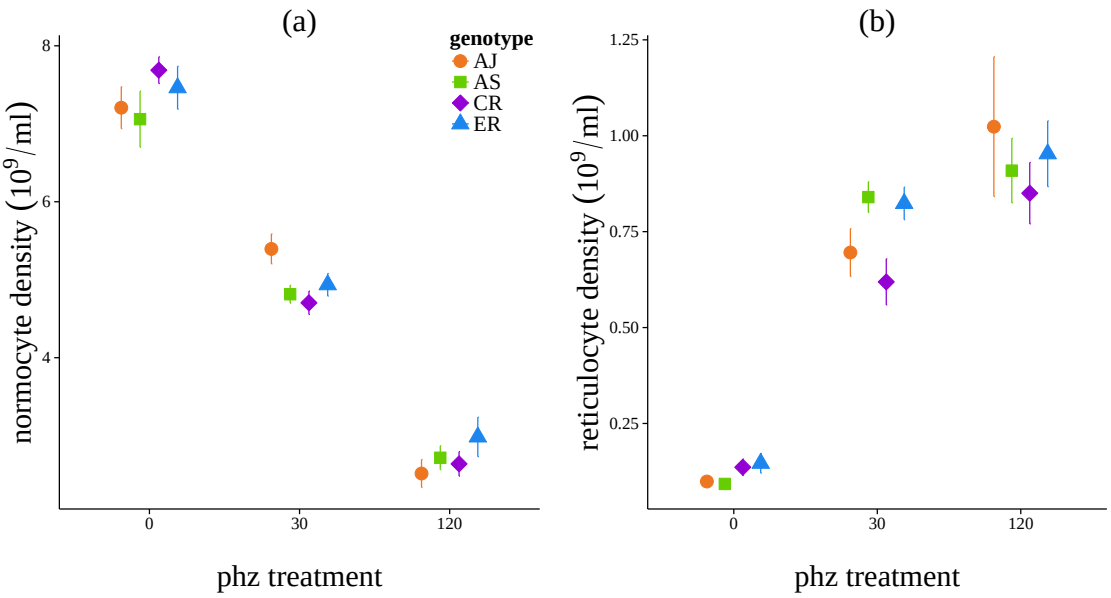


Figure 4.2. Mean ± standard error of the mean (SE) normocyte density (a) and reticulocyte density (b) on days 0–2 PI (n=68) by PHZ treatment (0 mg/kg, 30 mg/kg, 120 mg/kg) and genotype. Normocyte and reticulocyte densities are significantly different between PHZ treatments but not between genotypes.

adjusted for variation in asexual density, the interaction between genotype and PHZ treatment explains 84% of the variance in gametocyte densities. To assess whether each genotype employs a different conversion rate strategy, we tested whether any genotypes could be grouped together without causing significant change in model deviance. The responses of AJ and ER did not differ significantly from each other ($F(3, 54) = 0.08$, $p = 0.970$) but all other genotypes follow significantly different reaction norms. In response to increasing PHZ doses, AS increases its gametocyte density most, followed by CR, and finally AJ / ER. Also, mean pairwise differences between genotypes are significantly greater in PHZ-treated mice, with ∼6 fold greater variation between genotypes expressed in the 120 mg/kg environment compared to 0 mg/kg ($F(2, 15) = 11.47$, $p < 0.001$, figure 4.4).

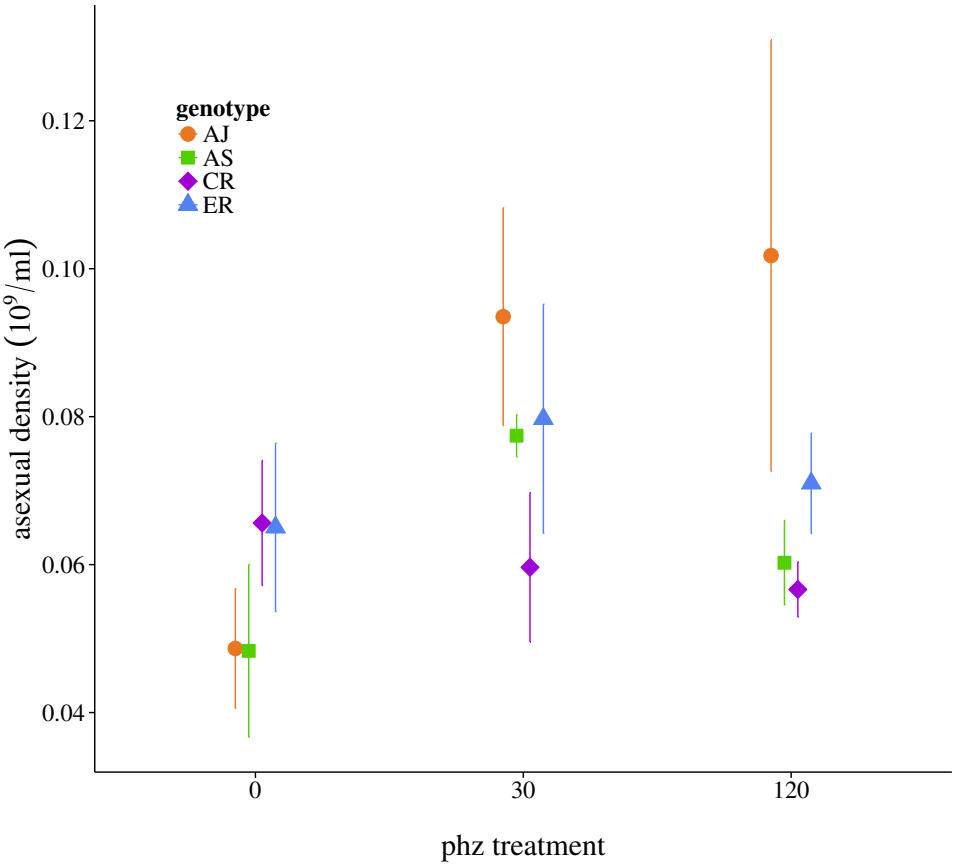


Figure 4.3. Mean±SE density of the asexual populations (days 1 and 2 post infection) that are the source population for the gametocytes measured, for different genotypes and PHZ treatments.

### 4.3.3 Conversion rate and RBC resources

Since PHZ was used only as a means to modify the RBC environment, we expect that parasites do not detect and respond to PHZ itself, but to its effects on the density and/or age structure of RBC resources. Since measurements of total red blood cell density and the proportion of red blood cells that are reticulocytes (frequency) are independently determined, we fitted them as explanatory variables (instead of PHZ treatment), together with other variables of interest such as genotype and asexual density, and all their interactions (model 1). Using deletion tests, this model was simplified to the interaction between the frequency of red blood cell types and genotype (Table D6 in appendix D). We then constructed and minimised three other models that differed according

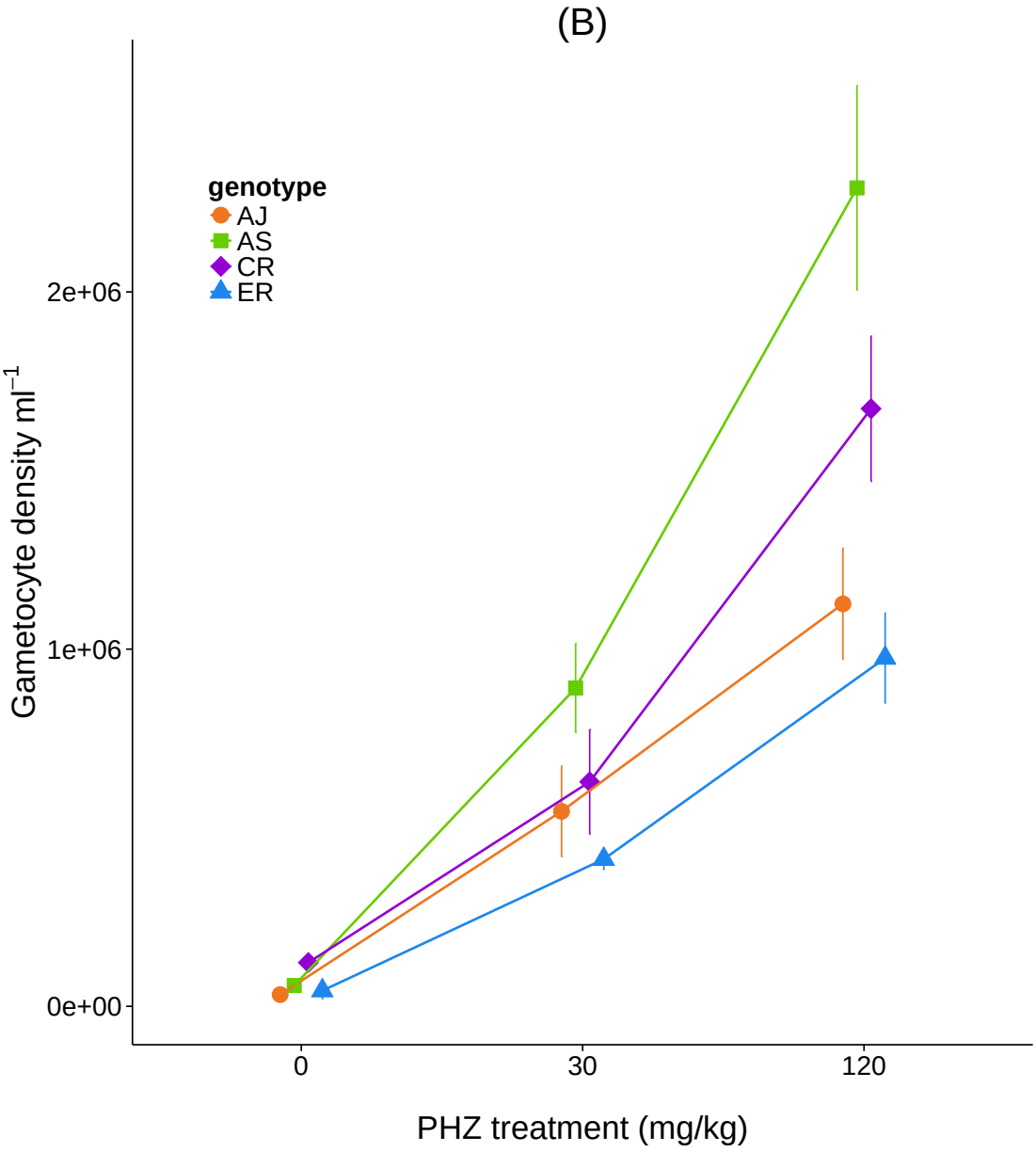


Figure 4.4. Reaction norms for conversion rate (mean ± SE gametocyte density for days 2 and 3 PI) of 4 genotypes across PHZ treatments (left to right along x axis represents a decrease in total red blood cell density and an increase in reticulocyte proportion). All genotypes increase their conversion as PHZ dose increases, but to different extents (note, there is no significant difference between AJ and ER). The points are dodged horizontally for clarity.

to variables inferred from our two independent measurements; reticulocyte density (model 2), normocyte density (model 3), or total RBC density (model 4). Comparison of the resulting four minimal models (using AIC) reveals that the model containing the frequency-by-genotype interaction is the best overall (model 1, table D7). This model explains 81% of the observed variation in gametocyte densities (Table S4) and reveals a positive correlation between gametocyte density and the proportion of RBC that are reticulocytes, whose slope varies between genotypes (AS > CR > AJ = ER ; figure 4.5).

## 4.4 Discussion

We found that the RBC resource environment, perturbed by PHZ, can explain > 80% of variation in the conversion rate of malaria parasites: the higher the frequency of reticulocytes, the more parasites allocate to gametocytes. This pattern is broadly evident for all four genotypes examined but some genotypes are more sensitive than others, suggesting there is genetic variation for plasticity in conversion rates. We concentrated our analysis on the first few days of infection before the densities of the decision-making asexual cohorts could diverge between treatments. This avoids issues in previous studies where other variables associated with conversion, such as the density of asexual parasites or an adaptive immune response, co-vary with anaemia (Cameron et al. 2012; Pollitt et al. 2011b; Reece et al. 2005). Our experiment also helps to elucidate the long-standing question of which cues parasites use for gametocytogenesis (Carter et al. 2014): metrics for the density of red blood cells, which is the most obvious measure of anaemia, do not correlate as strongly with conversion rate as the proportion of RBC that are reticulocytes, suggesting that parasites respond to RBC age structure. Because encounter rates between merozoites and host RBC of different ages during invasion are directionally proportional to the frequency, not the density, of RBC ages, encounter rates with different RBC ages could be a proximate mechanism for parasites to assess their RBC environment. It remains possible that instead, parasites respond to some unknown correlate

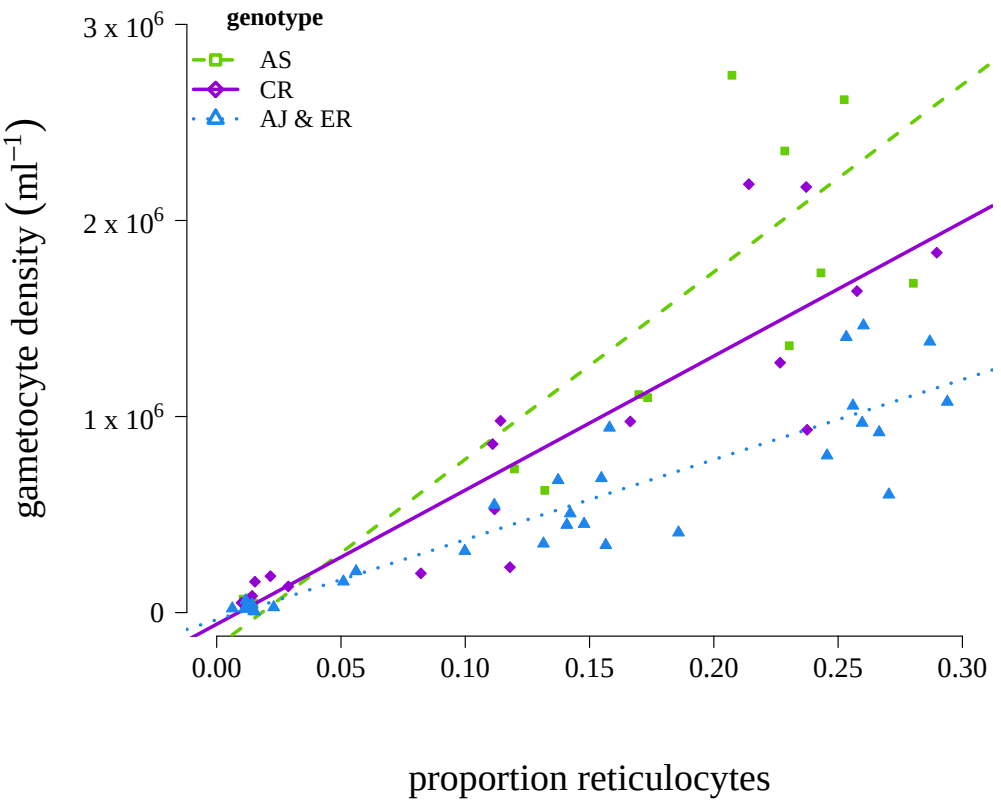


Figure 4.5. Gametocyte density for each mouse (mean of days 2 and 3 PI) correlates with RBC age structure (the mean proportion of RBC that are reticulocytes across days 0, 1 and 2 PI for each mouse). Regression lines illustrate the reaction norms for the different genotypes (note, there is no significant difference between AJ and ER). Data from the control group cluster around the origin (median reticulocyte density: 0.014), data from the 30mg/kg group span a reticulocyte density from 0.06 to 0.19 (median 0.14), and a dose of 120mg/kg produced a range between 0.21 and 0.29 (median 0.25).

of PHZ treatment, but we expect this is unlikely, given the diversity of data reporting positive associations between anaemia and correlates of conversion that do not involve PHZ Nacher et al. 2002; Price et al. 1999; von Seidlein et al. 2001.

Why do parasites increase their conversion rate in response to an increase in reticulocytes? The first possibility is that the environment has improved because resource availability has increased and parasites are able to allocate to gametocytes without unduly compromising survival inside the host (i.e. relaxing the need for reproductive restraint). Alternatively, parasites may perceive that the risk of the infection being cleared (by resource limitation, an immune response or host death) is sufficiently high that they should make a terminal investment. We favour the former explanation: it is unlikely that parasites interpret an influx of reticulocytes as a lethal situation since we observe that no hosts died during the experiment, no infections were cleared and asexual growth rate over the duration of the experiment was not compromised (see chapter 5). At first, it seems counterintuitive that anaemia could translate into an improvement of environmental quality; indeed, host-adaptive explanation for "bystander killing" of uninfected red blood cells implies that anaemia should facilitate the clearance of infection (Buffet et al. 2010; Cromer et al. 2009; Metcalf et al. 2011, 2012). However, the erthropoietin-mediated feedback during anaemia brings in reticulocytes which could be a cue for an imminent improvement in the environment, although this seems unlikely since the presence of reticulocytes generally also correlates with the appearance of adaptive immunity. Thus, parasites may simply find reticulocytes to be superior cells compared to normocytes. If a high proportion of reticulocytes is beneficial to parasites, then a terminal investment strategy would be maladaptive. There are several lines of evidence suggesting that malaria parasites respond to the differential resource qualities of reticulocytes and normocytes. For parasite species that strongly prefer reticulocytes, like *P. berghei*, an increase in replication rate and conversion rate in response to PHZ treatment has been reported (Cromer et al. 2006;

Gautret et al. 1996). Even though *P. chabaudi* is thought to be a generalist and able to infect a wide range of RBC ages, reticulocytes may be a better resource than normocytes for *P. chabaudi* for several non-mutually exclusive reasons: (1) reticulocytes carry a more diverse set of cell surface receptors to interact with rhoptry proteins on invading merozoites (Khan et al. 2001; Preiser et al. 1999); (2) reticulocytes are a metabolically more diverse resource (Srivastava et al. 2015); (3) reticulocytes could be particularly well suited for the development of gametocytes because their longer life span matches the longer maturation time and life span of gametocytes compared to asexuals (Bousema and Drakeley 2011; Ohnishi and Nishimura 2001; Trager 2005); and (4) an infected reticulocyte may produce more merozoites than a normocyte (Mideo et al. 2008b), possibly due to their larger size or reduced oxidative stress (Rifkind and Nagababu 2013). Such mechanisms may also explain observations of higher growth rates and conversion rates of *P. chabaudi* in mice pre-treated with erythropoietin, which increases reticulocyte frequency without affecting total RBC density Reece et al. (2005).

We observed three different reaction norms for conversion rate across the four genotypes that we examined. These genotypes vary in virulence and the most virulent genotypes (AJ, ER) are less plastic than the less harmful genotypes (CR, AS). Virulence is generally regarded as a fitness-related trait for parasites: virulent genotypes are better competitors against conspecifics sharing the host, and are more likely to survive drug treatment and immune responses (Barclay et al. 2012; Råberg et al. 2006; Schneider et al. 2012, 2008). Thus, it is not clear why genetic variation for virulence is maintained in natural malaria populations (Mackinnon and Read 2004). Because gametocyte density is positively correlated to transmission success, our results suggest that genotypes of low virulence could achieve greater transmission when hosts mount an erythropoietic response and so, compensate for the fitness costs of low virulence. If this holds for human malaria parasites, it suggests that co-infections that cause anaemia (e.g. helminth infections, Sanya et al. 2017) could select for less virulent malaria genotypes. Further, anaemic patients may require additional transmission-reducing measures because they may be more infectious than their counterparts (Bousema and

Drakeley 2011; Nacher et al. 2002).

# Chapter 5

# Plasticity and genetic variation in traits underpinning asexual replication rate of the rodent malaria parasite, *Plasmodium chabaudi*

## Abstract

The replication rate achieved by malaria parasites during infections is the result of both host-and parasite-controlled factors and their interactions. For malaria parasites, this includes host control of immunity and red blood cell (RBC) resources and how efficiently parasites evade immunity and utilise RBCs. Chapter 4 reveals that malaria parasites do not only plastically increase their investment into non-replicating transmission forms (conversion rate) in response to the development of host anaemia but also increase their asexual replication rate. Here, we ask how parasites achieve faster replication rates by measuring plasticity in a number of traits that could underpin replication rate: burst size (the number of merozoites produced per schizont), the duration of the asexual

cycle, and invasion preference for different ages of RBCs. We quantify these traits for four different genotypes of *P. chabaudi* to test whether their contributions to replication rate are genotype-specific. We find plasticity in all of the traits measured and genetic variation for most. However, it is plasticity in burst size that contributes the most (and in a genotype-specific manner) to the increase in replication rate observed in anaemic environments. The ability of parasites to plastically adjust reproductive (transmission) strategies has long been studied, but plasticity in traits involved in in-host survival are rarely considered. Thus, we reveal a new layer of complexity in host-parasite interactions by showing that traits involved in the exploitation of RBC are phenotypically plastic and vary according to parasite genetics.

## 5.1 Introduction

All parasites are adapted to extract energy from their host and divert it towards their own survival and reproduction. For malaria (*Plasmodium*) parasites, within-host survival involves cycles of rapid asexual replication, first in the liver, and then in the blood. Whereas reproduction requires the production of sexual stages (gametocytes) in the host, which mate when taken up by a vector, in the host's blood stream, the vast majority of parasites are asexually replicating stages, adapted for foraging on host resources and serving as a source population for the production of gametocytes (Josling and Llinas 2015). Asexual stages are responsible for virulence to the vertebrate host. They can induce anaemia by using host red blood cells (RBCs) as a primary resource, they carry virulence factors (e.g. the *var* genes of *P. falciparum*) which can bind to epithelial receptors and cause sequestration-related pathologies such as cerebral or pregnancy-associated malaria, and they are the main target of immune responses which cause immunopathology if not properly regulated by the host (Dasari et al. 2014; Jakeman et al. 1999; Price et al. 2001). Asexual stages are the main target of antimalarial drugs, which parasites counter through a variety of mechanisms, including genetic resistance (White et al. 2014b) and adaptation

of life-history traits (Schneider et al. 2012, Chapter 2 and 3). Considering the central importance of asexual stages in malaria pathology and in the evolution of drug resistance, a better understanding of the factors underpinning asexual replication in the blood could enable novel interventions to be developed, for example, by revealing how to create faster clearance of parasites by drugs or vaccine-induced immunity, or better tolerance of the damage asexual stages cause.

The replication rate achieved by parasites during infections is the result of both host-and parasite-controlled factors and their interactions. Host control of immunity and red blood cell (RBC) resources mediates how permissive the within-host environment is to replication (Antia et al. 2008; Cromer et al. 2009; Haydon et al. 2003; Miller et al. 2010). For example, innate immune factors (e.g. cytokines, macrophages) play a significant role in the control of parasite replication in acute infections. Parasite factors that directly underpin replication rate include burst size (the number of merozoites bursting from a schizont), preferential invasion for certain RBC ages, the duration of the asexual cycle, the ability of parasites to avoid clearance by the spleen (e.g. through sequestration, Brugat et al. 2014), and the conversion rate (Carter et al. 2013). Conversion and replication are negatively related through a fundamental resource allocation trade-off: an increase in conversion is inevitably linked to a reduction in asexual stages during the next round of replication. However, as outlined in the introduction, replication rate and conversion rate can be positively correlated if an environmental variable favours asexual replication, thus allowing parasites to increase their state and consequently invest more into conversion. The positive correlation between replication rate and conversion under such circumstances makes it difficult to study the knock-on impact of the trade-off between conversion rate and replication rate. Therefore, this chapter focuses on explaining plasticity in replication rate by investigating the impact of traits that more directly underpin asexual replication rather than conversion.

Variation in parasite traits that directly underpin asexual replication (termed "asexual traits") has been observed between different species of *Plasmodium*

(Antia et al. 2008; Cromer et al. 2009; Mancio-Silva et al. 2017; Mideo et al. 2008b), and there is also evidence of intraspecific variation (Antia et al. 2008; Mideo et al. 2008b). Antia et al. (2008), for example suggest that different genotypes of *P. chabaudi* have different age preferences for the RBCs they infect. Genetic variation in invasion preference, burst sizes and parasite effects on host erythropoiesis are key factors thought to underpin virulence differences between genotypes (Mideo et al. 2008b). However, in addition to the presence of genetic variation, asexual traits can vary plastically (i.e. during infections) but this has not attracted much attention (Mideo and Reece 2012). This seems surprising, given the widely reported plasticity in conversion rates in malaria parasites (Carter et al. 2013; Mideo and Reece 2012). This may be, in part due, to the challenges of separating parasite control of traits from variation that is directly caused by the impact of environmental change (i.e. host control of parasite traits). For example, parasites have recently been demonstrated to replicate slower in nutrient limited hosts (Mancio-Silva et al. 2017). Is this purely because parasites are starving or could parasites also plastically reduce replication rate? The latter appears to be the case: Mancio-Silva et al. (2017) show that *P. berghei* actively modifies its transcriptome, regulated by a nutrient-sensing mechanism, to adjust its burst size depending on the nutritional status of the host. Such plastic modification of burst size may be adaptive and allow parasites to optimally use within-host resources, guarantee the production of high-quality merozoites or even to avoid host death (and thereby guarantee future transmission opportunities). Thus, there is evidence that parasites are, at least in part, responsible for varying their replication rate. If asexual stages plastically modify their traits in response to within-host conditions e.g. to take advantage of a new resource (such as reticulocytes) or to avoid exposure of vulnerable developmental stages within the asexual cycle to immunity or drugs (Mideo et al. 2013, Reece et al., *in press*), it would presumably confer a significant fitness benefit. Furthermore, different genotypes may react differently to a given within-host conditions (so called genotype by environment effects), allowing genetic variation to be exposed, and thus selection to act. Quantifying

genetic variation and plasticity involved in asexual traits, and how variation in these traits acts in concert, may unravel new intervention opportunities where targeting one trait may affect other traits through trade-offs or pleiotropy (Beeson et al. 2016).

In this chapter, we first conduct further analysis of the data obtained in chapter 4 to test how asexual replication rates of 4 different genotypes of *P. chabaudi* are affected by anaemic conditions. We then conduct a new experiment, using the same 4 genotypes as in chapter 4, to investigate plasticity in asexual traits by artificially inducing anaemia to cause substantial variation in the within-host environment, and quantifying traits that underpin replication rate (burst size, RBC age preference) during various degrees of host anaemia. We do not measure sequestration due to the lack of precise and practical tools available for this trait. Whilst changes in RBC density and age structure are the main effects of artificially induced anaemia, it is possible that our perturbation alters elements of the innate immune response, which could directly affect replication rate. To account for this, we also assay the concentrations of two key cytokines in malaria infections (Langhorne et al. 2008; Mohan et al. 1997; Taverne et al. 1987). We then combine the measurements for all traits in a model to examine their relative influences on replication rate. In summary, we found plasticity and genetic variation for all traits, and that replication rate is best explained by interactions involving burst size and the invasion rate of normocytes.

## 5.2 Methods

### 5.2.1 Parasites and hosts

We obtained C57BL/6 female mice (aged 6–8 weeks) in-house (University of Edinburgh) and *Plasmodium chabaudi* clones AJ, AS, CR and ER from the Edinburgh Malaria reagent repository (University of Edinburgh). *P. chabaudi* was isolated between 1948 and 1974 from African thicket rats, *Thamnomys* spp., in Central Africa (Killick-Kendrick 1978). After cloning, the parasite genotypes have been cryopreserved and undergone regular transmission through mosquitoes

to maintain their wild type phenotypes (Spence et al. 2013). The four *P. chabaudi* genotypes chosen span the diversity of conversion rates and virulences reported from previous experiments (Bell et al. 2006; Mackinnon and Read 1999; Pollitt et al. 2011b).

### 5.2.2 Experimental design

The experimental set-up was similar to the one used in Chapter 4, but uses only two doses of phenylhydrazine (PHZ). PHZ is a drug that causes the premature death of mature RBCs and, in consequence, induces an inflow of immature red blood cells (reticulocytes) through a feedback loop controlled by the hormone erythropietin (Berger 2007). We treated C57BL/6 mice with 0mg/kg (PBS) or 30mg/kg of PHZ and intravenously infected them with one of 4 genotypes of parasites 4 days later, at a dose of 5 x $10^6$ parasites. This gives 8 experimental groups with 7 mice in each. From day 1 post -infection (PI) to day 5 PI, we monitored mice daily at 8am by taking 2$\mu$l of blood from the tail to quantify RBC density (Coulter Technologies, Ferguson et al. 2003) and make a thin blood smear to quantify asexual stages, the age structure of RBC (measured as the proportion of RBC that were reticulocytes), and the proportion of infected reticulocytes by microscopy. At midnight on day 4 PI, we made a smear of each mouse to count the number of merozoites inside schizonts. From 8 am on day 3 PI to 8 am on day 5 PI, we made blood smears for each mouse every 8 hours to assess the relative abundance of the developmental stages parasites pass through during the asexual cycle (staging them as either ring stage, trophozoite or schizont) to estimate the duration of the asexual cycle. The pilot data that were analysed from this study stem from the experiment described in chapter 4, which has a near-identical experimental set-up as the one described here, albeit with an additional PHZ treatment category. Briefly, mice were treated with one of three PHZ doses 4 days before infection (120 mg/kg, n=23 mice; 30 mg/kgl, n=23; 0 mg/kg, control treatment, n=22) and on day 0 PI we infected each mouse with one of four clones (AJ, AS, CR or ER). Infection were monitored until day 5 PI.

### 5.2.3 Immune assays

In addition to the experimental mice, we also injected a separate set with PHZ or PBS (0 mg/kg: n=17, 30mg/kg: n=23) and sacrificed them 4 days later to assess whether anaemia affected the immune environment encountered by parasites on the day of infection. We obtained blood by cardiac puncture, centrifuged it twice for 10 minutes at 60,000rpm, collecting about 200ul of plasma. We shock-froze the plasma on dry ice and stored it at -80°C. We performed an ELISA to detect the levels of TNF$\alpha$ and INF$\gamma$ following the manufacturer's protocol (Invitrogen). TNF$\alpha$ and INF$\gamma$ are two key cytokines in malaria infection and kill asexual stages either directly or through the recruitment of macrophages (Langhorne et al. 2008; Mohan et al. 1997; Taverne et al. 1987). For INF$\gamma$, we used a range of 7 standards (included in the kit) ranging from 1.7pg/ml to 300pg/ml (as recommended by the manufacturer). For TNF$\alpha$, we used a range of 9 standards, ranging from 7.8pg/ml to 2000pg/ml (adding one additional concentration on each end of the range recommended in the kit). All standards and all samples were run in duplicates, and for each mouse we took the mean of the reading at an absorbance of 450nm, as recommended by the manufacturer. Samples with poor repeatability between duplicates were excluded. We used ELISA software (elisaanalysis.com) to fit standard curves and infer concentrations present in samples. Each plate contained multiple negative controls and non-specific binding assays (chromogen blanks). Samples that gave a reading below that of a negative control were excluded.

### 5.2.4 Quantification of traits

We calculated replication rate as the slope of parasitaemia against time between between day 1 PI and the timing of peak parasitaemia. We quantified burst size for between 10 and 15 schizonts on each smear. We derived the distribution of burst sizes for each genotype and each treatment combination, by tallying the counts of all mice in a given experimental group. To compare distributions of each genotype, we compared the proportion of schizonts that have over 6 merozoites between PHZ treatments and genotypes. We calculated invasion rates

for reticulocytes and normocytes separately using equations 1 and 2 in Mideo et al. (2011). These calculations take account the age structure and density of the available cells before and after invasion, as well as of the density of merozoites before invasion. We chose to calculate invasion rates on day 4 PI because this is when the proportion of infected reticulocytes is high enough to return reliable estimates. To estimate the density of merozoites, we multiplied the average burst size for each infection by the density of asexual parasites on day 3 PI. This model assumes that merozoite lifespan is equal across genotypes and PHZ treatments and that no death of uninfected RBCs takes place before or during parasite invasion, so that the total density we measure on day 4 PI is equal to the density of uninfected cells before invasion.

### 5.2.5 Statistical analysis

All statistical analysis was carried out using R (version 3.2.3). For replication rate, burst size and asexual cycle duration we constructed linear models relating the dependent variable of interest to genotype and PHZ treatment. We used maximum-likelihood based deletion tests to assess the effects of excluding each fixed effect. The dynamics (variation over time) of the proportion of reticulocytes, density of red blood cells and parasitaemia were analysed with linear mixed effect (LME) models, including PHZ treatment, genotype and day PI as fixed effects and mouse ID as a random effect. Reticulocyte and normocyte invasion rates were analysed in a single LME model, as done by Mideo et al. (2011), with host cell identity (normocyte or reticulocyte), genotype and PHZ as fixed effects and mouse ID as random effect. Since the proportional data obtained from this experiment (proportion of reticulocytes) are non-binomial, linear models were used with the pre-condition that the values satisfy linear modelling assumptions (Crawley 2012; Warton and Hui 2011). The significance of fixed effects in LME models was determined by comparing the model including and the model excluding the fixed effect of interest using a likelihood ratio test. In minimised models where genotype or an interaction with genotype remained, we tested which genotypes could be combined without significant loss of variance using deletion tests. Finally, we

constructed a linear model and sequentially removed non-significant fixed effects to estimate the relative importance of traits (burst size, cycle duration, invasion rates) contributing to replication rate and whether they differ among genotypes (through interactions). For each statistical model, were examined diagnostic plots of residuals to assess whether linear modelling assumptions are satisfied (Crawley 2012; Warton and Hui 2011).

## 5.3 Results

### 5.3.1 Pilot analysis: Anaemic hosts and asexual replication

An exploration of the data obtained in the experiment described in chapter 4 shows that all four genotypes of *P. chabaudi* increase their replication rate in mice treated with PHZ (Fig.5.1). Asexual replication rates in mice treated with 120 mg/kg are around two times higher and in mice treated with 30 mg/kg 1.6 times higher than in untreated mice (Fig.5.1B), with PHZ treatment being the only significant effect (PHZ effect on replication rate: $F(2, 65) = 37.5, p < 0.0001$, PHZ effect on dynamics over time post-infection: $\chi^2 = 20.6, p < .0001$). The interaction effect between genotype and PHZ treatment as well as the genotype effect on its own was non-significant for both replication rate and dynamics of parasitaemia, although not far from significance (table 5.1). Chapter 4, which relies on the same data set, shows that the four genotypes in this experiment also increase their conversion. Since we observe an increase in both replication and conversion rates, they must be causally linked to an environmental variable that benefits expansion of the asexual population in anaemic environments and so, allows more resources to be diverted to gametocytogenesis (Carter et al. 2013).

### 5.3.2 Perturbing the within-host environment

As required by the experimental design, all four genotypes experienced non-overlapping RBC environments between PHZ treatments (Fig.5.2 and

Table 5.1. Statistical analysis of asexual replication rate and dynamics of parasitaemia, from data generated in chapter 4. Significant values are marked with a *.

| | |
|---|---|
| **Asexual replication rate** | |
| G by PHZ | $F(6, 56) = 2.10, p = 0.068$ |
| G | $F(3, 62) = 2.26, p = 0.090$ |
| PHZ | $F(2, 65) = 37.53, p < 0.001$* |
| **Parasitaemia dynamics** | |
| G by PHZ by Day | $\chi^2(6) = 8.02, p = 0.237$ |
| G by PHZ | $\chi^2(6) = 7.45, p = 0.280$ |
| G by Day | $\chi^2((3) = 4.17, p = 0.244$ |
| G | $\chi^2(3) = 7.25, p = 0.064$ |
| Day by PHZ | $\chi^2(2) = 20.60, p < .0001$* |

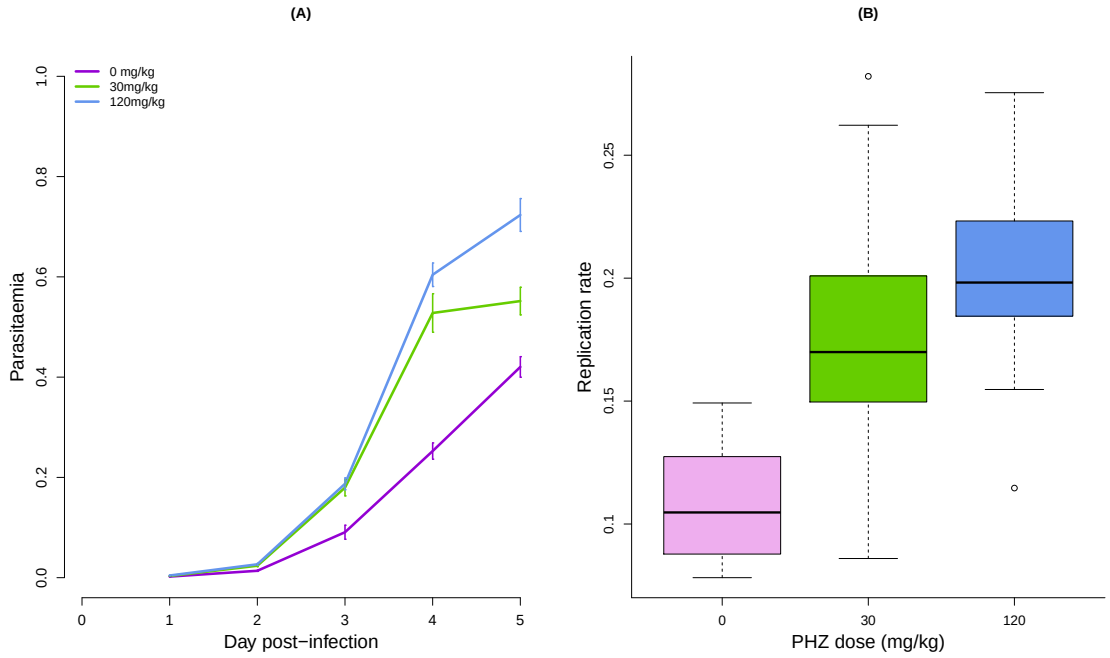


Figure 5.1. (A) Mean parasitemia ((±SE) over time post-infection for four genotypes of *P. chabaudi* (pooled because there was no significant difference between them) diverge in increasingly anaemic environments ($\chi^2 = 20.6, p < .0001$), generated through the injection of PHZ at 30mg/kg (green line) and 120mg/kg (blue), (B) Replication rate of asexual parasites (calculated as the difference in parasitaemia between day 1 and peak) is significantly higher in anaemic environments. Boxplot features here, and throughout the chapter, are: Horizontal line represents median, top of box is the 75th percentile, bottom of box the 25th percentile, whiskers 1.5 times the interquartile range, and dots are outliers.

Fig.5.3). On the day before infection, PHZ had significantly reduced RBC density and significantly increased the proportion of cells that were reticulocytes (Fig.5.2 A and B, table 5.2). Total RBC density in control hosts was 8.42 billion cells (±0.20) per ml, compared to 6.87 billion (±0.22) in PHZ-treated hosts. Reticulocyte proportion was increased from 0.023 (±0.006) to 0.077 (±0.007) by PHZ. The RBC environments remained significantly different between PHZ treatments during the experiment ($\chi^2(3) = 12.29, p < 0.001$). Further, the proportion of reticulocytes was not influenced by which genotype a mouse

was destined to be infected with ($F(3, 54) = 0.73, p = 0.537$) or a genotype by environment interaction ($F(3, 51) = 1.83, p = 0.154$). We found a small but significant genotype by day interaction for the dynamics of red blood cell densities (table 5.2), driven by deviation between AJ and AS infections after day 3 PI. Overall however, parasites within PHZ or control groups all encountered very similar environments upon and during infections.

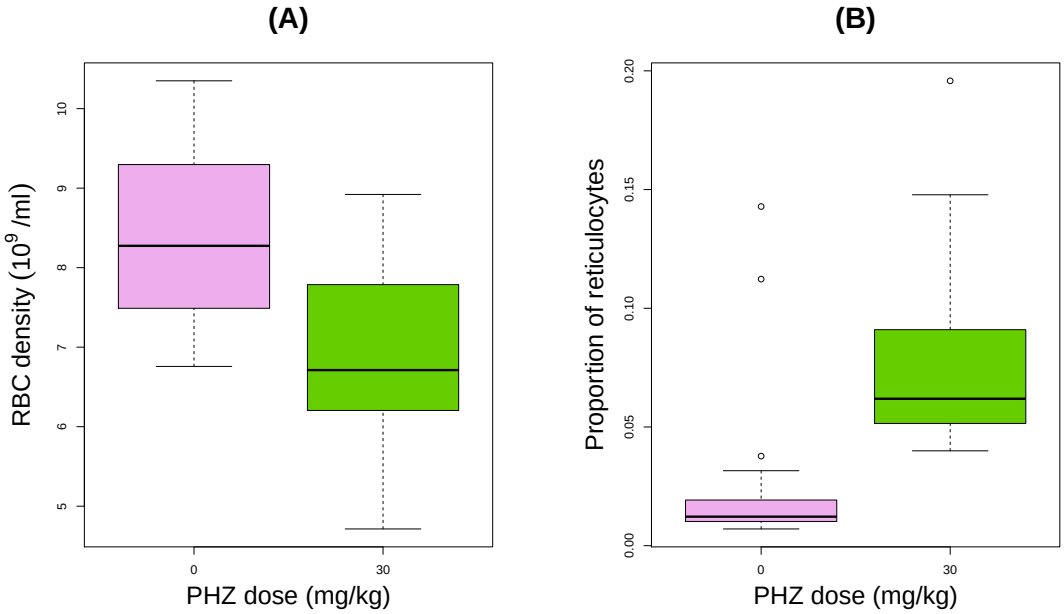


Figure 5.2. (A) Boxplots of the environment that parasites encountered on the first day of infections in terms of total RBC density and (B) the proportions of those cells that are reticulocytes.

### 5.3.3 Cytokines in PHZ-treated mice

The standards in each assay yielded the expected concentrations of INF$\gamma$ and TNF$\alpha$.With the exception of two mice, all mice in each treatment group had undetectable levels of both cytokines. For INF$\gamma$, one mouse was excluded because of poor repeatability between duplicates, and the two mice where INF$\gamma$ was detected had very low concentrations (2.79g/ml and 0.299pg/ml in a PHZ-treated and a control mouse, respectively). Similarly, the two TNF$\alpha$ positive mice had

Table 5.2. Statistical models for the initial perturbation of RBC density and reticulocyte proportion by phenylhydrazine (PHZ). Values of variables on day before infection (-1) and dynamics during infection. Significant terms, not eliminated from the model, are marked with an asterisk (*). G stands for genotype, day for day post-infection

| | | |
|---|---|---|
| **RBC density** | | |
| Day -1 PI | G by PHZ | $F(3, 51) = 2.01, p = 0.125$ |
| | G | $F(3, 54) = 1.62, p = 0.196$ |
| | PHZ | $F(1, 54) = 26.79, p < 0.001$* |
| Dynamics | G by PHZ by Day | $\chi^2(3) = 2.98, p = 0.39$ |
| | G by PHZ | $\chi^2(3) = 5.95, p = 0.114$ |
| | G by Day | $\chi^2(3) = 8.66, p = 0.034$* |
| | Day by PHZ | $\chi^2(3) = 12.29, p < 0.001$* |
| **Proportion of reticulocytes** | | |
| Day -1 PI | G by PHZ | $F(3, 51) = 1.83, p = 0.154$ |
| | G | $F(3, 54) = 0.73, p = 0.537$ |
| | PHZ | $F(1, 54) = 33.95, p < 0.001$* |
| Dynamics | G by PHZ by Day | $\chi^2(3) = 1.99, p = 0.572$ |
| | G by PHZ | $\chi^2(3) = 1.22, p = 0.748$ |
| | G by Day | $\chi^2(3) = 3.76, p = 0.288$ |
| | Genotype | $\chi^2(3) = 1.38, p = 0.710$ |
| | Day by PHZ | $\chi^2(1) = 48.98, p =< .0001$* |

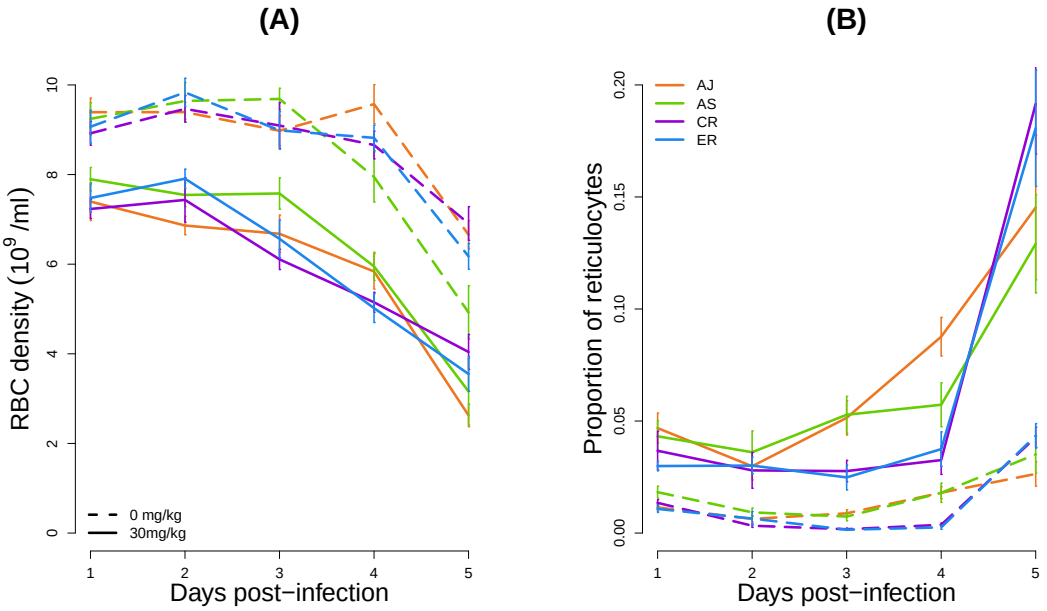


Figure 5.3. Mean (±SE) RBC density and proportion of reticulocytes for each genotype during infections according to their environment (PHZ or control). Within PHZ groups, the dynamics differ in a minor but significant manner between genotypes for RBCs but not for the proportion of reticulocytes.

very low levels (15.6pg/ml and 20pg/ml in a PHZ-treated and a control mouse, respectively).

### 5.3.4 Replication rate

The dynamics for parasitaemia and replication rate are affected by PHZ treatment in a genotype-specific manner (genotype by PHZ interactions; parasitaemia: $\chi^2(3) = 18.83, p < 0.001$; replication: $F(3, 51) = 5.81, p = 0.002$). Specifically, parasitaemia dynamics of AJ and CR show the largest difference between PHZ and control infections (Fig.5.4). In PHZ-treated mice, replication rates are significantly faster than those of controls, multiplying by the following (Fig.5.5): AJ x 1.99, CR x 2.3, ER x 1.14, and AS x 1.02. In a PHZ-treated host, AJ has a significantly higher replication rate than CR ($t(9.17) = 2.91, p = 0.017$) and ER ($t(8.8) = 5.29, p < 0.001$), but all other comparisons are not

significantly different. In the control hosts, only AS and CR differ significantly ($t(7.9) = 6.78, p = 0.001$). Overall, the interaction between genotype and the RBC environment explains 52.8 % of the variation observed in replication rates.

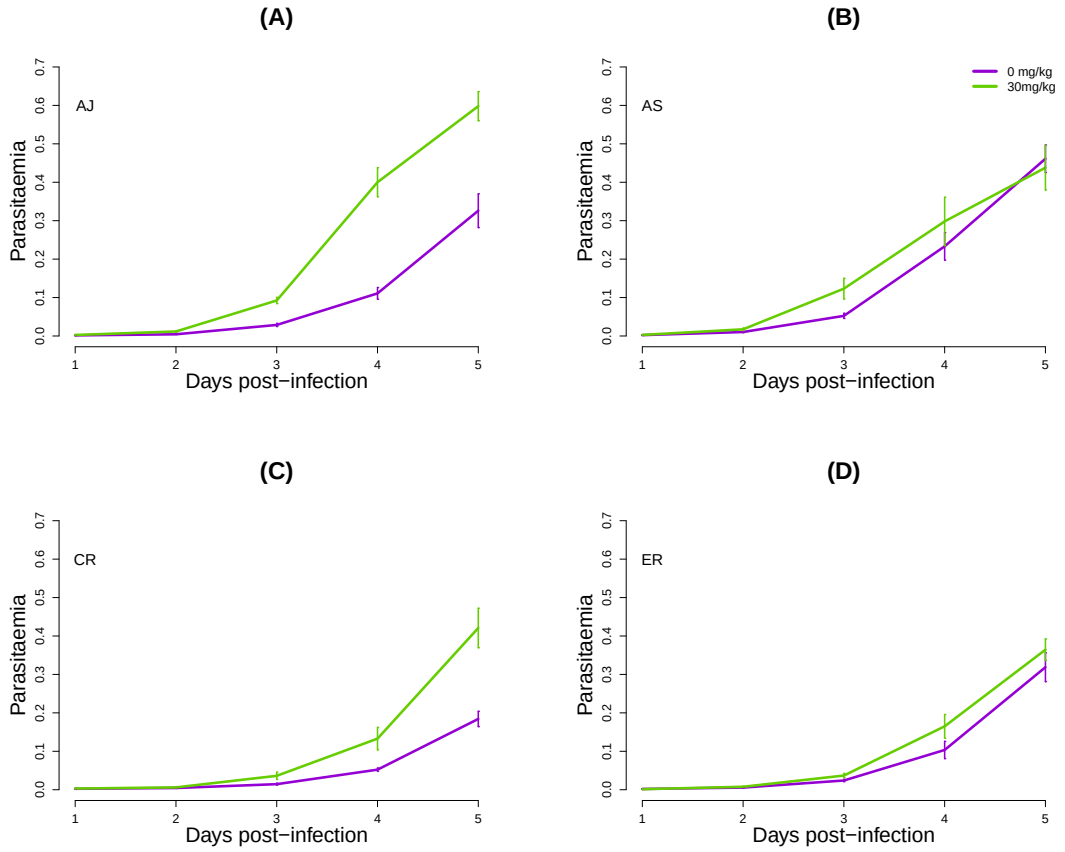


Figure 5.4. Mean parasitaemia (±SE) during infection for each genotype. Parasites in PHZ-treated environments (30 mg/kg, green) tend to have faster replication rates than those in control environments (0 mg/kg, purple).(A):AJ, (B):AS, (C):CR, (D):ER.

### 5.3.5 Burst size

We find that burst size increases in PHZ-treated hosts ($F(1, 49) = 64.782, p < 0.001$, Fig.5.6), and differs among genotypes ($F(3, 51) = 4.47, p = 0.008$), but there is no significant genotype by PHZ environment effect ($F(1, 49) = 64.782, p < 0.001$, see table 5.3 for effect sizes). Genotypes AJ, AS and ER can be combined without any significant decrease in the amount of variance

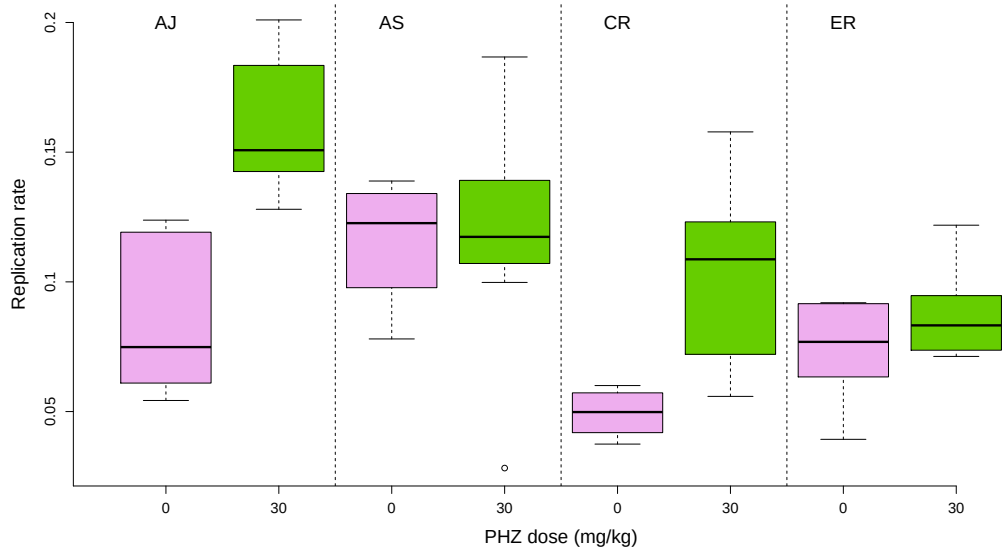


Figure 5.5. Boxplots of replication rates for each genotype in PHZ and control hosts. Whereas replication rate increases strongly in PHZ-treated environments for some genotypes (AJ and CR), others are less responsive to PHZ-treatment (AS and ER).

explained by the model ($F(51, 53) = 1.52, p = 0.228$), i.e. only CR has overall a lower burst size, in both PHZ and control environments. The minimal model (replication $\sim$ genotype + PHZ) explains up to 57.6% of the observed variance in burst sizes ($R^2 = 0.544$).

We also looked at the distribution of burst sizes to determine how the most frequent burst size class changes between treatments and genotypes. From this analysis we excluded 3 mice because less than 10 mature schizonts could be found on the blood smears. The distributions reveal that the mode shifts from 6 to 8 between PHZ and control hosts, for all genotypes and that schizonts with more than 6 merozoites are significantly more common in PHZ hosts (Figure 5.7, effect of PHZ on the proportions of schizonts that have a burst size over 6 merozoites: $F(3, 51) = 65.83, p < 0.001$). There is a significant genotype effect ($F(1, 51) = 2.94, p = 0.042$) but no significant genotype by environment

Table 5.3. **Mean burst size (±SE) by genotype and treatment**

| Treatment | Genotypes | | | |
|---|---|---|---|---|
| | AJ | AS | CR | ER |
| PHZ | 7.81 (±0.15) | 7.29 (±0.29) | 7.10 (±0.16) | 7.58 (±0.25) |
| Control | 6.70 (±0.15) | 6.55 (±0.26) | 6.05 (±0.05) | 6.45 (±0.14) |
| PHZ/Control | 1.17 | 1.11 | 1.17 | 1.17 |

effect ($F(3, 48) = 0.09, p = 0.9634$). In PHZ-treated hosts, genotypes have the following percentage of schizonts with burst sizes above 6 merozoites per schizont: AJ: 70%, AS:69%, CR:59%, ER: 70%, which contrasts with the control treatment where burst sizes $> 6$ reach 38% in AJ, 30% in AS, 13% in CR, and 33% in ER.

To investigate whether this can be explained simply by different burst sizes for parasites infecting reticulocytes or normocytes, we used a linear model to correlate the percentage of schizonts with more than 6 merozoites to the number of infected reticulocytes observed earlier that day, for each PHZ-treated mouse. Although there is a significant positive correlation between the number of infected reticulocytes, and the percentage of high burst size schizonts ($F(1, 25) = 6.64, p = 0.016$, $R^2 = 0.178$), a 1:1 correspondence fits those data significantly worse than the observed correlation ($F(2, 25) = 241.25, p < 0.001$). This suggests that the number of infected reticulocytes in the blood is only a minor determinant for the frequency of high-burst size schizonts (with less than 20% of variance explained). Indeed, the proportion of infected reticulocytes in PHZ-treated hosts was on average only 1.1% (±0.2) yet parasitaemias exceeded 23%, suggesting it is unlikely that burst size is only determined by the age of the RBC a parasite resides in.

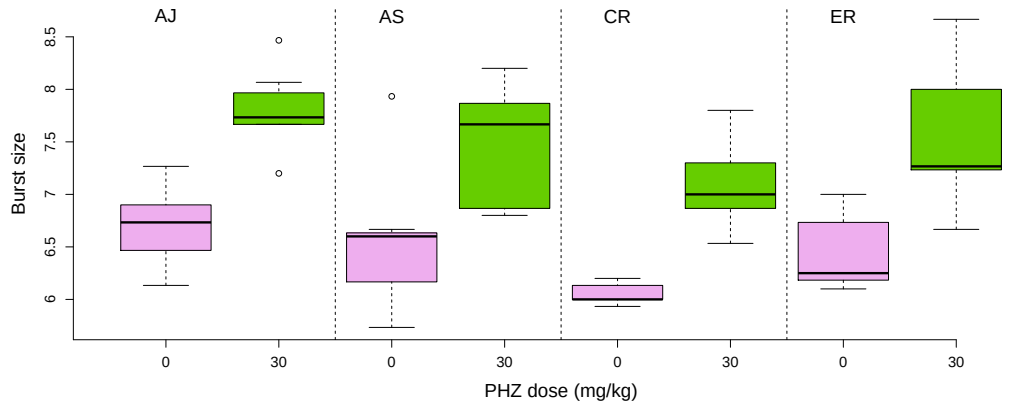


Figure 5.6. Boxplots of burst size for each genotype and treatment combinations. Burst size increases for each genotype in a PHZ-treated environment (green box).

### 5.3.6 Invasion rate

The method used to calculate invasion rates can cause some estimates to be negative due to associated random measurement error between variables. In our data set, 6 out of 56 mice returned negative rates for both reticulocytes and normocytes and were therefore excluded from the analysis. However, excluding or including these datapoints affected the conclusions from our statistical model only quantitatively, not qualitatively. We find that invasion rates do not vary across different PHZ environments depending on genotype identity (RBC age x PHZ x genotype, $\chi^2(3) = 5.17, p = 0.159$). However, if we pool PHZ and control mice, we find genetic variation for invasion rates of reticulocytes and normocytes (genotype x RBC age: $\chi^2(3) = 9.07, p = 0.028$, Fig.5.8A). This interaction is driven by the difference between CR and ER, which are the only two genotypes that cannot be grouped without a significant loss of variance ($\chi^2(2) = 9.07, p = 0.0107$). Specifically, across both PHZ environments, AJ and AS have no detectable RBC age preference but CR tends to prefer reticulocytes and ER prefers normocytes. Finally, although there may be no differences in invasion rate between genotypes in PHZ environments, reticulocytes and normocytes differ in their propensity to invasion in PHZ and control mice (RBC age x PHZ: $\chi^2(3) = 11.98, p = 0.017$),

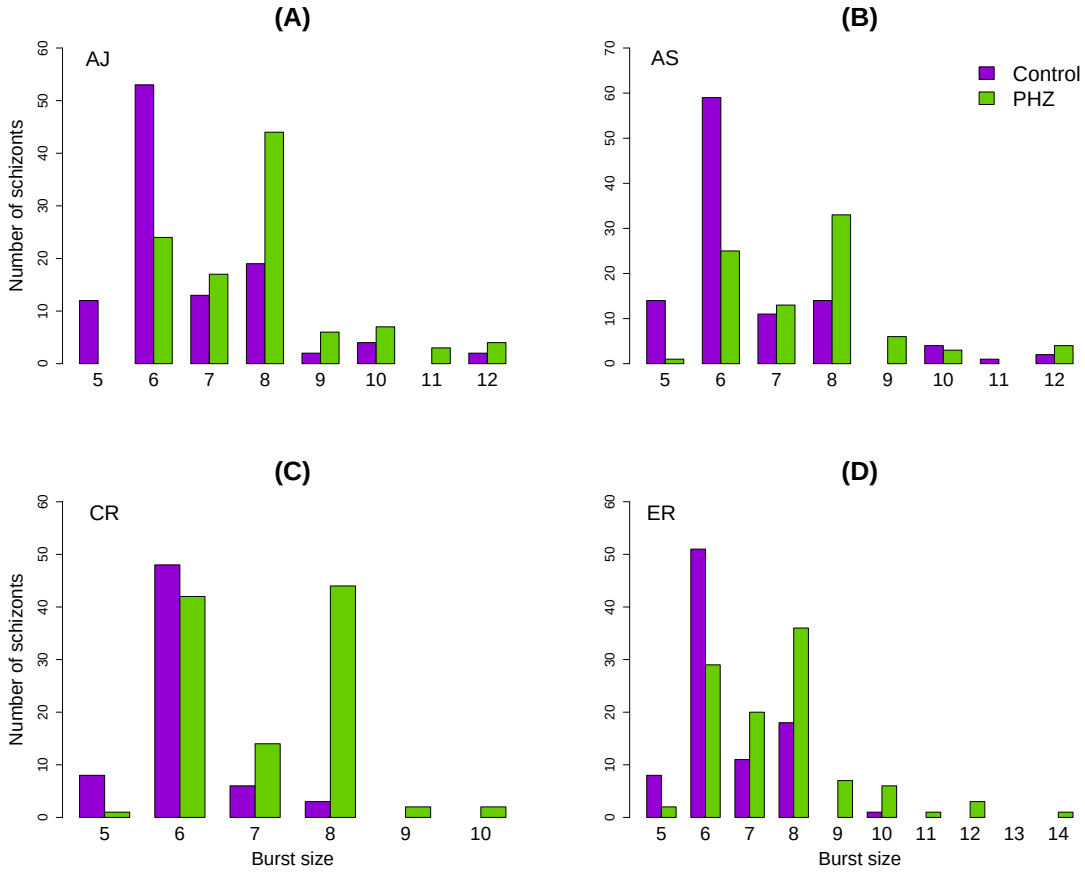


Figure 5.7. Distributions of burst sizes in schizonts, with schizont counts summed for all mice in a given treatment by genotype combination for (A) AJ, (B) AS, (C) CR and (D) ER. The percentage of schizonts that have a burst size of more than 6 merozoites significantly increases in PHZ-treated mice ($F(3, 51) = 65.83, p < 0.001$) and also varies between genotypes ($F(1, 51) = 2.94, p = 0.042$).

but all genotypes are affected equally by this effect. Specifically, invasion rates are generally lower in PHZ hosts, especially for reticulocytes (Fig. 5.8B). Thus, in a PHZ-treated host, normocytes are preferentially invaded over reticulocytes (by a factor of 1.85), whereas there is no apparent preference in a control environment.

### 5.3.7 Relating replication rate and parasite traits

We constructed a model to test the relative importance of traits and their interactions contributing to replication rate and whether they vary between genotypes. The maximal model simplifies to the effect of genotype ($F(3, 49) =$

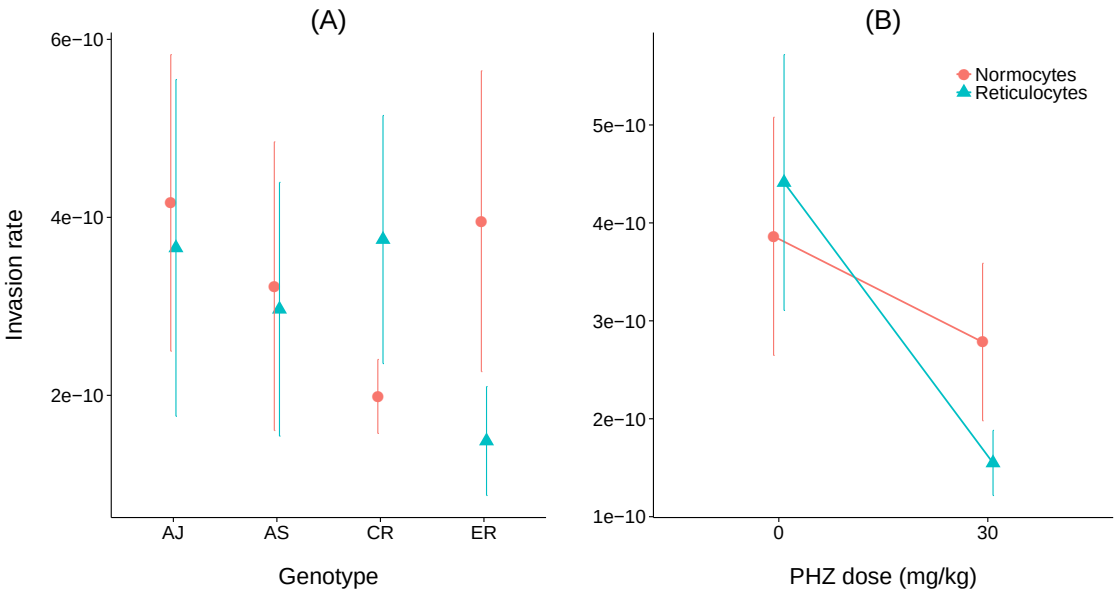


Figure 5.8. Mean (±SE) for invasion rates of normocytes (mature RBC) and reticulocytes (immature RBC) for (A) all genotypes and (B) for different PHZ treatments (all genotypes combined).

$10.38, p < 0.001$ ) and burst size ($F(1, 47) = 7.64, p = 0.008$) (table 5.4) but with no significant interaction between the two ($F(3, 43) = 1.89, p = 0.145$). All interactions between asexual traits as well as the effect of invasion rates can be deleted without significant loss of the variance explained. Replication rate increases with burst size for all genotypes ($F(1, 47) = 7.64, p = 0.008$), and some genotypes have faster growth rates than others, with AJ and AS generally achieving higher growth rates than CR and ER (see first section of results in this chapter). Overall, genotype-specific effects and burst size explain nearly 39.2% of the variance observed in replication rates.

## 5.4 Discussion

The understanding of parasite exploitation traits is inherently challenging because quantitative and qualitative characteristics of an infection are the result of both host and parasite effects (Mideo et al. 2008b). We have examined the potential for complexity by asking whether parasite traits are phenotypically

Table 5.4. Linear model relating replication rate to asexual traits and their interactions, with "G" standing for genotype, "Normo.Inv" and "Retic.Inv" for normocyte and reticulocyte invasion, and "Burst" for burst size.

| | |
|---|---|
| **Full model**: Replication rate $\sim$ G x Normo.Inv x Retic.Inv x Burst | |
| **Minimal model**: Replication rate $\sim$ G + burst | |
| **Non-significant terms deleted from model** | |
| G x Burst x Retic.Inv x Norm.Inv | $F(3, 22) = 0.91, p = 0.455$ |
| Burst x Norm.Inv x Retic.Inv | $F(1, 23) = 0.03, p = 0.870$ |
| G x Norm.Inv x Retic.Inv | $F(3, 26) = 0.38, p = 0.769$ |
| G x Burst x Retic.Inv | $F(3, 29) = 0.64, p = 0.597$ |
| G x Burst x Norm.Inv | $F(3, 32) = 0.50, p = 0.686$ |
| Norm.Inv x Retic.Inv | $F(1, 33) = 0.003, p = 0.953$ |
| Burst x Retic.Inv | $F(1, 34) < 0.001, p = 0.984$ |
| G x Retic.Inv | $F(1, 34) < 0.001, p = 0.984$ |
| Burst x Norm.Inv | $F(1, 38) = 0.79, p = 0.379$ |
| G x Norm.Inv | $F(3, 41) = 2.03, p = 0.126$ |
| Retic.Inv | $F(1, 42) = 0.10, p = 0.748$ |
| Norm.Inv | $F(1, 43) = 0.003, p = 0.953$ |
| G x Burst | $F(3, 43) = 1.89, p = 0.145$ |
| **Significant terms** | |
| G | $F(3, 49) = 10.38, p < 0.001$ |
| Burst | $F(1, 47) = 7.64, p = 0.008$ |

plastic and vary according to within-host conditions. First, we show that PHZ-induced anaemia generates an apparently beneficial environment for parasites in which they can increase their replication and conversion rate (see also chapter 4), and that this does not seem to be caused by a more benign immune environment created by PHZ. We found evidence for plasticity and also of genetic differences for the traits investigated, in anaemic hosts: i) burst size of schizonts increases for all genotypes, ii) whereas there are genetic differences in red blood cell host preference, all genotypes tend to prefer normocytes in a reticulocyte-rich, anaemic environment. Overall however, the data suggest the major contributing trait to replication rate, in addition to genotype identity, is burst size. However, we note that in previous experiments using the same dose of PHZ, all genotypes investigated here increased their replication rate to a greater extent (see figure 5.1 and chapter 6). The difference is likely explained by a reduction in efficacy of the older stocks of PHZ used here. However, the smaller effect of PHZ reported here suggests that our results are conservative, but potentially too conservative to detect major contributions of other traits to replication rate.

The increase in burst size is small (on average 1 merozoite more per schizont), but thanks to exponential growth at the beginning of infection, even a small, early increase in burst size can make a large contribution to within-host replication. We suggest that this increase is achieved through a shift in the distribution of burst sizes, with schizonts producing more than 6 merozoites prevailing in anaemic infections. However, the derived distributions may be anticonservative because the pooled schizont counts comprise repeated measures from each mouse. Nonetheless, they are indicative of a shift that requires further exploration. Previous studies have suggested that burst size in *P. chabaudi* is directly related to growth conditions inside reticulocytes (Mideo et al. 2008b). Also, *P. berghei*, another rodent malaria species that is restricted to infecting reticulocytes, produces, on average, two times more merozoites per schizont compared to *P. chabaudi* (Killick-Kendrick 1978). More recent work, however, has noted that burst size in *P. chabaudi* is hard to explain by the age of host

RBC alone (Mideo et al. 2011). Further, if parasites increase their burst size to make use of reticulocytes, it is hard to explain why preference for reticulocytes decreases in anaemic mice, unless some invasion-related constraint applies (e.g. Khan et al. 2001). Here we confirm that growth inside reticulocytes *per se* is not the cause of increased replication: schizonts with higher burst size (around 70%) vastly outnumber infected reticulocytes (around 1%). This suggests that parasites actively adjust their burst size, while mainly growing in normocytes, in response to anaemic conditions. Recently, *P. berghei* has been reported to actively (through differential gene expression) increase its burst size and replication rate in response to nutrients inside the host blood, suggesting that this plastic strategy may be widespread among malaria species (Mancio-Silva et al. 2017). Through a range of experiments, the authors were able to pinpoint the parasite genes involved in nutrient sensing. In our case, we have yet to identify the cause or cue to which parasites increase replication because it is unlikely that PHZ causes hosts to forage more.

Explaining the increased replication in anaemic hosts can be approached from two perspectives. The first set of explanations are teleological: parasites increase their burst size at the present timepoint to profit from some event in the future. For example, one could view an increase in burst sizes as a "parasites in a rush"-scenario, i.e. as part of a terminal investment strategy where parasites "hurry" to increase their state, followed by high conversion rates, to make optimal use of a dying host. The corollary of these teleological arguments is however that they assume that parasites restrain their burst size (and replication rate) during the majority of normal infections. Furthermore, evidence from this, other experiments (chapter 4) and the fact that parasites increase conversion in parallel, suggest a second, causal perspective: parasite increase their burst size in response to the appearance of additional energetic resources or nutrients. Due to the fundamental trade-off between conversion and replication, both can only be increased simultaneously if the state of the parasite population increases, i.e., if available resources for the within-host parasite population increase or parasite killing decreases (discussed in more detail in chapter 4). We

propose that as yet unidentified factors in an anaemic within-host environment create favourable conditions in which parasite state and reproductive investment increase simultaneously. A reduction in immune killing in PHZ treated hosts is unlikely because the rapid ageing of RBC and subsequent clearance should, if anything, stimulate an inflammatory state. This includes macrophage expansion in the spleen (Naughton et al. 1990), formation of reactive oxygen species (McMillan et al. 2005) and oxidisation of cellular components (Beutler 2001), and decreased membrane deformability (Berger 2007). We were unable to detect any such response in plasma cytokines to PHZ or anaemia, but quantification of macrophages or immune-cell stimulation assays with parasite antigen (Langhorne et al. 2008) may provide further clarification.

Each malaria parasite species can be characterised by its preference for certain RBC ages. Here, we confirm that *P. chabaudi* is very much a generalist. Genotype-specific preferences have also previously been reported, although they differ qualitatively from those presented here (e.g. reticulocyte preference for AS, Mideo et al. 2011, normocyte preference for AJ, Antia et al. 2008). RBC preference is important determinant of whether parasites are capable of successfully setting up an infection (Antia et al. 2008; Mideo et al. 2011; Okada et al. 2015). Although our model cannot confirm the importance of preference on the realised replication rate, we show that preference can plastically change depending on the within-host environment, with no apparent preference in a control mouse but a preference for normocytes in anaemic mice. If reticulocytes really are a superior host cell for *P. chabaudi*, this switch in preference seems counterintuitive. One potential adaptive explanation would be active avoidance of reticulocytes to allow the host to recover and for parasite to establish a chronic infection, and thus benefitting from future transmission opportunities (McQueen and McKenzie 2006). This explanation suffers from such restraint being maladaptive in genetically mixed infections (which are the norm). More likely, the switch in preference is the outcome of a constraint defined by the age composition of the within-host environment. Work on the PY235 gene family of the rodent parasite *P. yoelli* suggests that age composition of the

RBC environment could epigenetically affect the invasion receptor profile of merozoites- and by extension the way they can exploit their resource environment (Khan et al. 2001; Preiser et al. 2002). Since *P. chabaudi* carries a homologous gene family (PCH235, Grüner et al. 2004) similar mechanisms may operate. One may therefore expect that the presence of reticulocytes, which carry a more diverse set of cell-surface receptors than normocytes, could also promote a higher diversity of invasion receptors among merozoites, which in turn may lead to better invasion of normocytes too (Khan et al. 2001).

In conclusion, we have shown the presence of plasticity of traits underpinning asexual replication in response to anaemia. A plastic increase in burst size seems the most likely contributor to the increased replication rate observed in anaemic environments. Future work should explore in more detail whether changes in asexual cycle duration contribute to increasing replication rate or whether they are related to burst size through trade-offs. For example, the production of a certain invasion receptor profile at the surface of merozoites may trade-off with the number of merozoites produced in a focal schizont. Finally, it is important to determine whether anaemia is also a favourable environment for parasites in natural human infections because the development of anaemia in natural infections may not always coincide with the appearance of anti-parasite immunity. Anaemia is one of the most common and lethal malaria-associated complications (Menendez et al. 2000) so it is crucial to determine whether anaemia can enhance virulence of malaria parasites by promoting their replication rate.

# Chapter 6

# Costs and consequences of plasticity: rates of conversion and asexual replication in *Plasmodium chabaudi*

## Abstract

Phenotypic plasticity allows organisms to match trait values to diverse selection pressures, mediated by different environments. Such adaptive phenotypic plasticity may be of special importance to parasites, which experience frequent environmental changes during their life-cycle, between individual hosts and during infections. However, the evolution of adaptive phenotypic plasticity is thought to be limited by costs and constraints. Such limits to the evolution of plasticity are rarely explored empirically. In previous chapters we have shown that malaria parasites plastically increase both their asexual replication and gametocyte conversion rate in anaemic hosts. A key question is whether these traits can be efficiently switched back to their control values if the within-host environment returns to normal. Any failure in doing so could be evidence of a constraint in plasticity and thus limit its adaptive significance. Here we show that

if parasites are transferred from an anaemic host into an uninfected, control host, they adjust asexual replication rate (and a key trait underpinning it, burst size) as well as conversion rate to the values observed in parasites transferred between control hosts. Furthermore, we do not detect any longer-term fitness costs of having previously adjusted asexual or sexual traits in anaemic environments. Thus, we suggest that malaria parasites are highly plastic and rapidly able to match their phenotypes to within-host conditions. However, we also suggest future research to test whether costs may be paid at other levels.

## 6.1 Introduction

The ability of organisms to alter aspects of phenotype in response to changes in the environmental conditions they experience (phenotypic plasticity) is commonly observed in multicellular organisms (Pigliucci 2001; Scheiner 1993; Schlichting and Smith 2002). But, the notion that phenotypic plasticity is central to the fitness of parasites is relatively recent and remains controversial in medicine and parasitology (Kochin et al. 2010a; Thomas et al. 2002). This is due to the longstanding assumption that parasites, since they live inside their hosts, are sheltered from the rapidly changing conditions of the exterior environment, such as weather, temperature and predators, and that there is a high autocorrelation in conditions experienced during infections and between hosts (Sukhdeo and Sukhdeo 1994, 2002). In contrast, increasing evidence suggests that parasites are confronted with fast changing conditions during infections, that individual hosts vary considerably over time, and that within-host environmental variation impacts upon parasite fitness. Phenotypic plasticity of parasites may be adaptive on at least three scales of biological organisation. Parasites encounter diverse within-host environments during complex life-cycles, between different host individuals and within individual infections. First, parasites with complex life cycles that include multiple types of host (including vectors), encounter different within-host environments, which generally comprise hosts from different phyla. For example, for the tapeworm *Schistocephalus*

*solidus*, the immune system of the intermediate host (the copepod *Macrocylops albidus*) poses different challenges, compared to that of its final vertebrate host (the three-spines stickleback *Gasterosteus aculeatus* (Hammerschmidt and Kurtz 2005). Some life-cycles of parasites may even comprise a free-living stage, such as *Strongyloides ratti*, which can plastically alter its developmental route either into free-living, sexual adults or into newly infective stages (Fenton et al. 2004). Some species of bird malaria parasites (e.g. *Plasmodium* SGS1), are found to infect and transmit successfully from a large variety of bird species (Drovetski et al. 2014), each of which may differ in the within-host environment they provide (Hellgren et al. 2009).

Second, despite some degree of autocorrelation across conspecific hosts, individuals vary (e.g. age, sex, condition, immunocompetence, other infections) in ways that impact survival and transmission of parasites. For example, European Siskins differ in their transcriptional response to infections of *P. ashfordi* and the parasites show an equally diverse transcriptional profile that varies highly between infections of different individuals of a same host species (Videvall et al. 2017, 2015).

Third, within-host conditions change rapidly as infections progress. This includes altered resources available to parasites (Cromer et al. 2009), attack by immune responses (Miller et al. 2010), interactions with co-infecting parasites (Pollitt et al. 2011b; Ramiro et al. 2016), and drug treatment (Chapter 2). For example, *Strongyloides ratti* parasitic nematodes modify their generally stable transcriptome during late infection of immunised individuals, supposedly, to avoid expulsion out of the host intestine (O'Meara et al. 2010).

As the examples above illustrate, phenotypic plasticity has been reported for a diverse range of parasites and traits of parasites, with many of them appearing to be adaptive (Gomez-Diaz et al. 2012; Kochin et al. 2010a; Thomas et al. 2002). However, like all traits, phenotypic plasticity is subject to costs, limits and constraints, but these concepts are rarely studied (Auld et al. 2010). For example, the evolution of plastic strategies are limited by the presence of genetic

variation and shaped by past selection or drift (evolutionary history, Schlichting and Pigliucci 1998). There are also a range of costs and constraints specific to plasticity. First, there are production costs, i.e. the cost of maintaining the sensory machinery required to monitor changes in the environment and produce the plastic phenotype (Auld et al. 2010). Second, plastic traits are not independent of other (plastic or fixed) traits due to allometry or genetic linkage, with which they may trade-off (Auld et al. 2010). Third, costs may arise due to the production of a suboptimal phenotype or due to a time lag between the environment changing and producing the optimal phenotype. For example, plasticity of many developmental traits is constrained by a window of sensitivity, i.e. where a trait is only responsive to the environment during early development and becomes fixed for the remaining lifetime of the individual (e.g. maternal effects). Thus, there is a reasonable risk that this trait will be at a suboptimal value at some point during an organism's lifetime. Finally, current trait values may be constrained by past plastic modifications of that trait, resulting in long-term costs of plastic responses (Murren et al. 2015). The latter may be mediated by epigenetic repercussions, a constraint in energy that can be allocated to altering a trait, or the footprint of the physiological state of the organism (Gomez-Diaz et al. 2012).

In multicellular non-parasitic organisms, costs of plasticity have been mostly observed in the form of fitness costs of phenotype-environment mismatches (Beaman et al. 2016; van Kleunen and Fischer 2005), which can be very high. In contrast, for parasites, studies of constraints on plasticity have focussed on "trans-host effects" (in analogy to transgenerational effects). This concerns the effect of conditions that parasites experience in one host (and their potential plastic response to them) on trait expression (and fitness) in the next host (Davies et al. 2001; Gower and Webster 2004; Hammerschmidt and Kurtz 2005). For example, the apicomplexan mosquito parasite *Ascogregarina taiwanensis* is more virulent if its previous host was well-fed (Tseng 2006), which can reduce fitness of the parasite if it kills the next host too quickly (Frank 1996). The same phenomenon has been observed for the bacterium *Pasteuria ramosa*, in its

host *Daphnia magna* (Little et al. 2007). Similarly, high parasite density within the intermediate host can reduce the body size of parasitic worms, affecting their survival in, and transmission success from, the final host (Steinauer and Nickol 2003, Fredensborg and Poulin 2005). Although, yet to be investigated, we suggest that a similar process could take place within a single host: a plastic reaction against one feature of the within-host environment may constrain or trade off against responses to future changes in the within host environment.

Studying the cost, limits, and constraints on phenotypic plasticity in parasites is fraught with the inherent difficulty of distinguishing how much of the trait change in the host is directly due to the parasite and how much is directly caused the host. For example, to what extent do parasitic worms living in high densities control their growth and to what extent do other direct host effects such as nutrient limitation and immunity play a role (Cornet 2011)? Such issues are compounded if the plastic response of parasites feeds back to further alter the environmental character eliciting the change in phenotype. Further, the extent to which parasites can realise a change in phenotype (the optimal vs actual trait value) can be affected by the constraints and opportunities of the new environment.

In this chapter, we explore the consequences of plasticity in the rodent malaria parasite *P. chabaudi*. *P. chabaudi* is capable of facultative adjustment of the ratio of male and female transmission forms in response to the number of coinfecting conspecific strains, following Hamilton's Local Mate Competition theory (Reece et al. 2008), and parasites plastically modify their investment into transmission forms in the manners consistent with life-history theory (Carter et al. 2013). Asexual replication rate is also phenotypically plastic and sensitive to anaemia (Fig.6.1). While there is considerable evidence that plasticity in transmission strategies is under parasite control (Chapter 4, Carter et al. 2013; Kafsack et al. 2014; Sinha et al. 2014), asexual replication rate is clearly the net effect of interactions between multiple parasite and host traits. However, chapter 4, chapter 5 and recent work (Mancio-Silva et al. 2017) suggest that parasites have control over traits involved in replication rate, such as burst size

(the number of merozoites produced by a mature schizont). Most experiments into parasite plasticity are designed to reveal plasticity following perturbations of the environment, few ask if and how parasites traits revert back to a trait value associated with the previous environment. We undertake the latter, focusing on how asexual replication rate and investment into transmission forms (conversion rate) vary when parasites are transferred from anaemic to healthy hosts. Anaemic within-host environments may have lasting effects on parasite traits (Fig.6.1). For example, the age composition of red blood cells (RBC) within a host affects the diversity of invasion receptors among the released progeny of asexual parasites (merozoites), thereby potentially also affecting their invasion pattern (Khan et al. 2001). This occurs because mature red blood cells (normocytes) and immature red blood cells (reticulocytes) carry different receptors and invasion receptors on parasites are partly epigenetically inherited from the parent. Variation in the repertoire of invasion ligands may explain observations of both genetic variation and plasticity in RBC age preference (Antia et al. 2008; Mideo et al. 2011) and may also cause a time-lag until the pool of invasion receptors among merozoites has adjusted to the RBC age profile of new within-host environment.

## 6.2 Methods

### 6.2.1 Parasites and hosts

We obtained C57BL/6 female mice (aged 6–8 weeks) in-house (University of Edinburgh) and the *Plasmodium chabaudi* clone AS from the Edinburgh Malaria reagent repository (University of Edinburgh). *P. chabaudi* was isolated between 1948 and 1974 from African thicket rats, *Thamnomys* spp, in Central Africa Killick-Kendrick (1978). After cloning, the genotype AS has been cryopreserved and undergone regular transmission through mosquitoes to maintain its wild type phenotypes (Spence et al. 2013). This experiment comprises two sets of hosts. The first set ("initial hosts") were treated on day -4 post infection (PI) with 30 mg/kg of PHZ (n=14) or 0 mg/kg (PBS, control treatment, n=11) and injected

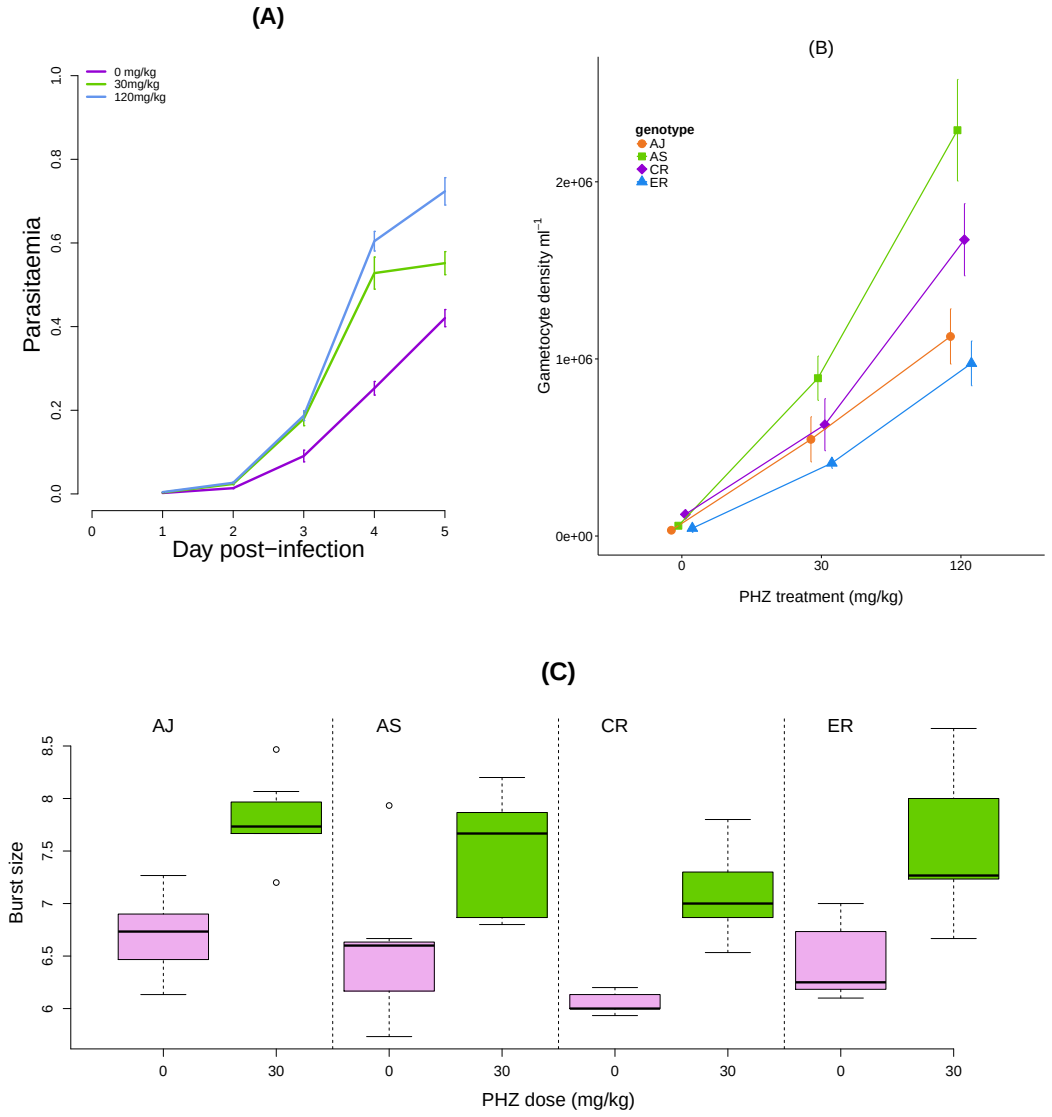


Figure 6.1. **Plasticity in parasite traits in response to host anaemia**. (A) Mean (±SE) parasitaemia during infections for all 4 genotypes combined (as no significant differences between genotypes were observed) for different levels of anaemia, induced by PHZ injection. (B) Mean (±SE) conversion rate increases with increasing doses of PHZ but to different extents for different genotypes (genotype by environment interaction). (C) Burst size increases for all genotypes in anaemic environments (green) compared to control environments (purple). Boxplot features here, and throughout the chapter, are: Horizontal line represents median, top of box is the 75th percentile, bottom of box the 25th percentile, whiskers 1.5 times the interquartile range, and dots are outliers.

intravenously with 5 x $10^6$ AS parasitized RBC on day 0 PI. On day 4 PI, we transferred 5 x $10^6$ parasitized RBC ("asexual parasites") from each initial host into a randomly chosen mouse that was not treated with either PHZ or PBS (the final, "common garden" hosts, n=25). For simplicity, we designate the mice that received their parasites from a PHZ-treated mouse as an "ExPHZ" host (n=14) and mice that received their parasites from a control mouse as an "ExControl" host (n=11). Note, the initial and final hosts are paired by a one-to-one passage, thus data points across host types compare the same parasites in each type of environment (Fig.6.2). We monitored the initial hosts for 5 days PI and the final hosts for 16 days PI by taking 2$\mu$l of blood to quantify RBC density, making a thin blood smear to assess asexual stages and RBC age structure, and collecting 10$\mu$l blood for RT-qPCR to quantify gametocytes that are more than approximately 35 hours old, using the gene *CG2* whose expression is limited to gametocytes (Wargo et al. 2007b, see chapter 4 for further details). For initial and final hosts, burst size was assessed by taking the mean number of merozoites counted from the first 30 mature schizonts observed on blood smears taken shortly before schizogony on day 4 PI, (initial hosts: n=8, 4 per treatment, final hosts: n=25, ExPHZ: n=14, ExControl: n=11).

### 6.2.2 Analysis

All calculations and statistical analysis were carried out in R version 3.2.3. We used linear mixed effect models, including the mouse ID as a random effect. All derived test-statistics stem from a maximum likelihood-based comparison of the model including and excluding the fixed effect of interest. All other comparisons were made using Welsh's t-test and checking for normality of residuals. The within-host environment in initial and common garden hosts was characterised by following the density of total red blood cells (i.e. the combined density of normocytes and reticulocytes) as well as the age structure (i.e. the proportion of RBC that are reticulocytes). Since the latter are non-binomial data, linear models were used with the pre-condition that the values satisfy linear modelling assumptions (Warton and Hui 2011). Asexual replication was measured by peak

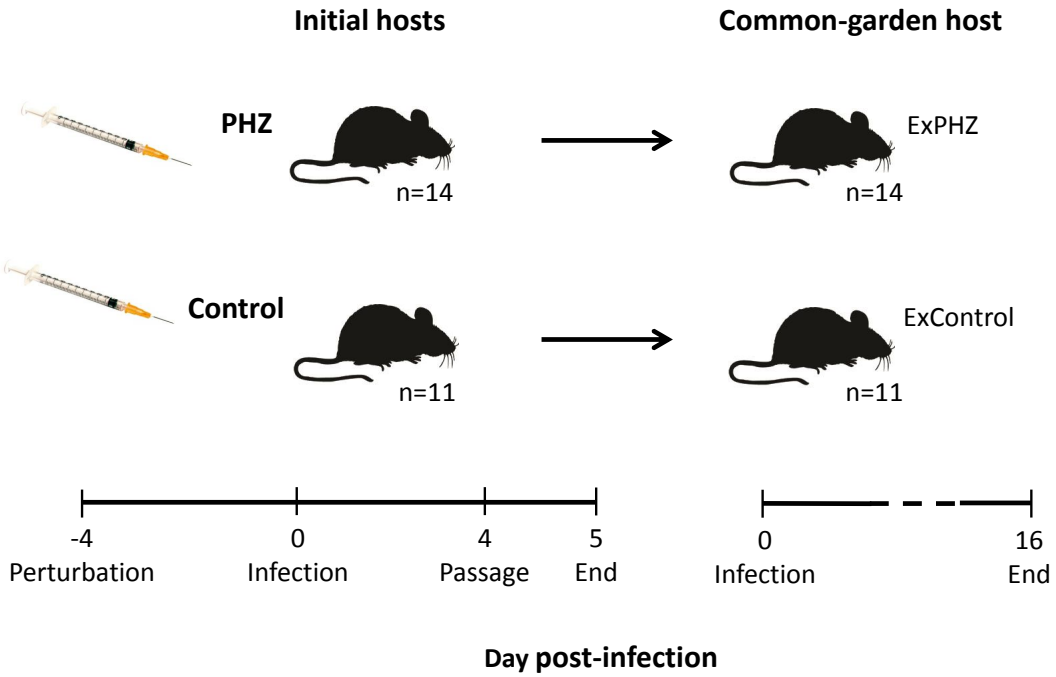


Figure 6.2. **Experimental set-up**. Initial hosts were treated with 30mg/kg of PHZ or PBS (control) on day -4 PI and infected with *P. chabaudi* AS on day 0. On day 4 PI 5 x $10^6$ asexual parasites were transferred from each initial host into common garden hosts to initiate a new infection, which was followed for 16 days. In all sets of hosts we measured peak parasitaemia, replication rate, burst size (on day 4 PI), peak gametocytaemia, cumulative gametocytaemia, and variation in density over time (dynamics) of asexual parasites and gametocytes.

parasitaemia, the rate of parasitaemia increase from day 1 to peak (from now on called replication rate), as well as the burst size of mature schizonts. We also followed the dynamics of the densities of asexual parasites for the duration of infections, calculated as daily parasitaemia multiplied by daily total RBC density. For each statistical model, were examined diagnostic plots of residuals to assess whether linear modelling assumptions were satisfied (Crawley 2012; Warton and Hui 2011). In common garden hosts, infections were followed for 16 days post-infection which allowed us to use to estimate conversion, relying on

the spline-based model of Greischar et al. (2016b). We also compare peak and cumulative gametocyte densities in both initial and common garden hosts.

## 6.3 Results

### 6.3.1 Comparisons of within host environments

Our experimental design involved four different groups of host: control or PHZ-treated "initial" hosts and Excontrol or ExPHZ "common garden hosts". To meet the assumptions of the design, the control and PHZ-treated "initial" hosts must differ in terms of anaemia, whereas the control "initial" hosts and the ExControl and ExPHZ "common garden hosts" must not (i.e. there should be an interaction between initial and final host types). This interaction is highly significant for the dynamics of RBC density ($\chi^2(4) = 27.97, p < 0.001$, Fig.6.3) and the proportion of reticulocytes ($\chi^2(4) = 17.35, p = 0.002$, Fig.6.4) during infections. Likewise, this interaction shapes the summary variables relating to anaemia; minimum RBC density ($F(1, 47) = 10.33, p = 0.002$) and the maximum proportion of reticulocytes ($F(1, 47) = 20.45, p < 0.001$, see table 6.1 for pairwise comparison between host types). As expected, mice grouped as either PHZ treated or not (Control, ExControl and ExPHZ) strongly differ in these variables. PHZ-treated mice had an average minimum RBC density of 3.35 ($\pm$0.64) billion cells per ml and an average maximum reticulocyte proportion of 0.17 ($\pm$0.02). This compares to 6.11 ($\pm$0.23) billion RBCs and a reticulocyte proportion of 0.04 ($\pm$0.006) for mice not treated with PHZ. Pairwise comparisons between each group confirm that parasites experienced different within-host environments in PHZ-treated mice compared to mice that did not receive PHZ (Control, ExPHZ and ExControl, table 6.1).

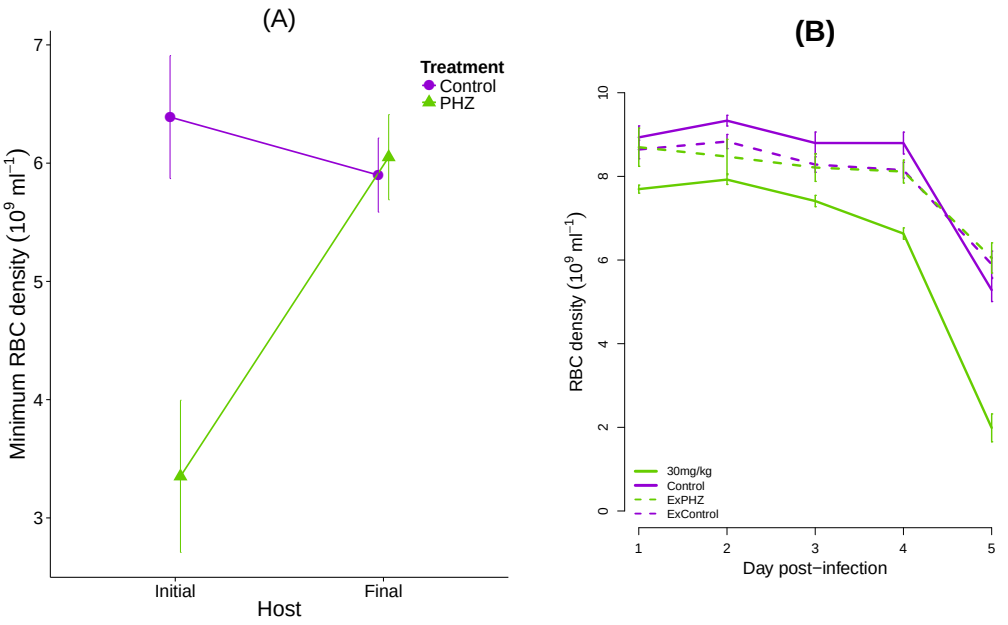


Figure 6.3. (A) Average minimum RBC density (±SE) differs between PHZ treated and not treated ("Control", "ExPHZ" and "ExControl") mice, (B) Mean (±SE) RBC density follows significantly different patterns between mice that received PHZ (30mg/kg) and those did not receive PHZ ("Control", "ExPHZ" and "ExControl", $\chi^2(4) = 27.97, p < 0.001$.

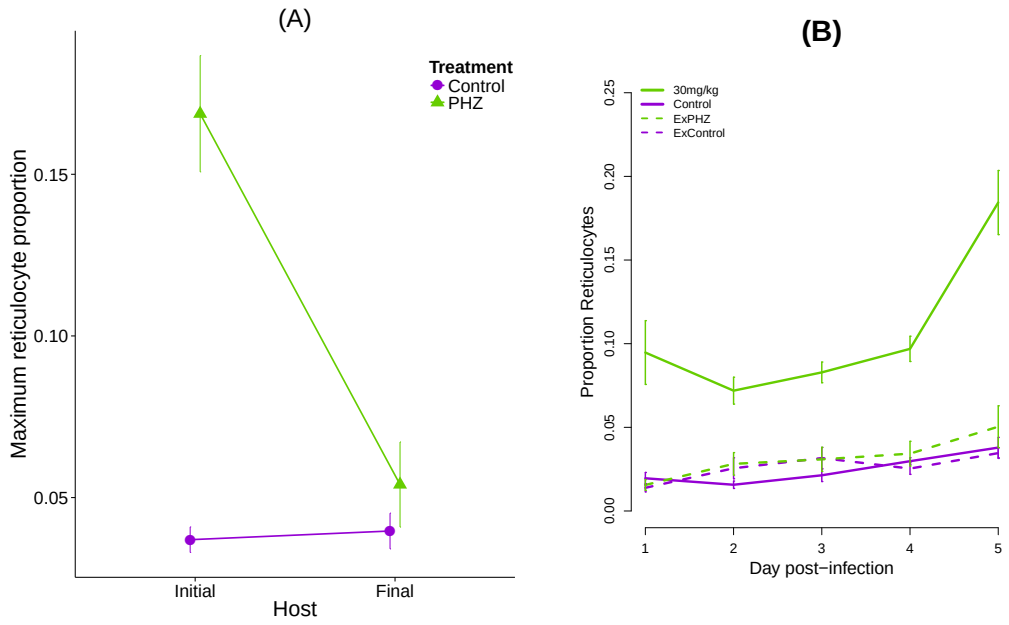


Figure 6.4. (A) Average maximum proportion of reticulocytes (±SE) differs between PHZ treated and not treated ("Control", "ExPHZ" and "ExControl") mice, (B) Mean (±SE) proportion of reticulocytes follows significantly different patterns between mice that received PHZ (30mg/kg) and those did not receive PHZ ("Control", "ExPHZ" and "ExControl").

Table 6.1. Pairwise statistical comparisons of minimum RBC density and maximum proportion of reticulocytes between host types.

**Minimum RBC density**

| | | Initial | Common Garden | |
|---|---|---|---|---|
| | | **Control** | **ExControl** | **ExPHZ** |
| Initial | **PHZ** | $t(15) = -7.63, p < 0.001$ | $t(18.8) = -8.54, p < 0.001$ | $t(21.7) = -8.3, p < 0.001$ |
| | **Control** | | $t(15.8) = -1.48, p = 0.159$ | $t(18.8) = -1.69, p = 0.107$ |
| Common Garden | **ExControl** | | | $t(23) = -0.32, p = 0.754$ |

**Maximum proportion of reticulocytes**

| | | Initial | Common Garden | |
|---|---|---|---|---|
| | | **Control** | **ExControl** | **ExPHZ** |
| Initial | **PHZ** | $t(10.7) = 7.29, p < 0.001$ | $t(9.5) = 7.7, p < 0.001$ | $t(16.3) = 5.86, p < 0.001$ |
| | **Control** | | $t(9.2) = 0.48, p = 0.643$ | $t(17.7) = -0.90, p = 0.380$ |
| Common Garden | **ExControl** | | | $t(14.6) = -1.22, p = 0.240$ |

### 6.3.2 Parasite traits vary between PHZ-treated and control "initial" hosts

The second of assumption of our design is that parasites modify their traits in PHZ-treated and control "initial" hosts in the manners previously described. As expected, we find that the dynamics of asexual density varies significantly between treatments ($\chi^2(6) = 75.43, p < 0.0001$), peak parasitaemia is on average 1.4 times higher and replication rate on average 1.5 times higher in PHZ-treated mice compared to control mice (table 6.2). Furthermore, burst size was significantly higher in PHZ-treated mice with on average 7.96 ($\pm 0.08$) merozoites per schizont compared to 6.93 ($\pm 0.19$) in control hosts (table 6.2). Also as expected, we find that the dynamics of sexual stage gametocytes differs between treatments ($\chi^2(1) = 30.59, p < 0.001$), giving rise to a peak gametocyte density 3.4 times, and a cumulative density that is 4.3 times, higher than in control hosts. We thus confirm that all traits of interest are significantly different in PHZ-treated and control initial hosts.

### 6.3.3 Trait values in the common garden hosts

Having confirmed that initial hosts provide significantly different within-host environments and that parasites traits are adjusted between these environments, we focus on parasite traits in the final hosts. We did not detect any significant differences in asexual stage traits between ExPHZ or ExControl parasites (Fig.6.5). This includes, asexual replication rate, peak parasitaemia and burst size (table 6.3) and the dynamics of asexual densities ($\chi^2(15) = 12.45, p = 0.6445$, Fig.6.6A). Also, we did not detect any significant differences in sexual stage traits between ExPHZ or ExControl parasites (Fig.6.7). Conversion rates did not follow significantly different dynamics between treatments ($\chi^2(13) = 8.17, p = 0.832$, Fig.6.6B) though ExPHZ parasites tended to reach higher peak gametocyte densities (table 6.3). Overall fitness, measured as cumulative gametocyte density, did not differ significantly between ExPHZ and ExControl parasites (Fig.6.7B, table 6.3).

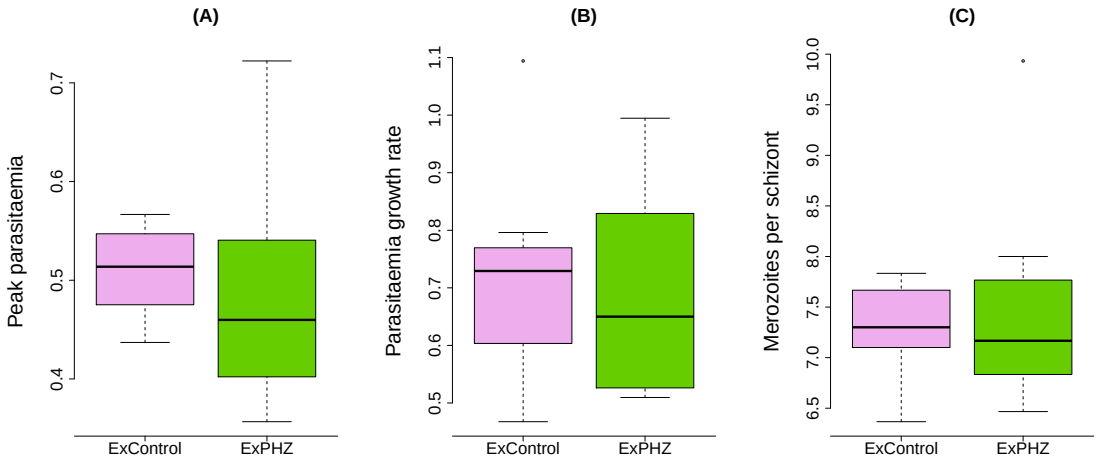


Figure 6.5. (A) Boxplots for peak parasitaemia, (B) replication rate (C) and the mean number of merozoites per schizont in common garden hosts, with green indicating parasites that previously experienced anaemic conditions (ExPHZ) and purple for parasites stemming from control hosts (ExControl).

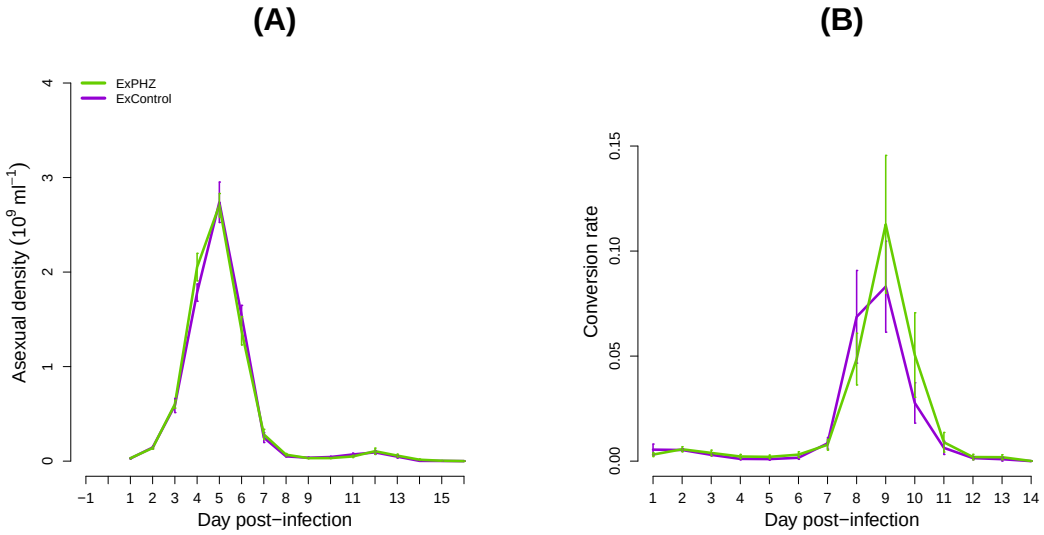


Figure 6.6. (A) Mean(±SE) density of infected RBC cells and (B) conversion rate into gametocytes in common garden hosts. Parasites stem from anaemic hosts (ExPHZ, green) or control hosts (ExControl, purple).

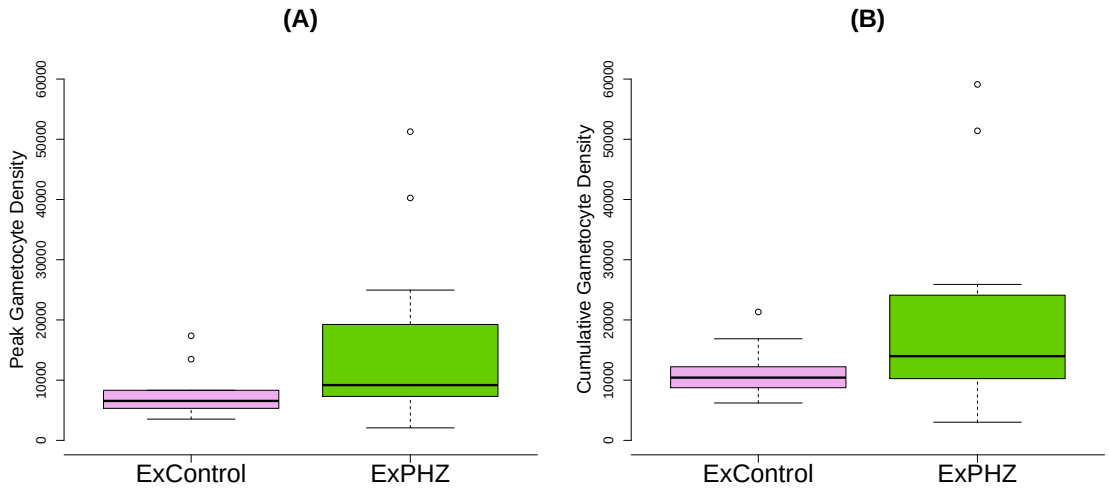


Figure 6.7. Box-plots for peak gametocyte density (A) and cumulative gametocyte density (B), both proxies for parasite fitness, in common garden hosts.

Table 6.2. Traits in initial host (mean ± SE) and trait comparisons between host types.

| | PHZ | Control | Statistics |
|---|---|---|---|
| Peak parasitaemia (prop. of RBCs infected) | 0.63 (±0.04) | 0.44 (± 0.04) | $t(19.0) = 3.40, p = 0.003$ |
| Replication rate | 1.28 (±0.08) | 0.84 (±0.06) | $t(21.9) = 4.31, p < 0.001$ |
| Burst size (No of merzoites per schizont) | 7.97 (±0.08) | 6.93 (±0.20) | $t(4.1) = -4.84, p = 0.007$ |
| Peak Gametocytaemia (density $\mu l^{-1}$) | 12875 (±2043) | 3836 (±789) | $t(16.3) = 4.12, p < 0.001$ |
| Cum. Gametocytaemia (density $\mu l^{-1}$) | 21997 (±3594) | 5051 (±963) | $t(14.8) = 4.55, p < 0.001$ |



Table 6.3. Traits in final,"common garden" hosts (mean ± SE) and trait comparisons between host types.

| | ExPHZ | ExControl | Statistics |
|---|---|---|---|
| Peak parasitaemia (prop. of RBCs infected) | 0.48 (±0.03) | 0.51 (±0.015) | $t(19.5) = 0.88, p = 0.40$ |
| Replication rate | 0.67 (±0.69) | 0.713 (±0.053) | $t(18.0) = 0.40, p = 0.68$ |
| Burst size (n of merzoites per schizont) | 7.38 (±0.23) | 7.32 (±0.132) | $t(19.9) = -0.25, p = 0.80$ |
| Peak Gametocytaemia (density $\mu l^{-1}$) | 15660 (±3829) | 7982 (±1342) | $t(16.1) = -2.01, p = 0.061$ |
| Cum. Gametocytaemia (density $\mu l^{-1}$) | 19935 (±4398) | 11330.7 (±1454.7) | $t(16.0) = 1.27, p = 0.22$ |

## 6.4 Discussion

In this experiment, we ask whether mounting phenotypically plastic responses to a perturbation of the within host environment results in longer-term costs, limits, or constraints, for future plastic responses. We harness the diversity of parasite responses to anaemia to examine whether parasites revert to the trait values observed in control hosts when their environment returns to normal. For example, because the age composition of RBCs also determines invasion receptor expression of merozoites, we expected knock-on effects of the preceding within-host environments leading to a time-lag in asexual replication rate adjustment. Furthermore, any production costs related to higher gametocyte investment in an anaemic environment could potentially affect conversion in the following, non-anaemic environment and manifest itself as a reduction in cumulative gametocyte density (fitness).

However, we find that that *P. chabaudi* moving from anaemic to common garden (control hosts) appears to match the trait values adopted by parasites transferred from control to common garden hosts and does not pay any measurable fitness costs. One of the preconditions for plasticity to evolve is that the costs of plasticity should be low (Auld et al. 2010; Murren et al. 2015). Rapid plastic modification of survival and transmission traits may indeed be a general feature of plasticity in parasites, selected by rapidly changing within-host environments. For example, the parasitic nematode *Heligmosomoides polygyrus* plastically increases expression of two immunomodulatory genes in an inflammatory environment, but reverts to the original expression in control host, even after experiencing upregulated immune environments for four generations (although egg output increases) (Guivier et al. 2017). However, the traits investigated here could be affected by both parasite and host factors. Burst sizes could for example be entirely host-determined if higher nutrient content inside reticulocytes directly leads to better asexual growth. Similarly, if there is some host factor (e.g. a specific nutrient like LysoPC, Marti et al, unpublished) that promotes or dissuades gametocyte production in certain red blood cell

ages, conversion rates would be largely host-driven. Furthermore, if asexuals or gametocytes experience different clearance rates by the immune system, this could mistakenly be interpreted as a change in parasite-driven conversion, whereas it is clearly host-driven. In such cases, it would be trivial to show that parasite traits match those of control parasites. However, chapter 4 and chapter 5 have established that plasticity in these traits are at least in part, controlled by parasites. For example, the anaemia-driven increase in burst size is not directly related to growth inside reticulocytes but is a response to the presence of reticulocytes in the within-host environment. That plasticity in conversion rates is under parasite-control is uncontested; parasites respond adaptively to a number of within-host variables and *P. chabaudi* shows genetic variation for plasticity in this trait (Cameron et al. 2012; Carter et al. 2013, 2014; Mideo and Reece 2012; Pollitt et al. 2011b). We can therefore, with some confidence, ascribe the changes in trait values between anaemic and control environments to parasite-driven plasticity.

Life-history theory predicts that conversion to gametocytes should be dependent on state, which in malaria parasites is related to asexual replication traits (Carter et al. 2013). This is because a positive state (e.g. characterised by high asexual replication rates) also allows parasites to invest more into gametocytes. However, a very low state selects for high investment (terminal investment), so the relationship between state and conversion is non-linear. Chapter 4 and 5 suggest that increased conversion in anaemic hosts is most likely due to an increase in state (because asexual replication rates are also higher in anaemic hosts). The data presented here confirms this because state and conversion change in parallel when parasite move from an anaemic to a health host. The precise cues that parasites use to make decisions about conversion rate and asexual replication are unclear, but parsimony suggests the same decision-making pathway for each trait, or that environmental information is detected and then fed into each decision-making pathway. Candidate information includes facilitated invasion of reticulocytes (Khan et al. 2001), reticulocytes as

a superior source of nutrients (Srivastava et al. 2015), or more efficient nutrient uptake in anaemic environments in general. In chapter 5, we showed that there is no preliminary evidence that immunity is less effective (and therefore more permissive to asexual replication) in anaemic hosts.

Auld et al. (2010) reports that the majority of studies find local rather than global costs of plasticity. Consider, for example, *Daphnia* as an illustration for local, genotype-specific costs: a genotype that produces a long spine in response to the presence of predators may suffer costs (e.g. due to movement inhibition) compared to a genotype that produces a short spine, as long as a short spine is sufficient to protect against that particular type of predator (Murren et al. 2015). In contrast "global costs" are costs related to the maintenance of the plastic machinery and so, are constitutively present across all environments. Global costs, for example, have been demonstrated for the snail *Physa heterostropha*: families with the greatest morphological plasticity in response to predator pressure experience reduced growth compared to less plastic families (Dewitt et al. 1998). In malaria, global costs would manifest themselves as a constitutive cost paid in each environment by parasite genotypes with the steepest reaction norm. Genetic differences in plasticity in *P. chabaudi* have been reported for sex ratio adjustment (Reece et al. 2008), asexual traits (chapter 5), and conversion rates (chapter 4, Cameron et al. 2012; Pollitt et al. 2011b), all of which could be used as a starting point to test for global costs, i.e. to test whether more plastic genotypes vary in fitness-related components across multiple host environments. These genetic differences in reaction norms could also be used to test for local costs, i.e. by comparing genotypes with different responses in a focal environment (e.g. anaemic mice). The strong genotype by environment effects in conversion rates means that reproductive strategies are probably better suited to test for local and global costs of plasticity than asexual traits, which show only little or no genetic variation in reaction norm (chapter 4 and 5).

Our experiment does not reveal any local costs but it is possible that by using naive hosts as the common-garden environment, we may have placed the parasites into a too favourable environment that compensates for any costs. If costs

and constraints are imposed in stressful environments (Chevin and Hoffmann 2017), future work should examine parasites when competing with con-specific genotypes, in semi-immune hosts, or during drug treatment. Furthermore, we only exposed parasites to a single environmental change (anaemic to healthy hosts) and so, costs and limits may emerge after parasites experience a sequence of different within-host conditions, especially if they are stressful. For example, Steinger et al. (2003) report that sib-families of the plant *Sinapis arvensis* that show greater plasticity in specific leaf area also suffer larger fitness costs in a shady environment than in full light conditions. Similarly, plasticity in internode length in *Ranunuculus reptans* is more costly in competitive than under benign conditions (van Kleunen et al. 2000).

Finally, since we used only one genotype of *P .chabaudi*, we are cautious in generalising and recommend the search for costs of plasticity to be extended other genotypes, including other types of local costs as well as global costs. PHZ-induced anaemia as a way of modifying the within-host environment to induce plastic life-history changes in *P. chabaudi* has several key advantages: i) compared to other manipulations, it has a strong effect on life-history traits (especially conversion rates), giving considerable power to detect potential costs, ii) anaemia does not directly cause asexual death, thus facilitating parasite-centric calculation of replication and conversion rates, iii) the perturbation caused by PHZ has similar magnitude of effects to real infections, so that the plastic responses of parasites should mirror what happens during the development of, and recovery from, anaemia in natural infections. If indeed the lack of cost, limits or constraints proves general, interventions targeted at plastic traits of malaria parasites (e.g. transmission blocking interventions, Ramiro et al. 2011) will remain a major challenge.

# Chapter 7

# General discussion

A significant part of research in malaria is devoted to molecular mechanisms and genetics of pathogenesis, with the important ambition of finding new control strategies, including the development of vaccines and new drugs (Burrows et al. 2013; Flannery et al. 2013). New drugs are urgently required due to the evolution of resistance to all drugs within a few years of their widespread use (Read and Huijben 2009) and the state of affairs with vaccine development suggests that sterilising immunity is unlikely to be achieved. However, intervention strategies to date have not taken into account the baseline resistance mechanisms that are available to parasites. These mechanisms consist of life-history traits that all parasite genotypes possess, including conversion rate, virulence, and quiescence. Increasing evidence (some of which is provided by this thesis) suggests these traits are phenotypically plastic. Thus, flexibility in these traits could allow parasites to partially compensate from the fitness costs of drugs, even in absence of more commonly considered drug resistance mutations (Mideo and Reece 2012).

My thesis focuses on how plasticity in gametocyte conversion rates can be adjusted by malaria parasites to optimally balance survival and transmission inside a variable within-host environment. Thus, my work provides insights into the ecological conditions that alter the conversion rate decisions made by parasites (chapter 2 and 4), and underpin parasite life-histories in perturbed within-host environments (chapter 2, 3 and 5). This chapter puts my findings into

an evolutionary context and suggests potential applications of my findings and future research directions. Because this thesis utilises two different within-host environmental perturbations, anaemia and drug treatment, I will discuss empirical chapters, i.e. relating to anaemia (chapter 4, 5, 6) and theoretical chapters, i.e. relating to drug treatment (chapters 2 and 3) separately.

## 7.1 Anaemia and life-history traits of malaria parasites

Host anaemia, i.e. when red blood cell density is low, is a situation that malaria parasites are likely to encounter in almost every infection, as a consequence of resource use by parasites (bursting of RBCs) but also by host-controlled destruction of uninfected red blood cells (Jakeman et al. 1999). In natural infections, the development of anaemia is also associated with the onset of adaptive immunity. Increased gametocyte densities in anaemic hosts have been reported from a number of field studies (Nacher et al. 2002; Price et al. 2001), but explanations of how more gametocytes appear and why parasites might produce more has been controversial and unsatisfactory. In chapters 4, 5 and 6, I use an experimental procedure to generate an anaemic environment, characterised by a low red blood cell density and a high proportion of immature red blood cells (reticulocytes), without causing any significant changes in immunity (chapter 5) and make the following observations relating to plasticity of life-history traits:

i) four different genotypes of *P. chabaudi*, increase their conversion rates in response to anaemia, but with strong genetic variation in their reaction norms (i.e. genotype by environment effect). They respond to the age structure of red blood cells, rather than to measures of red blood cell density (chapter 4), suggesting they detect information about the frequency of RBC ages.

ii) the replication rate of asexual stages in all four genotypes also increases in anaemic environments (chapter 4), which is mainly driven by an increase in the number of merozoites produced per schizont (chapter 5).

iii) other traits of asexual parasites also change in anaemic environments, e.g.

the preference for different ages of RBC. The extent of genetic variation and G x E for this trait varies (chapter 5).
iv) parasites that modify their replication rate and conversion rate in an anaemic environment are able to rapidly return to the trait value observed in control infections when placed back into a non-anaemic environment. This suggests there are no long term effects or fitness costs related to a past plastic response to anaemia (chapter 6).

I explain the increase in both replication rate and conversion rate in anaemic hosts in the context of "affluent investment", i.e. since the environment is clearly highly permissive to asexual replication, parasites can afford a greater commitment of asexual stages to sexual stages. To my knowledge, this is the first time that affluent investment has been invoked to explain conversion rates. All previous examples of conversion rate modification have been assumed to be responses to suboptimal within-host conditions in which parasites adopt reproductive restraint (Carter et al. 2014; Pollitt et al. 2011b; Reece et al. 2010, Schneider *in prep*) or terminal investment (Buckling et al. 1999, 1997, Schneider, *in prep*). Previously though, Reece et al. (2005) explained an increase in gametocytogenesis in reticulocyte-rich environments of EPO-treated mice as a mechanism to assure transmission from stressful anaemic environments (fertility insurance), which inherently assumes some fitness cost of parasites growing inside an anaemic environment. Thus, I suggest that these data sets are revisited to examine the state of the asexual populations for *P. chabaudi* and other *Plasmodium* species that use a range of host cell ages, such as *P. falciparum* (e.g. Gautret et al. 1996; Trager 2005; Trager et al. 1999).

An increase in gametocyogenesis in response to reticulocytes has also been reported for *P. berghei*, another rodent malaria parasite (Carter et al. 2013). Similarly, the asexual replication rate also increases because *P. berghei* shows a strong preference for reticulocytes (Cromer et al. 2009) and increases its burst size in them (Killick-Kendrick 1978). Thus, it would be of interest to test

how affluent investment plays out in malaria parasites with different host cell preferences (e.g. *P. vinckei* is restricted to normocytes, *P. yoellei* is restricted to reticulocytes). Given that rodent malaria species span the range of preferences observed in human parasites, they are a useful model for questions about RBC use. Future work should also investigate what factor(s) pertaining to anaemia facilitate increased replication since this cannot be explained simply by parasites developing inside reticulocytes (chapter 5). Furthermore, identifying the cue(s) associated with an increase in the frequency of reticulocytes (chapter 4) will enable the consequences of plasticity in conversion and replication rates for future transmission and within-host survival to be determined.

Finally, an important question to address is whether the mechanisms we describe here are also responsible for the increased gametocyte densities observed during anaemia in natural infections (Nacher et al. 2002; Price et al. 1999; von Seidlein et al. 2001). In natural infections, anaemia is also associated with change in other within-host environmental variables, such as adaptive immunity, thus imposing additional stress on parasites (Miller et al. 2010). There is thus a possibility that higher gametocytaemias in natural infections originate in a different context than we propose here, e.g. through a terminal investment rather than affluent investment. Alternatively, anaemia and immunity may also interact in an additive or antagonistic manner on replication and conversion decisions. Given that anaemia is one of the main causes of death from malaria (WHO 2016), it is important to understand how within-host parasite populations behave, including whether they can cause more virulence (through increased replication) and transmit better from anaemic patients. Given the observation of genetic variation and G x E in *P. chabaudi* traits, assessing genetic variation in human parasites is warranted, since this shapes the evolutionary potential of response to host anaemia.

## 7.2 Drug treatment and conversion

In contrast to anaemia, drug treatment is a selection pressure that has entered the selection landscape of parasites relatively recently (Mackinnon and Marsh 2010). Even so, the frequent evolution of resistance against antimalarial drugs is a reminder that new selection pressures may also quickly alter selection on life-history strategies of parasites. In my thesis, I dedicate two chapters to the theoretical exploration of how drug pressure shapes conversion rates.

In chapter 2, I show that parasites could use altered conversion profiles to increase their survival and transmission in the face of drugs. Such drug-adapted conversion profiles are characterised by reproductive restraint in the case of low and medium drug doses- to compensate for the asexual parasites lost by drugs, but parasites opt for early terminal investment in the case of high-dose drug treatments. These findings concur with the verbal model of the u-shaped curve, proposed by Pollitt et al. (2011b) and Carter et al. (2013), which predicts that malaria parasites should invest less resources into transmission with increasing within-host stresses, but invest all their resources into transmission either when within-host stresses become so strong that the infection will be cleared (e.g. clearance by adaptive immunity or drugs) or when the host dies.

The u-shaped curve was originally proposed as reaction norm model- i.e. how conversion should plastically vary according to changing within-host conditions. In chapter 2, I was constrained by the modelling framework and instead of truly plastic conversion rate responses, I explored the characteristics of constitutive, drug-adapted conversion profiles, i.e. conversion rates vary during infections but a particular pattern is hard-wired into the parasites genome depending on the drug dose that has selected for its evolution. These constraints were overcome in chapter 3, where I modified the modelling framework and the optimisation routine of the model in chapter 2. I allow parasites to alter conversion rates from the pattern followed in control infections to a different pattern when drugs are administered. I show that plastic conversion rates are also characterised by reproductive restraint and terminal investment in response to different doses of

drugs. These responses allow parasites to reach a considerably higher fitness after drug treatment than parasites that do not adjust their conversion rate, and plastic conversion strategies to allows parasites to achieve higher fitness if less than 70-85% of hosts are drug-treated in a population. To our knowledge, chapter 2 and 3 provide the first formal theoretically predictions for how the transmission strategies of malaria parasites should vary in the face of drugs.

An obvious next step is to extend the modelling routine to include other types of drugs, especially those that affect other parasite stages. For example, it would be highly interesting to test whether parasites could use plastic conversion to overcome transmission blocking drugs, which have received a lot of attention as a new approach to malaria eradication (Sinden 2017). The model could also be modified to explore other within-host stresses. For example, Greischar et al. (2016a) use the same model to show that parasites benefit from reproductive restraint in coinfections, providing theoretical support to analogous experimental findings (Pollitt et al. 2011b). Our model could also easily be adapted to explore the effects of anaemia (e.g. by integrating the experimental findings from this thesis), immune killing and how multiple stresses act in concert to affect conversion rates. Finally, a framework that allows for variation in the probability that an infection may be terminated at any point in time as a function of the intensity of within-host stress will be a useful next step. This is because current optimisation routines cause a large effect of the timepoint of infection (day post-infection) on conversion, which is ultimately driven by the defined duration of infection in models. Adding such inherent uncertainty would improve the applicability of the modelling framework because in natural infections, parasites may, to some extent, have to gamble when deciding between restraint or terminal investment, if it is not obvious whether a given stress (e.g. a strong drug treatment) will end the infection. With such a framework, one could elaborate theoretical predictions of conversion rates for each within-host stress to develop reaction norms for the duration of different kinds of infections and so, better interpret future experimental outcomes and the design of future experiments.

There is also a clear need for more experimental work on the effect of drugs on parasite conversion rates. A main challenge is the difficulty of reliably measuring conversion rates in vivo under high drug-induced death rates of asexual parasites (Greischar et al. 2016b). The contradicting experimental evidence of terminal investment, where both high drug doses and subcurative doses appear to cause a steep increase in conversion (Buckling et al. 1999, 1997) in in vivo infections, should be re-assessed with better methods to estimate conversion. Up to now, evidence for reproductive restraint in response to drugs has come from *Plasmodium* cultures (Reece et al. 2010), thus circumventing problems of measuring conversion rates in vivo. A new empirical effort that focusses on a range of drug doses to test simultaneously for reproductive restraint and terminal investment in *P. chabaudi* is particularly promising because it uses state-of-the art methods to estimate conversion under drug pressure, and also considers potential interactions with the RBC resource environment (Schneider et al, *in prep*).

Finally, natural infections have revealed that plastic modification of a life-history trait (e.g. cell dormancy during artemisinin treatment, Hott et al. 2015; Teuscher et al. 2010) can help protect parasites from drugs. However, whether conversion rate modification plays an important role in natural settings remains unknown. This knowledge could be key to interpreting treatment-failure in the absence of genetic drug resistance (Cohen et al. 2013) or the context in which de-novo resistance mutations arise. Indeed, the question of what drives the rapid evolution of drug resistance mutations in *P. falciparum* is yet to be answered. Plasticity in conversion rates may be part of the explanation: something that benefits within host survival during drug treatment may facilitates the emergence and spread of resistant variants. I therefore recommend that studies of natural malaria infections look for evidence of restraint or terminal investment, including follow-ups to account for the possibility of resurgence following reproductive restraint. Given that drugs are administered upon detection of infections in humans, relevant follow-up samples could be relatively easily obtained.

## 7.3 Life-history

The results of my thesis also provide new insights for the understanding of life-history theory in general, and plasticity in resource allocation to reproduction, confirming *P. chabaudi* as an useful testing ground for life-history theory. For example, I provide experimental evidence for state-dependent reproductive investment, a research area dominated by theoretical explorations but lacking in empirical tests (McNamara and Houston 2008). The main reason why state-dependent life-history theory is hard to test empirically is the difficulty of measuring what constitutes "state" and "residual reproductive value" in a given species (McNamara and Houston 1999, Clutton-Brock 1984). In malaria parasites however, the question of state is radically simplified, since it can be directly related to the number of asexual stages of a given genotype. This also constitutes a good proxy for RRV, since every asexual parasite could be committed towards gametocytogenesis in the following replication cycle. Furthermore, identifying the cues used by parasites to make their life-history decisions (Carter et al. 2014) would make *P. chabaudi* a more powerful model system. The ability to perturb cues separately from the environmental changes they signal will enable experiments that "trick" parasites in making a suboptimal decisions, thus revealing the fitness consequences of plastic strategies. A related outstanding issue is understanding how plastic decisions are coordinated across a disparate within-host parasite population. Recent research suggests cell to cell communication occurs via the production of DNA containing microvesicles that are released into the blood (Mantel et al. 2016, 2013; Regev-Rudzki et al. 2013). Depending on which mechanisms are used, one may expect delays or energetic costs to be associated with the production and detection of information required to make plastic decisions (see chapter 6). Once environment-sensing mechanisms have been uncovered, the genetic tractability of *P. chabaudi* compared to many multicellular taxa should facilitate the identification of molecular mechanisms involved in sensing and processing information, and the production of different phenotypes. The potential to investigate plasticity in life histories from molecular

mechanisms to phenotypes to epidemiology would make malaria parasites a very powerful model system.

# Chapter 8

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# Appendix

## A Chapter 2: Supplementary figures

Huijben et al. (2013) parameterised a model for the action of pyrimethamine against *Plasmodium chabaudi* in mice, finding that the dose of drugs affected the duration of drug action. We show this relationship (i.e., solutions to Equation 9 of the main text) in Figure A.1. A schematic of the full drug action model is presented in Figure A.2. In Figure A.3, we explore the effects of using a different fitness function on the predicted optimal patterns of investment in the absence of drug treatment and with a medium dose drug treatment. Finally, Figure A.4 shows the fitnesses achieved by different strategies in different environments (i.e., untreated or treated hosts). In each case, the optimal strategy predicted for a given environment outperforms the predicted optimal strategies for other environments.

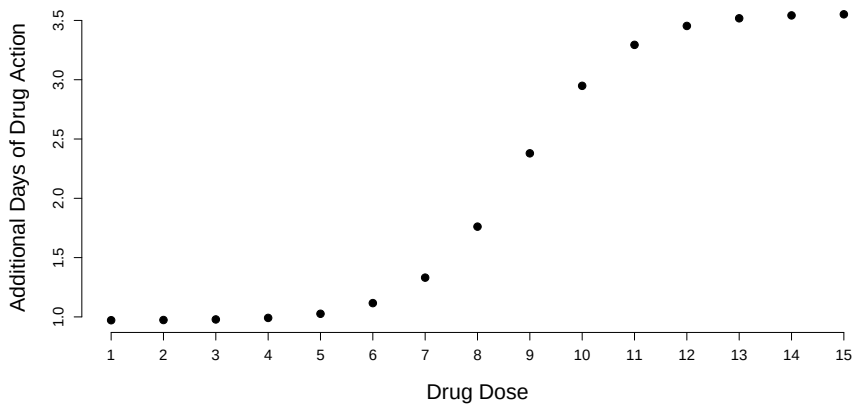


Figure A.1. Drug dose affects duration of drug-related parasite killing, but not the rate at which parasites are killed. Shown are the additional days of drug action, beyond the days when drugs are administered, when drugs are predicted to still be "active" (as defined in Huijben et al. 2013). Drug dose is expressed in mg/kg.

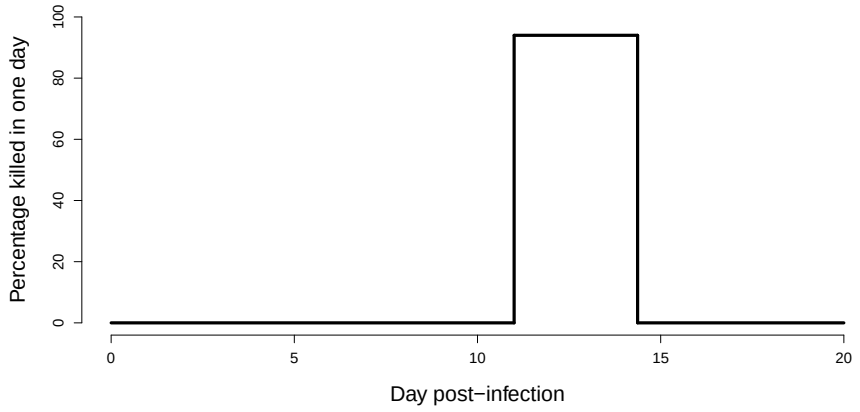


Figure A.2. Schematic of drug action in our model, a stylized version of how pyrimethamine acts against *P.chabaudi*. In this example, drug treatment is composed of two doses of 9 mg/kg, administered on day 11 and 12. The last dose determines how long the drugs will persist in the host after treatment, here an additional ∼2.4 days of drug action. Before and after drug action, drug-related killing is zero.

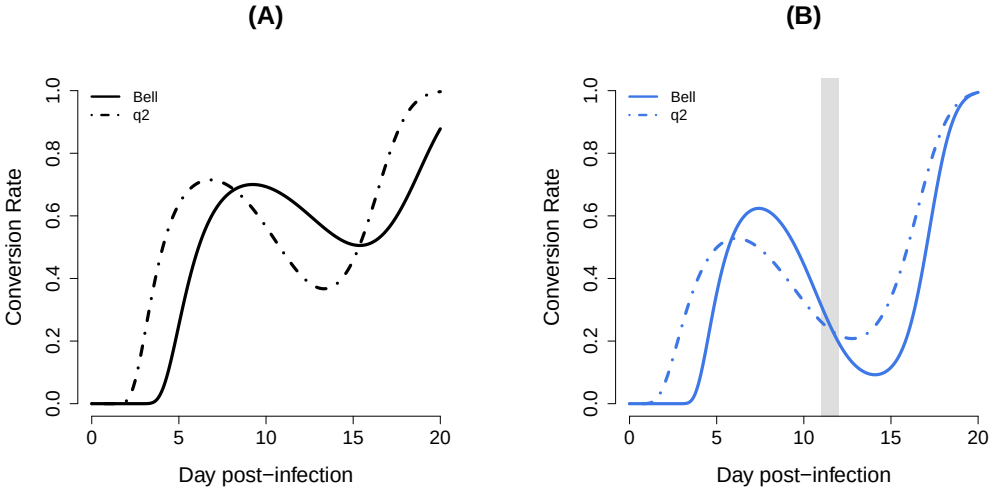


Figure A.3. The optimal pattern of conversion over the course of infections, using equation "q2" in Huijben et al. (2010), rather than equation 11 of the main text to define fitness. (A) The black line shows the predicted best response in an untreated infection for the q2 fitness equation and the fitness equation proposed by Bell et al. (2012), used in this paper and marked "Bell".(B) When infections are treated with a moderate drug dose (blue line, 8 mg/kg), parasites do better by reducing conversion, for both fitness functions. Drugs are administered on the days denoted by the grey bar.

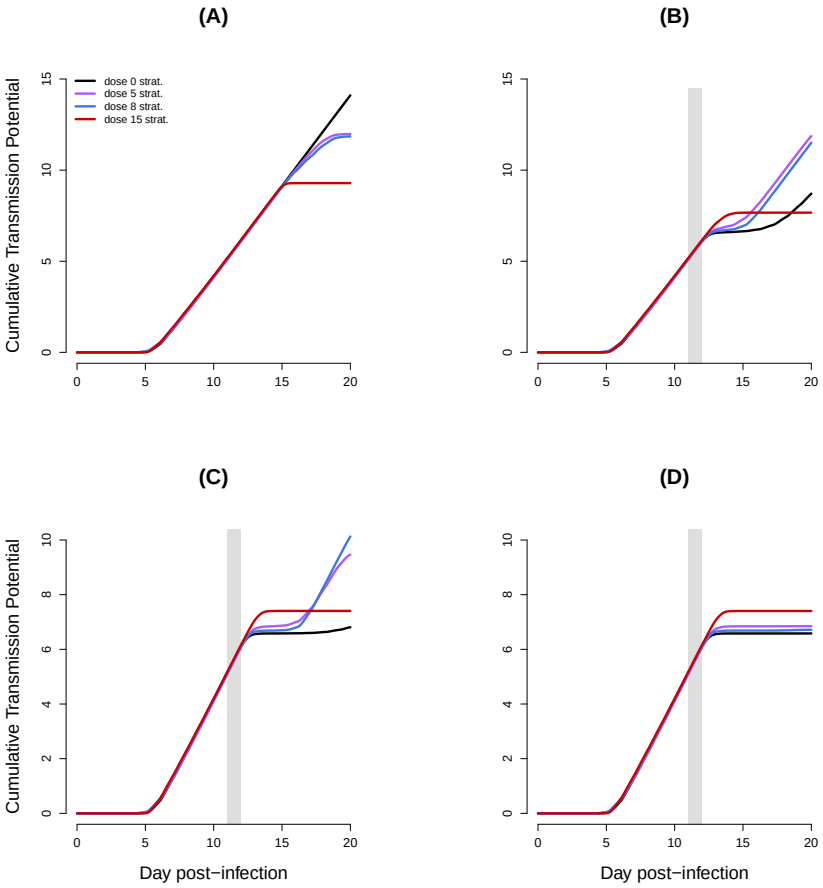


Figure A.4. The cumulative transmission potential of different drug-adapted strategies in untreated hosts (A), hosts treated with 5 mg/kg of drugs (B), 8 mg/kg (C), and 15 mg/kg (D). For each drug treatment, the putative optimal strategy against that dose outperforms the putative optimal strategies from other doses. Grey bars denote the days of drug treatment.

# B Chapter 2: Fitness calculations

Imagine the following set of fitnesses for a non-drug adapted and a drug-adapted pattern of transmission investment (first subscript 0 or $D$, respectively) of malaria parasites in untreated and treated host (second subscript 0 or $D$, respectively)

$$\begin{aligned} w_{0,0} &= a \\ w_{0,D} &= a - d \\ w_{D,0} &= a - c \\ w_{D,D} &= a - d + b \end{aligned} \tag{B.1}$$

where $d$ is the reduction in fitness of the non-drug adapted strain due to drug treatment (i.e., the drug effect), $c$ is the reduced fitness of the drug-adapted strain in an untreated host (i.e., cost to "resistance"), and $b$ is the increase in fitness achieved by the drug-adapted strain in the presence of drugs (i.e., the benefit of "resistance").

We can write the expected fitness of the two different strategies in a host population, where a proportion, $f$, of hosts receive drug treatment:

$$\begin{aligned} \mathrm{E}[w_0] &= f w_{0,D} + (1-f)\, w_{0,0} \\ \mathrm{E}[w_D] &= f w_{D,D} + (1-f)\, w_{D,0}. \end{aligned} \tag{B.2}$$

Substituting the fitness expressions from B.1 into B.2 and rearranging, we find that the drug-adapted strategy has a higher fitness when

$$f > \frac{c}{c+b}. \tag{B.3}$$

Put another way, the drug-adapted strategy will be favoured when the ratio of the benefits to costs of the strategy is greater than the relative frequency of encountering an untreated host:

$$\frac{b}{c} > \frac{1-f}{f}. \tag{B.4}$$

In Table B.1 we list the cumulative transmission potential (as predicted by our model), over a 20-day simulated infection, for each of the predicted drug-adapted

strategies, in the presence and absence of drug treatment, as well as the non-drug adapted strategy in each of these environments. From these values we can plot the expected fitness of different strategies (i.e., solutions to Equations B.2) over different values of $f$ (Figure B.5). We see that over a range of $f$ values, the non-drug adapted strategies performs better on average than the drug adapted strategy, for all drug doses, but above a given $f$ value, the drug-adapted strategy will be favoured. From the fitness values, we can also calculate $b$ and $c$ for each of the drug-adapted strategies (Table B.2). Plugging these costs and benefits into equation B.3, gives rise to the frequencies of drug treatment required to favour the drug-adapted over the non-drug adapted strategies reported in the main text (i.e., the intersection of the lines in Figure B.5).

Table B.1. Estimated fitness values (i.e., cumulative transmission potential) for different transmission investment strategies in different host environments, as predicted by the model presented in the main text.

| **Strategy** | **Environment (drug dose)** | | | |
|---|---|---|---|---|
| | 0 | 5 | 8 | 15 |
| 0 | 14.1 | 8.7 | 6.8 | 6.6 |
| 5 | 11.98 | 11.8 | | |
| 8 | 11.84 | | 10.1 | |
| 15 | 9.28 | | | 7.4 |

Table B.2. Calculated benefits, $b$, and costs, $c$, of drug-adapted strategies.

| **Strategy** | **Effects of 'resistance'** | |
|---|---|---|
| | $b$ | $c$ |
| 5 | 3.1 | 2.12 |
| 8 | 3.3 | 2.26 |
| 15 | 0.8 | 4.82 |

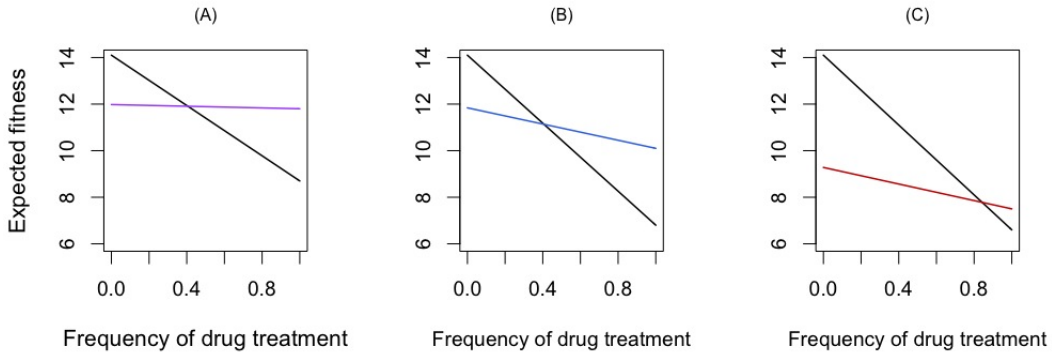


Figure B.5. Expected fitness for different transmission investment strategies in a host population treated with a particular drug dose (A: low; B: medium; C: high) at a given frequency. Lines show the weighted average of fitness achieved in untreated and treated infections (i.e., solutions to Equations B.2). Black lines represent the transmission investment strategy predicted to be best in the absence of drug treatment (the "non-drug adapted" strategy); coloured lines represent the transmission investment strategy predicted to be best in the face of a low drug dose (purple), medium drug dose (blue) or high drug dose (red).

# C Chapter 3: Supplementary figures

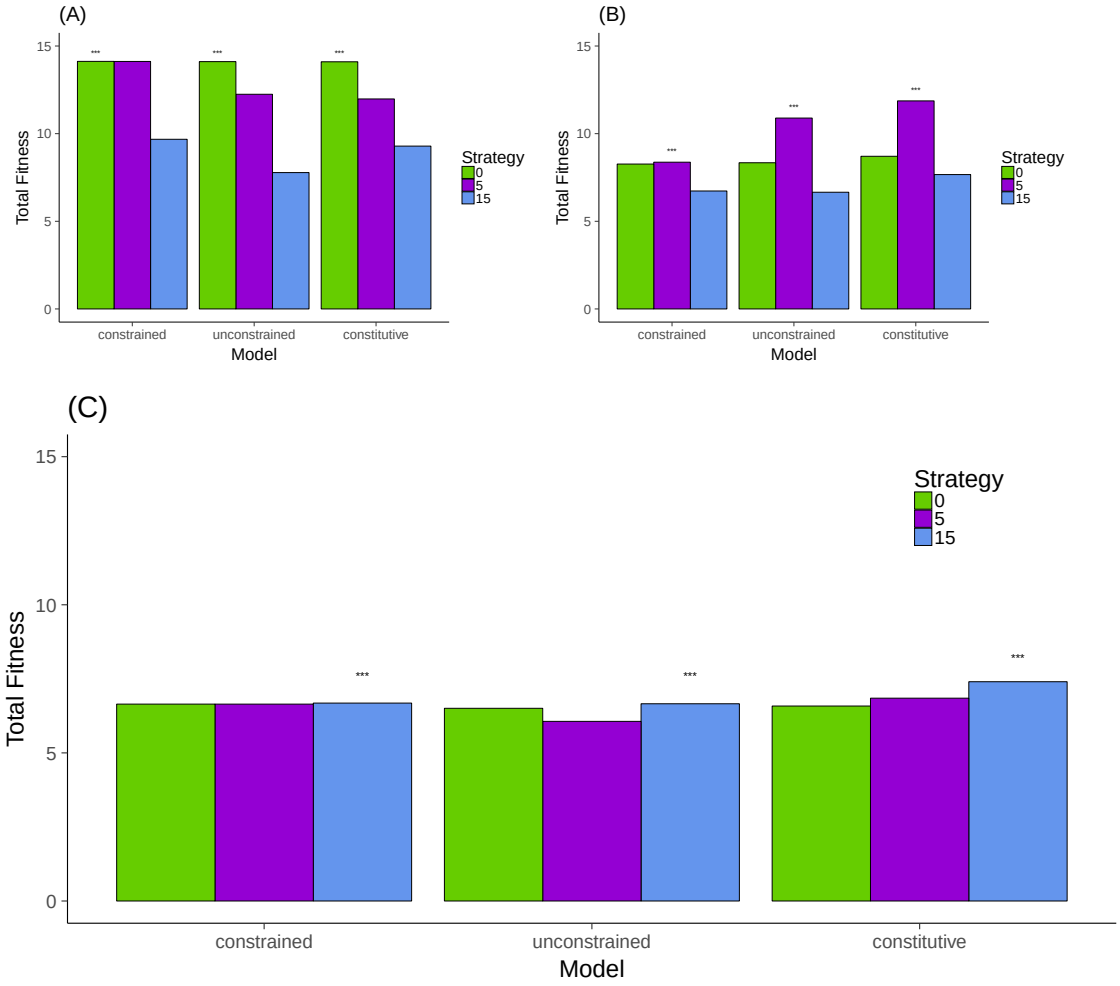


Figure C.6. The performance of each drug-specific strategy for each modelling approach (constrained, unconstrained and constitutive strategies) in the 3 drug environments (A: untreated infections, B: 5mg/kg, C:15mg/kg). (***) indicate the best performing strategy.

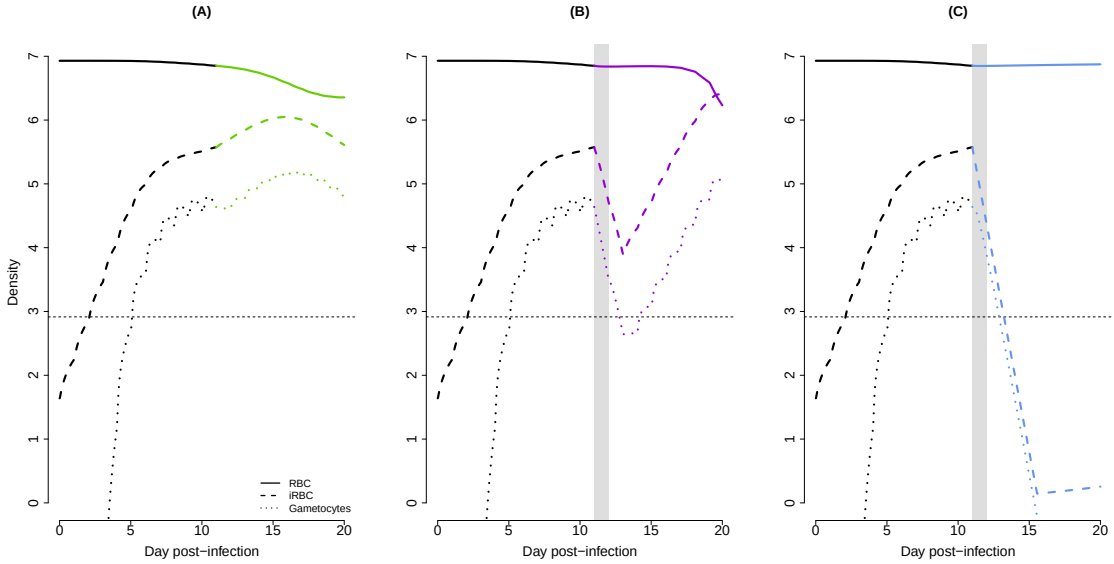


Figure C.7. Density of RBCs, iRBCs and gametocytes for time-constant conversion post-drug treatment in (A) untreated infections, (B) a drug dose of 5 mg/kg and (C) a drug dose of 15 mg/kg. The grey area indicates the days of drug treatment.

# D Chapter 4: Supplementary information and figures

## D.1 Pilot experiment: Modifying the RBC environment.

We first carried out a pilot experiment to determine which doses of phenylhydrazine (PHZ) create statistically distinguishable RBC resource environments in the blood. Phenylhydrazine results in the clearance of circulating red blood cells (Savill et al. 2009) and the resulting anaemia stimulates the release of immature RBC (reticulocytes) from the bone marrow. We injected one of four doses of 30 mg/kg, 60 mg/kg, 120 mg/kg of PHZ dissolved in PBS or 0 mg/kg (control, PBS only), via the peritoneal cavity, into 6 mice for each treatment dose. We monitored both normocyte (mature RBC) and reticulocyte density by flow cytometry and thin blood smears (Coulter Counter, Beckman Coulter) once or twice a day for 14 days (Ferguson et al. 2003). We asked whether the doses of PHZ created different total RBC densities

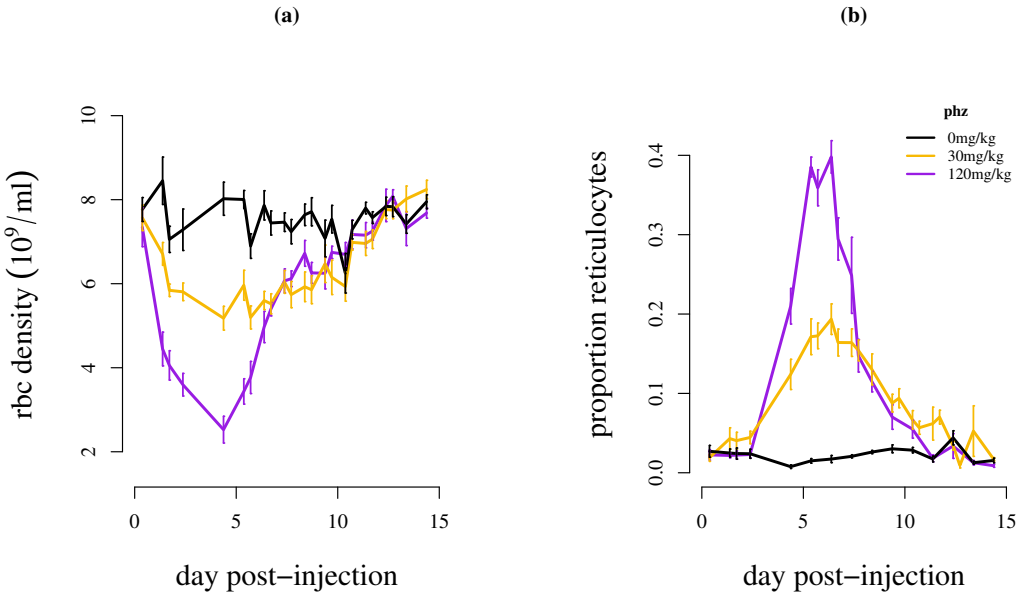


Figure D.8. The effect of PHZ doses on total RBC density (a) and the proportion of RBCs that are reticulocytes (b) starting from the day of administration of PHZ (day 0). All mice recover to normal levels after 15 days. Data are the mean and standard error of the mean (SE) for 6 mice per treatment.

and age structures, when these differences occurred, and how long they persisted. We found that PHZ reduces red blood cell density below the levels of controls in a dose-dependent manner and concomitantly increases the proportion of reticulocytes in the blood (figure D.8). We found that 120 mg/kg, 30 mg/kg and 0 mg/kg (control) generated significantly different environments in terms of both total red blood cell density and reticulocyte proportion (figure D.9) two days after injection and that these differences between doses persisted for 5 more days. The environment following treatment with a 60 mg/kg dose overlapped with that of other doses and was therefore not included in the main experiment.

## D.2 Figures and models

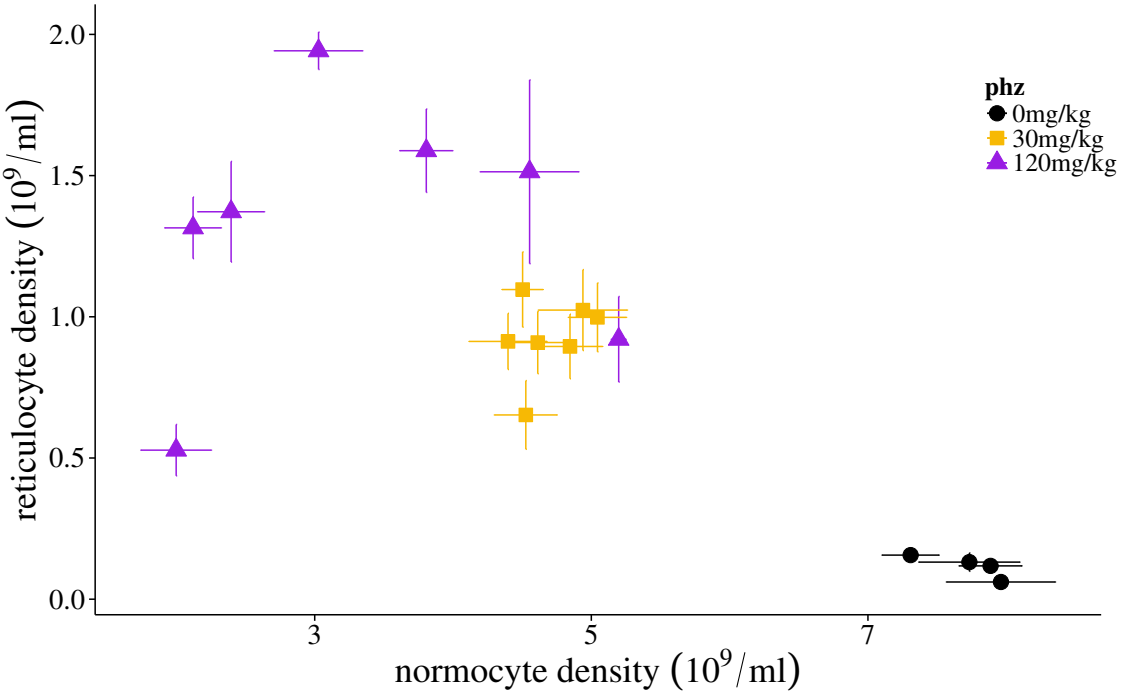


Figure D.9. Results of the pilot study to determine which doses of phenylhydrazine (PHZ) create mutually exclusive environments (normocyte density and reticulocyte density) to expose parasites to. Each point corresponds to the mean±SE of all mice in that treatment for one time point post PHZ injection. Only the time points corresponding to day 0 – day 3 post-infection in the main experiment are shown (i.e. days 4–7 post-injection in pilot experiment)

Table D.3. Summary of statistical models examining the effect of PHZ treatment and parasite genotype on the cumulative density (day 0,1,2 PI) of normocytes, reticulocytes and frequency (proportion of those cell types). Frequency was recorded as the proportion of reticulocytes. All models were minimised and test-statistics, degrees of freedom and p-values were derived using maximum-likelihood-based deletion tests, following Crawley (2013).

| | |
|---|---|
| **Normocytes** | |
| **Significant terms in minimal model** | |
| PHZ | $F(2, 65) = 282.38$, $p < 0.001$ |
| **Non-significant terms deleted from model** | |
| Genotype | $F(3, 62) = 0.68$, $p = 0.567$ |
| PHZ x Genotype | $F(6, 56) = 1.09$, $p = 0.381$ |
| **Reticulocytes** | |
| **Significant terms in minimal model** | |
| PHZ | $F(2, 65) = 231.87$, $p < 0.001$ |
| **Non-significant terms deleted from model** | |
| Genotype | $F(3, 62) = 2.48$, $p = 0.069$ |
| PHZ x Genotype | $F(6, 56) = 2.19$, $p = 0.057$ |
| **Frequency** | |
| **Significant terms in minimal model** | |
| PHZ | $F(2, 65) = 450.23$, $p < 0.001$ |
| **Non-significant terms deleted from model** | |
| Genotype | $F(3, 62) = 0.75$, $p = 0.526$ |
| PHZ x Genotype | $F(6, 56) = 1.31$, $p = 0.269$ |

Table D.4. Summary of statistical model of cumulative asexual parasite growth (day 1 and 2 PI) by parasite genotype and PHZ treatment. The model was minimised and all test-statistics, degrees of freedom and p-values were derived using maximum-likelihood-based deletion tests, following Crawley (2013).

| **Asexuals** | |
|---|---|
| **Non-significant terms deleted from model** | |
| PHZ | $F(2, 65) = 2.81$, $p = 0.067$ |
| Genotype | $F(3, 62) = 1.62$, $p = 0.193$ |
| PHZ x Genotype | $F(6, 56) = 1.73$, $p = 0.132$ |

Table D.5. Summary of statistical model of how conversion varies by PHZ treatment, parasite genotype and asexual density, using gametocyte density as a proxy for conversion. The model was minimised and all test-statistics, degrees of freedom and p-values were derived using maximum-likelihood-based deletion tests, following Crawley (2013).

| **Gametocytes** | |
|---|---|
| $R^2$=0.84, df=51 | |
| **Significant terms in minimal model** | |
| PHZ x Genotype | $F(6, 51) = 4.34$, $p = 0.001$ |
| Asexuals | $F(1, 51) = 4.20$, $p = 0.046$ |
| **Non-significant terms deleted from model** | |
| PHZ x Asexuals x Genotype | $F(6, 40) = 0.22$, $p = 0.968$ |
| Genotype x Asexuals | $F(3, 46) = 0.34$, $p = 0.799$ |
| PHZ x Asexuals | $F(2, 49) = 0.50$, $p = 0.610$ |

Table D.6. Summary of statistical models of how conversion varies with within-host physiological variables, using gametocyte density as a proxy for conversion. The proportion of reticulocytes (frequency) and total red blood cell density are the focus of model 1, reticulocyte density (model 2), normocyte density (model 3), and total red blood cell density (model 4). For each model, the density of asexuals was also controlled for. All models were minimised and all test-statistics, degrees of freedom and p-values were derived using maximum-likelihood-based deletion tests, following Crawley (2013).

| **Model 1** | |
|---|---|
| **Significant terms in minimal model** | |
| Genotype x Frequency | $F(3, 56) = 7.50$, $p < 0.001$ |
| **Non-significant terms deleted from model** | |
| Genotype x Frequency x Total Density x Asexuals | $F(3, 32) = 0.55$, $p = 0.652$ |
| Frequency x Total Density x Asexuals | $F(1, 35) = 0.05$, $p = 0.828$ |
| Genotype x Total Density x Asexuals | $F(3, 36) = 0.22$, $p = 0.880$ |
| Genotype x Frequency x Asexuals | $F(3, 39) = 0.31$, $p = 0.820$ |
| Genotype x Frequency x Total Density | $F(3, 42) = 0.49$, $p = 0.689$ |
| TotalDensity x Asexuals | $F(1, 45) = 1.46$, $p = 0.232$ |
| Frequency x Asexuals | $F(1, 46) = 0.28$, $p = 0.601$ |
| Genotype x Asexuals | $F(3, 47) = 0.18$, $p = 0.911$ |
| Frequency x Total Density | $F(1, 50) = 0.74$, $p = 0.393$ |
| Genotype x Total Density | $F(3, 51) = 0.39$, $p = 0.761$ |
| Asexuals | $F(1, 54) = 2.14$, $p = 0.149$ |
| Total Density | $F(1, 55) = 0.03$, $p = 0.869$ |

| Model 2 | |
|---|---|
| **Significant terms in minimal model** | |
| Reticulocytes x Genotype | $F(3, 56) = 3.74$, $p = 0.016$ |
| **Non-significant terms deleted from model** | |
| Genotype x Reticulocytes x Asexuals | $F(3, 48) = 0.93$, $p = 0.433$ |
| Reticulocytes x Asexuals | $F(1, 51) = 0.03$, $p = 0.856$ |
| Genotype x Asexuals | $F(3, 52) = 0.98$, $p = 0.407$ |
| Asexuals | $F(1, 55) = 0.00$, $p = 0.999$ |
| **Model 3** | |
| **Significant terms in minimal model** | |
| Normocytes x Genotypes | $F(3, 55) = 6.54$, $p < 0.001$ |
| Asexuals | $F(1, 55) = 6.17$, $p = 0.016$ |
| **Non-significant terms deleted from model** | |
| Genotype x Normocytes x Asexuals | $F(3, 48) = 0.10$, $p = 0.958$ |
| Normocytes x Asexuals | $F(1, 51) = 0.40$, $p = 0.530$ |
| Genotype x Asexuals | $F(3, 52) = 0.48$, $p = 0.696$ |
| **Model 4** | |
| **Significant terms in minimal model** | |
| Genotype x Total Density | $F(3, 55) = 5.27$, $p = 0.003$ |
| Asexuals | $F(1, 55) = 9.09$, $p = 0.004$ |
| **Non-significant terms deleted from model** | |
| Genotype x Total Density x Asexuals | $F(3, 48) = 0.13$, $p = 0.943$ |
| Total Density x Asexuals | $F(1, 51) = 0.56$, $p = 0.459$ |
| Genotype x Asexuals | $F(3, 52) = 0.91$, $p = 0.440$ |

Table D.7. Comparison of the minimal models described in table S4, ranked by AIC from lowest to highest.

| **Minimal Model** | **DF** | **$R^2$** | **AIC** |
|---|---|---|---|
| **Model 1** | | | |
| Gametocytes ~ Frequency + Genotype + Frequency x Genotype | 56 | 0.81 | 1830.9 |
| **Model 3** | | | |
| Gametocytes ~ Normocytes + Genotype + Normocytes x Genotype + Asexuals | 55 | 0.79 | 1840.0 |
| **Model 4** | | | |
| Gametocytes ~ Total Density + Genotype + Total Density x Genotype + Asexuals | 55 | 0.74 | 1844.9 |
| **Model 2** | | | |
| Gametocytes ~ Reticulocytes + Genotype + Reticulocytes x Genotype | 56 | 0.69 | 1867.6 |

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# Phenotypic plasticity in reproductive effort: malaria parasites respond to resource availability

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The trade-off between survival and reproduction is fundamental in the life history of all sexually reproducing organisms. This includes malaria parasites, which rely on asexually replicating stages for within-host survival and on sexually reproducing stages (gametocytes) for between-host transmission. The proportion of asexual stages that form gametocytes (reproductive effort) varies during infections—i.e. is phenotypically plastic—in response to changes in a number of within-host factors, including anaemia. However, how the density and age structure of red blood cell (RBC) resources shape plasticity in reproductive effort and impacts upon parasite fitness is controversial. Here, we examine how and why the rodent malaria parasite *Plasmodium chabaudi* alters its reproductive effort in response to experimental perturbations of the density and age structure of RBCs. We show that all four of the genotypes studied increase reproductive effort when the proportion of RBCs that are immature is elevated during host anaemia, and that the responses of the genotypes differ. We propose that anaemia (counterintuitively) generates a resource-rich environment in which parasites can afford to allocate more energy to reproduction (i.e. transmission) and that anaemia also exposes genetic variation to selection. From an applied perspective, adaptive plasticity in parasite reproductive effort could explain the maintenance of genetic variation for virulence and why anaemia is often observed as a risk factor for transmission in human infections.

## 1. Introduction

Parasites are exposed to rapid and extensive variation in the environmental conditions they experience inside their hosts and vectors, both during infections and across different hosts (e.g. dynamic immune responses, competition with other parasites, fluctuations in the availability and quality of resources). Therefore, parasites, like any organism which experiences frequent environmental change, could use phenotypic plasticity—the ability to match phenotypes to different environmental conditions—to maintain their fitness in the face of changing conditions [1,2]. However, because parasites have traditionally been viewed as organisms with inflexible strategies, their environmental sensing mechanisms are assumed to be directed towards maintaining homeostasis [3]. Thus, adaptive plasticity in parasites has generally been overlooked in evolutionary biology and is controversial in applied bioscience [4,5]. For malaria parasites (*Plasmodium* spp.), there is mounting evidence that allocation to within-host growth versus between-host transmission is phenotypically plastic [6,7]. Malaria parasites rely on asexually replicating stages for within-host survival and on sexually reproducing stages (gametocytes) for between-host transmission. Therefore, for malaria and all other parasites that use distinct stages for transmission and within-host replication (e.g. trypanosomes), between-host transmission is equivalent to

'reproduction' of an infection and within-host replication determines the 'survival' of an infection [6,8,9]. The proportion of asexual stages in each cycle of replication that commit to forming gametocytes represents the parasite's reproductive effort (called the 'conversion rate' in parasitology). Why conversion rates are generally low and highly variable in malaria parasites are long-standing questions in parasitology [6,10] and explaining plasticity in reproductive effort is a major aim of evolutionary biology [11–13].

The fundamental life-history trade-off between survival and reproduction has attracted much theoretical and empirical attention [14–17]. The consequences of diverting energetic resources from maintenance of the organism into reproduction means that organisms must balance their current reproductive effort against their prospects for survival and future reproduction. For most organisms, a reproductive event comes with several types of costs (e.g. egg production, parental care, competition for mates), many of which can affect an organism's survival [18]. For example, breeding-associated immobility or the need for increased foraging to feed offspring can expose parents to increased predation risk [19,20]. For malaria parasites, allocating cells to become gametocytes comes at the instant cost of a reduced number of asexual stages, needed to perpetuate the infection. Theory predicts that, compared with older organisms, whose residual reproductive value (RRV, the age-specific expectation of future offspring) is lower, young organisms should allocate less into current reproduction ('reproductive restraint') so as to minimize risk to their survival prospects [15]. By contrast, reproductive effort should increase as the probability of future reproductive success decreases [13], and in the last reproductive event of their life, i.e. when the RRV is very small, organisms should allocate all of their remaining energy to reproduction ('terminal investment').

In addition to the age of the organism, the current environment and an organism's physiological characteristics are also expected to affect reproductive effort. Accounting for these effects, summarized as 'state variables', has given rise to theory that complements age-based life-history theory [17,21–23]. For example, terminal investment may not just be observed when age is the main reason for an organism's death, but also if external factors cause the probability of future reproduction to be near zero [24]. Conversely, if current conditions are not conducive to reproduction (e.g. resources are limited), an organism may be selected to exert reproductive restraint and delay reproduction until environmental conditions improve [25–27]. Finally, state-based and age-based life history interact because physiological state varies over an organism's lifetime [11,28]. In summary, an optimally behaving organism is expected to adjust its reproductive effort over successive bouts of reproduction according to interactions between environmental conditions, energetic reserves and expected lifespan. The predictions of age- and state-based theories are supported by empirical data from diverse laboratory models and natural systems [15,16,29–32], confirming the fitness benefits of adjusting reproductive effort in relation to circumstances.

Life-history theory has also been applied to explain plasticity in the reproductive effort of malaria parasites, which appear to adjust conversion rates in response to information about their within-host environment and the density of clone-mates within the host [33–39]. A clonal parasite population inside a host is the selective equivalent of a single organism [40] and can experience considerable variation in within-host environments during infections and in different

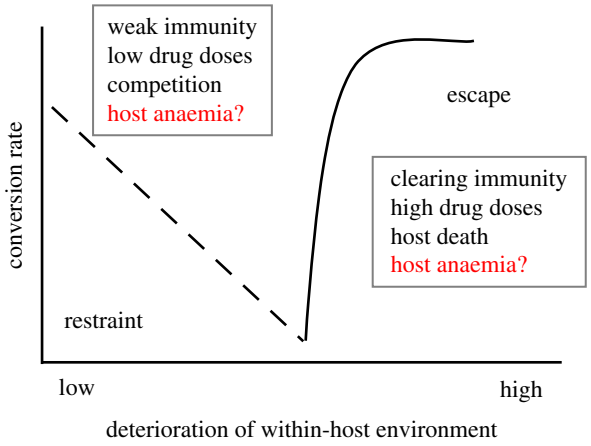


**Figure 1.** Cartoon of a reaction norm for conversion rate against conditions experienced by parasites inside the mammalian host, adapted from [6]. Importantly, the *x*-axis does not represent time since infection, but a given stress, or combination of stressors, experienced at any point during the infection that reduces the condition/state of parasites. The dotted line represents a decrease in conversion rate ('restraint') as the parasites experience a loss of condition/state, but the exact functional form of this is not known. If the within-host environment has deteriorated substantially and recovery of condition/state is unlikely or impossible parasites should make a terminal investment by putting all resources into transmission ('escape'). Factors in the boxes denote circumstances thought to induce reproductive restraint or terminal investment, but the effect of variable red blood cell resources during anaemia is unclear. (Online version in colour.)

hosts. Characteristics of the in-host environment can affect state, which for malaria parasites is associated with asexually replicating stages because they make up the bulk of parasite biomass and produce gametocytes. Environmental conditions thought to affect parasite state are immune attack, drug treatment, competition with con specific parasite strains and a variable supply of red blood cells (RBCs) [6]. Parasites should therefore prioritize allocation to asexual stages to maintain the infection when faced with a moderate loss of state because prolonging in-host survival rewards parasites with future transmission opportunities [7,34,41,42]. By contrast, parasites should allocate all remaining resources to transmission when faced with a situation where in-host survival is unlikely (e.g. clearance by drugs or strong immunity) [6,37,38,43] or when the infection is likely to end due to host death. Finally, an increase in state (e.g. due to an enrichment of the environment) allows parasites to allocate more to gametocytes, as within-host survival is not at risk. These strategies can be represented in a reaction norm for conversion rate which illustrates how the circumstances that parasites find themselves in determine the allocation made to gametocytes at each cycle of replication (figure 1).

Mounting evidence suggests that malaria parasites have evolved to adjust conversion rates according to circumstances—including changes in the supply of an essential resource—RBCs [35,44–46]. Most studies report an increase in gametocyte densities when hosts become anaemic. Only two studies link elevated gametocyte densities to higher conversion in response to anaemia [35,45], and most findings are based on post hoc correlations of observational data in which confounding changes in asexual densities cannot be accounted for [44,46,47]. Moreover, it is not known whether increased conversion occurs because parasites are making a terminal investment (e.g. due to RBC limitation or because an anaemic

host has a high mortality risk), or because the in-host environment has improved and parasites can afford to reproduce. These contradictory hypotheses emerge because anaemia is complex. The population of circulating RBCs within the host is characterized quantitatively by their overall density (i.e. number of RBCs per millilitre blood) and qualitatively by their age structure (the frequency of immature RBCs, termed reticulocytes, versus mature RBCs, termed normocytes). The density and/or frequency of reticulocytes and normocytes matters because different parasite species preferentially invade different age classes [6,48]. A further complication arises because parasites may not be selected to respond to anaemia *per se*, but simply use it as a proxy for the appearance of immune responses that have a major effect on state [49–51].

Here, we avoid confounding changes in immune responses and variation in asexual density to ask how parasites adjust conversion rates in response to the in-host environmental characteristic anaemia. Our approach enables us to examine several different parasite genotypes of the rodent malaria parasite *Plasmodium chabaudi* to assess genetic and genotype-by-environment influences on conversion rate. We find that all genotypes increase their conversion in response to anaemic conditions and that different genotypes do so to different extents. Further analysis suggests that parasites respond to the frequency of reticulocytes versus normocytes and that parasites increase conversion because they are taking advantage of an increase in resources rather than making a terminal investment.

## 2. Material and methods

We pre-treated hosts with different doses of a drug that modifies the age structure (measured as the proportion of reticulocytes) and overall density of RBCs in the blood (phenylhydrazine, PHZ), then inoculated mice with parasites of one of several genotypes. We then measured the density of asexual parasites and gametocytes to infer the conversion rate.

### (a) Parasites and hosts

We obtained C57BL/6 female mice (aged six to eight weeks) in-house (University of Edinburgh) and *P. chabaudi* clones AJ, AS, CR and ER from the Edinburgh Malaria Reagent Repository (University of Edinburgh). *Plasmodium chabaudi* was isolated between 1948 and 1974 from African thicket rats, *Thamnomys* spp., in Central Africa [52]. After cloning, the parasite genotypes have been cryopreserved and undergone regular transmission through mosquitoes to maintain their wild-type phenotypes [53]. The four *P. chabaudi* genotypes chosen span the diversity of conversion rates and virulence reported from previous experiments [34,54,55]. Four days after PHZ treatment (see below), hosts were infected intravenously with $5 \times 10^6$–$1 \times 10^7$ parasitized RBCs.

### (b) Perturbing red blood cell resources in the within-host environment

We used PHZ to generate different RBC resource environments in the blood. Phenylhydrazine causes the clearance of circulating RBCs [56] and the resulting anaemia stimulates the release of immature RBCs (reticulocytes) from the bone marrow. We found in a pilot study that injecting 120, 30 and 0 mg $kg^{-1}$ (control) of PHZ generated non-overlapping environments in terms of both total RBC density and reticulocyte proportion (see electronic supplementary material, figures S1 and S2) 2 days after injection, and that these differences persisted for 5 more days (see the electronic supplementary material). The variation in total RBC density and reticulocyte proportion observed for these doses corresponds to environments typically encountered by parasites during infection: the control treatment emulates the initial environment encountered in a healthy host, the 120 mg $kg^{-1}$ treatment reflects conditions after the peak of infection when hosts are most sick, whereas the 30 mg $kg^{-1}$ treatment resembles the RBC environment in a recovering host.

### (c) Experimental design and data collection

Four days before infection (day -4 post infection, PI) we injected mice with 120 mg $kg^{-1}$ ($n = 23$ mice), 30 mg $kg^{-1}$ ($n = 23$) or 0 mg $kg^{-1}$ (control treatment, $n = 22$) PHZ and on day 0 PI we infected each mouse with one of the four clones. This gave us a fully cross-factored design with 12 different treatment combinations (4 genotypes × 3 PHZ treatments). From day 1 PI to day 3 PI, we monitored mice daily at 09.00 by taking 2 µl of blood to quantify RBC density [57], making a thin blood smear, and collecting 10 µl blood for RT-qPCR to quantify gametocytes approximately aged 35 hours or older (since bursting from a committed schizont) [42]. RNA extraction was performed as described in [58] with minor changes to the protocol (see the electronic supplementary material). We deliberately focused on the early infection dynamics to maximize the likelihood of observing parasite responses to the RBC environments they encountered upon infection, rather than confounding factors that develop as infections progress (e.g. immune responses, divergence in parasite densities between treatments). Blood smears were used to estimate the density of asexual parasites and the proportion of RBCs that were reticulocytes or normocytes. All host measures relating to the RBC environment were inferred from the age structure and total density of RBCs; the density of reticulocytes was estimated as the proportion of reticulocytes multiplied by the total RBC density and the density of normocytes as (1 − the proportion of reticulocytes), multiplied by total RBC density.

### (d) Estimation of conversion rate and statistical analysis

Data were analysed using R v. 3.0.2. We used ANOVAs to assess the effect of PHZ on the within-host environment and on asexual parasite density. We then carried out two analyses to ask how anaemia affects conversion rates. Using ANCOVAs, we first tested the effect of genotype and PHZ treatment on conversion while also controlling for asexual density. All test statistics, degrees of freedom and *p*-values reported are from maximum-likelihood-based deletion tests (i.e. comparing a model with and without the explanatory variable of interest) [59]. Second, we fitted four ANCOVA models that decomposed the effects of PHZ into the densities and frequencies of RBC types to identify which in-host environmental variables correlate most closely with conversion rates. These models were simplified using maximum-likelihood-based deletion tests as above and the minimal models were compared by AIC. To infer conversion rate, we compared the summed gametocyte densities on days 2 and 3 PI to the summed density of their source populations of asexual parasites whose densities were recorded on days 1 and 2 PI. In the absence of differences in asexual parasite density, analysing pooled gametocyte densities directly avoids the difficulties of accurately calculating conversion rate using PCR data [6,60]. Gametocyte densities on days 2 and 3 PI may include gametocytes produced in donor mice. However, as all mice within each clone group were infected from the same donor mouse and the number of gametocytes or sexually committed parasites in inocula is small, this is unlikely to influence differences between treatments.

## 3. Results

### (a) Modifying resources in the within-host environment

For the duration of experimental infections, hosts in the different PHZ treatment groups had significantly different

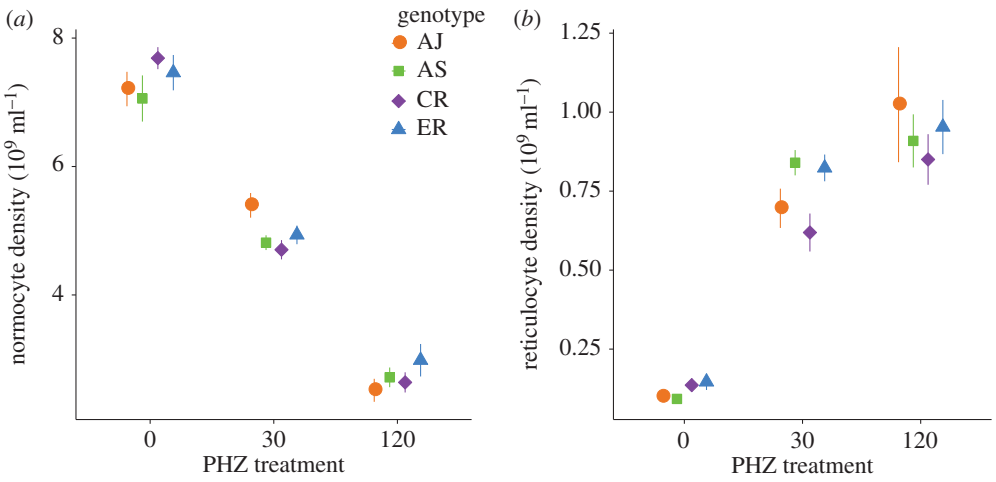


**Figure 2.** Mean ± standard error of the mean (SE) normocyte density (*a*) and reticulocyte density (*b*) on days 0–2 PI ($n = 68$) by PHZ treatment (0, 30, 120 mg kg$^{-1}$) and genotype. Normocyte and reticulocyte densities are significantly different between PHZ treatments but not between genotypes. (Online version in colour.)

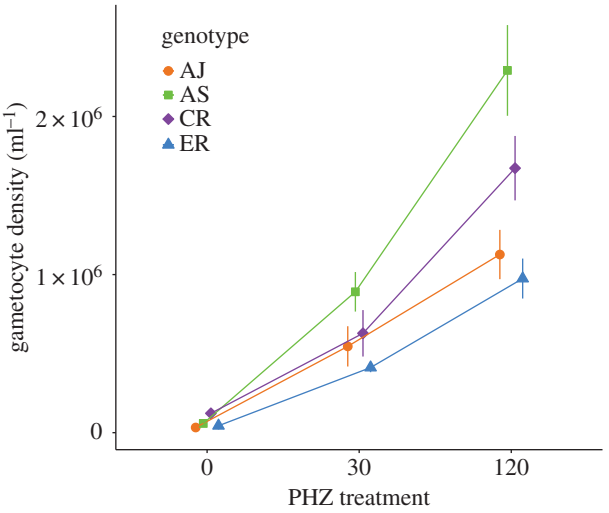


**Figure 3.** Reaction norms for conversion rate (mean ± SE gametocyte density for days 2 and 3 PI) of four genotypes across PHZ treatments (left to right along *x*-axis represents a decrease in total red blood cell density and an increase in reticulocyte proportion). All genotypes increase their conversion as PHZ dose increases, but to different extents (note, there is no significant difference between AJ and ER). The points are dodged horizontally for clarity. (Online version in colour.)

total RBC densities and age structures (figure 2; electronic supplementary material, table S1). As expected, normocyte density was reduced by PHZ in a dose-dependent manner, whereas the density and proportion of reticulocytes varied in the reverse fashion (figure 2). There are no genotype or genotype by PHZ treatment effects, i.e. within a given PHZ treatment, all genotypes encountered the same RBC environment (electronic supplementary material, table S1). The mean total RBC density from all genotypes combined (expressed as $10^9$ cells ml$^{-1}$) from day 0 PI to day 2 PI varied from 3.03(±0.18) to 4.57(±0.17) in the 120 mg kg$^{-1}$ treatment, from 5.63(±0.14) to 5.68(±0.13) in the 30 mg kg$^{-1}$ treatment and from 7.04(±0.34) to 7.71(±0.15) in the control treatment. The proportion of reticulocytes varied from 0.21(±0.02) to 0.27(±0.001) in the 120 mg kg$^{-1}$ treatment, from 0.12(±0.01) to 0.13(±0.008) in the 30 mg kg$^{-1}$ treatment and from 0.01(±0.002) to 0.02(±0.002) in the control treatment.

## (b) Conversion rate in perturbed within-host environments

Asexual density increases from day 1 to day 2 PI but there is no significant difference in their summed densities between genotypes within each PHZ treatment ($F(3, 62) = 1.62$, $p = 0.193$; electronic supplementary material, figure S3) or between PHZ treatments ($F(2, 65) = 2.81$, $p = 0.067$; electronic supplementary material, table S2). Thus, the summed gametocyte densities on days 2 and 3 PI reflect the conversion decision taken by asexuals on days 1 and 2 PI. However, we also control for asexual density in the following statistical models to account for any subtle variation between individual infections. The mean total gametocyte densities (over days 2 and 3 PI) vary significantly across PHZ treatments and genotypes. All genotypes increase their gametocyte densities (0 mg kg$^{-1}$ < 30 mg kg$^{-1}$ < 120 mg kg$^{-1}$) with increasing PHZ dose, but they vary in the magnitude of their responses (figure 3; electronic supplementary material, table S3). Once adjusted for variation in asexual density, the interaction between genotype and PHZ treatment explains 84% of the variance in gametocyte densities. To assess whether each genotype employs a different conversion rate strategy, we tested whether any genotypes could be grouped together without causing significant change in model deviance. The responses of AJ and ER did not differ significantly from each other ($F(3, 54) = 0.08$, $p = 0.970$) but all other genotypes follow significantly different reaction norms. In response to increasing PHZ doses, AS increases its gametocyte density most, followed by CR, and finally AJ/ER. Also, mean pairwise differences between genotypes are significantly greater in PHZ-treated mice, with approximately sixfold greater variation between genotypes expressed in the 120 mg kg$^{-1}$ environment compared with 0 mg kg$^{-1}$ ($F(2, 15) = 11.47$, $p < 0.001$, figure 3).

## (c) Conversion rate and RBC resources

As PHZ was used only as a means to modify the RBC environment, we expect that parasites do not detect and

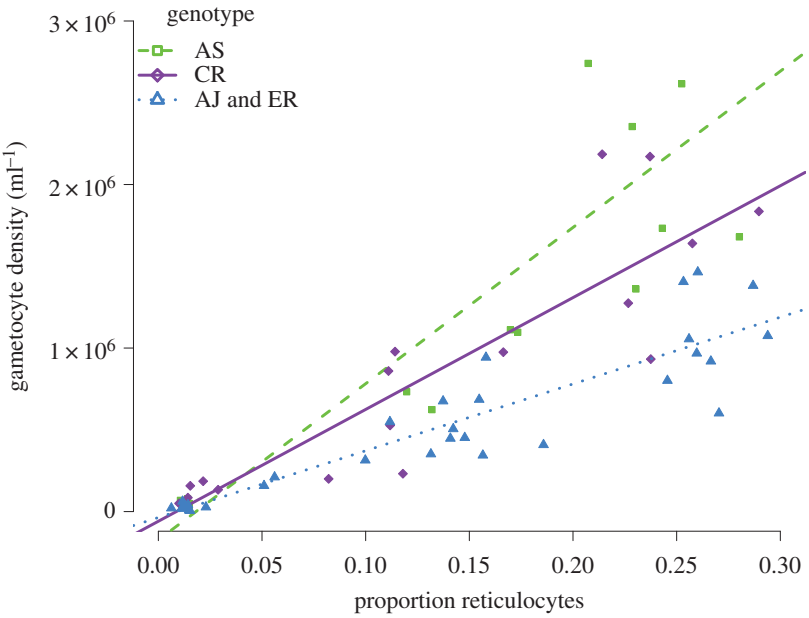


**Figure 4.** Gametocyte density for each mouse (mean of days 2 and 3 PI) correlates with RBC age structure (the mean proportion of RBCs that are reticulocytes across days 0, 1 and 2 PI for each mouse), and fitted lines illustrate the reaction norms for the different genotypes (note, there is no significant difference between AJ and ER). Data from the control group cluster around the origin (median reticulocyte frequency: 0.014), data from the 30 mg kg$^{-1}$ group span a reticulocyte frequency from 0.06 to 0.19 (median 0.14), and a dose of 120 mg kg$^{-1}$ produced a range between 0.21 and 0.29 (median 0.25). (Online version in colour.)

respond to PHZ itself, but to its effects on the density and/or age structure of RBC resources. As measurements of total RBC density and the proportion of RBCs that are reticulocytes (frequency) are independently determined, we fitted them as explanatory variables (instead of PHZ treatment), together with other variables of interest such as genotype and asexual density, and all their interactions (model 1). Using deletion tests, this model was simplified to the interaction between the frequency of RBC types and genotype (electronic supplementary material, table S4). We then constructed and minimized three other models that differed according to variables inferred from our two independent measurements; reticulocyte density (model 2), normocyte density (model 3) or total RBC density (model 4). Comparison of the resulting four minimal models (using AIC) reveals that the model containing the frequency-by-genotype interaction is the best overall (model 1, electronic supplementary material, table S5). This model explains 81% of the observed variation in gametocyte densities (electronic supplementary material, table S4) and reveals a positive correlation between gametocyte density and the proportion of RBCs that are reticulocytes, whose slope varies between genotypes (AS > CR > AJ = ER; figure 4).

## 4. Discussion

We found that the RBC resource environment, perturbed by PHZ, can explain more than 80% of variation in the conversion rate of malaria parasites: the higher the frequency of reticulocytes, the more parasites allocate to gametocytes. This pattern is broadly evident for all four genotypes examined but some genotypes are more sensitive than others, suggesting there is genetic variation for plasticity in conversion rates. We concentrated our analysis on the first few days of infection before the densities of the decision-making asexual cohorts could diverge between treatments. This avoids issues in previous studies where other variables associated with conversion, such as the density of asexual parasites or an adaptive immune response, covary with anaemia [34,35,45]. Our experiment also helps to elucidate the long-standing question of which cues parasites use for gametocytogenesis [33]: metrics for the density of RBCs, which is the most obvious measure of anaemia, do not correlate as strongly with conversion rate as the proportion of RBCs that are reticulocytes, suggesting that parasites respond to RBC age structure. Because encounter rates between merozoites and host RBC of different ages during invasion are directionally proportional to the frequency, not the density, of RBC types, encounter rates with different RBC ages could be a proximate mechanism for parasites to assess their RBC environment. It remains possible that instead, parasites respond to some unknown correlate of PHZ treatment, but we expect this is unlikely, given the diversity of data reporting positive associations between anaemia and correlates of conversion that do not involve PHZ.

Why do parasites increase their conversion rate in response to an increase in reticulocytes? The first possibility is that the environment has improved because resource availability has increased and parasites are able to allocate to gametocytes without unduly compromising survival inside the host (i.e. relaxing the need for reproductive restraint). Alternatively, parasites may perceive that the risk of the infection being cleared (by resource limitation, an immune response or host death) is sufficiently high that they should make a terminal investment. We favour the former explanation: it is unlikely that parasites interpret an influx of reticulocytes as a lethal situation because we observe that no hosts died during the experiment, no infections were cleared and asexual growth rate over the duration of the experiment was not compromised. At first, it seems counter-intuitive that anaemia could translate into an improvement of environmental quality; indeed, host-adaptive explanation for 'bystander killing' of uninfected RBCs implies that anaemia should facilitate the clearance of infection [61–64]. However, the erythropoietin (EPO)-mediated feedback during anaemia

brings in reticulocytes which could be a cue for an imminent improvement in the environment, although this seems unlikely because the presence of reticulocytes generally also correlates with the appearance of adaptive immunity. Thus, parasites may simply find reticulocytes to be superior cells compared with normocytes. If a high proportion of reticulocytes is beneficial to parasites, then a terminal investment strategy would be maladaptive. There are several lines of evidence suggesting that malaria parasites respond to the differential resource qualities of reticulocytes and normocytes. For parasite species that strongly prefer reticulocytes, like *P. berghei*, an increase in replication rate and conversion rate in response to PHZ treatment has been reported [65,66]. Even though *P. chabaudi* is thought to be a generalist and able to infect a wide range of RBC ages, reticulocytes may be a better resource than normocytes for *P. chabaudi* for several non-mutually exclusive reasons: (i) reticulocytes carry a more diverse set of cell surface receptors to interact with rhoptry proteins on invading merozoites [67,68]; (ii) reticulocytes are a metabolically more diverse resource [69]; (iii) reticulocytes could be particularly well suited for the development of gametocytes because their longer lifespan matches the longer maturation time and lifespan of gametocytes compared to asexuals [70–72]; and (iv) an infected reticulocyte may produce more merozoites than a normocyte [73], possibly due to their larger size or reduced oxidative stress [74]. Such mechanisms may also explain observations of higher growth rates and conversion rates of *P. chabaudi* in mice treated with EPO, which increases reticulocyte frequency without affecting total RBC density [45].

We observed three different reaction norms for conversion rate across the four genotypes that we examined. These genotypes vary in virulence and the most virulent genotypes (AJ, ER) are less plastic than the less harmful genotypes (CR, AS). Virulence is generally regarded as a fitness-related trait for parasites: virulent genotypes are better competitors against conspecifics sharing the host, and are more likely to survive drug treatment and immune responses [75–78]. Thus, it is not clear why genetic variation for virulence is maintained in natural malaria populations [79]. Because gametocyte density is positively correlated to transmission success, our results suggest that genotypes of low virulence could achieve greater transmission when hosts mount an erythropoietic response and so, compensate for the fitness costs of low virulence. If this holds for human malaria parasites, it suggests that co-infections that cause anaemia (e.g. helminth infections, [80]) could select for less virulent malaria genotypes. Further, anaemic patients may require additional transmission-reducing measures because they may be more infectious than their counterparts [44,72].

Ethics. All procedures were carried out in accordance with the UK Animals (Scientific Procedures) Act 1986.

Data accessibility. All data from this project can be obtained from our website http://reecelab.science or from S.E.R.

Authors' contributions. This project was conceived and planned by P.L.G.B. and S.E.R.; P.L.G.B., C.R., A.J.O. and P.S. carried out the experiments; P.L.G.B. and S.E.R. drafted the first version of the manuscript, and all authors contributed to the final draft.

Competing interests. We declare we have no competing interests.

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ORIGINAL ARTICLE

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# Altered life history strategies protect malaria parasites against drugs

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## Abstract

Drug resistance has been reported against all antimalarial drugs, and while parasites can evolve classical resistance mechanisms (e.g., efflux pumps), it is also possible that changes in life history traits could help parasites evade the effects of treatment. The life history of malaria parasites is governed by an intrinsic resource allocation problem: specialized stages are required for transmission, but producing these stages comes at the cost of producing fewer of the forms required for within-host survival. Drug treatment, by design, alters the probability of within-host survival, and so should alter the costs and benefits of investing in transmission. Here, we use a within-host model of malaria infection to predict optimal patterns of investment in transmission in the face of different drug treatment regimes and determine the extent to which alternative patterns of investment can buffer the fitness loss due to drugs. We show that over a range of drug doses, parasites are predicted to adopt "reproductive restraint" (investing more in asexual replication and less in transmission) to maximize fitness. By doing so, parasites recoup some of the fitness loss imposed by drugs, though as may be expected, increasing dose reduces the extent to which altered patterns of transmission investment can benefit parasites. We show that adaptation to drug-treated infections could result in more virulent infections in untreated hosts. This work emphasizes that in addition to classical resistance mechanisms, drug treatment generates selection for altered parasite life history. Understanding how any shifts in life history will alter the efficacy of drugs, as well as any limitations on such shifts, is important for evaluating and predicting the consequences of drug treatment.

**KEYWORDS**

gametocytes, life history evolution, nonclassical drug resistance, *Plasmodium*, pyrimethamine, transmission investment

## 1 | INTRODUCTION

Malaria parasites (*Plasmodium* spp.) remain one of the most severe and common causes of human disease (White, Pukrittayakamee, et al., 2014). Although interventions against malaria parasites have seen significant successes over the last 30 years (WHO 2015a), resistance has evolved to every antimalarial drug in widespread use (Hyde, 2005; White, 2004; WHO 2015a). In many cases, this resistance has been attributed to "classical" resistance mechanisms (sensu Schneider et al., 2012), including target site mutations or detoxification mechanisms (Hyde, 2002, 2005). However, changes in parasite behaviour, metabolism or life history, that is, "nonclassical" resistance

mechanisms (Schneider et al., 2012), offer additional threats to drug efficacy.

One potential mechanism for nonclassical resistance is evolving traits that give rise to higher within-host parasite densities; this may offer protection against drugs by increasing the likelihood that some (genetically identical) parasites survive treatment (White, 1998). Experimental rodent malaria infections confirm that more virulent parasite strains, with faster within-host replication, survive better in drug-treated hosts (Schneider, Chan, Reece, & Read, 2008; Schneider et al., 2012). But within-host densities are at least in part governed by a resource allocation trade-off in malaria and other sexually reproducing parasites: achieving higher within-host densities comes at the cost of producing fewer specialized sexual stages (gametocytes) that are required for transmission (Carter et al., 2013; Pollitt et al., 2011), as a parasite in a given infected host cell can follow only one of the two developmental routes. Transmission investment—by convention referred to as the conversion rate—varies plastically within artificial culture, increasing as conditions become more crowded (Bruce, Alano, Duthie, & Carter, 1990). While conversion rate can change plastically in response to changing environmental conditions, data suggest that there is parasite genetic variation for patterns of conversion (Pollitt et al., 2011; Birget, Repton, O'Donnel, Schneider, & Reece, 2017) and that this variation can be selected upon (reviewed in Bousema & Drakeley, 2011). It is well known, for example, that serial passage and culture experiments, which by their nature select for faster within-host replication, result in reduced transmission investment (Dearsly, Sinden, & Self, 1990; Sinha et al., 2014; reviewed in Carter et al., 2013). Similarly, artificial selection for attenuation in a related parasite, *Eimeria*, resulted in indirect selection for earlier investment in transmission, which translated into a substantial reduction in total transmission potential (McDonald & Shirley, 2009). Therefore, conversion rates represent an evolvable parasite trait essential to transmission, and the challenge is to explore if and how drug treatment might alter parasite strategies.

Malaria parasites appear to vary transmission investment in ways thought to be adaptive (Carter et al., 2013), and theory is an essential check on intuition regarding the fitness consequences of different strategies (Greischar, Reece, & Mideo, 2016). Models have shown that reducing transmission investment—though it might appear maladaptive (Taylor & Read, 1997)—can dramatically enhance parasite fitness by increasing the parasite numbers available to produce gametocytes later on and by improving persistence in the face of immunity and competing strains (Greischar, Mideo, Read, & Bjornstad, 2016a; Greischar, Read, & Bjørnstad, 2014; Koella & Antia, 1995; McKenzie & Bossert, 1998; Mideo & Day, 2008). It remains challenging to show experimentally that these predicted patterns are adaptive, actually improving parasite fitness in the face of environmental change, as techniques for forcing parasites to make alternative life history decisions are currently not available. However, the development of improved statistical methods now allows more accurate estimates of conversion rates in vivo (Greischar, Mideo, Read, & Bjornstad, 2016b), and theory is urgently needed to form clear expectations to compare with natural patterns. In contrast, conversion rates are comparatively easy to integrate into mathematical models by simply varying allocation to asexual growth and gametocyte production. Mathematical models demonstrate that changing allocation patterns can have significant impacts on parasite fitness (i.e., transmission potential) and can predict the optimal pattern in different environments (Greischar et al., 2014; Greischar et al., 2016a; Koella & Antia, 1995; McKenzie & Bossert, 1998; Mideo & Day, 2008). Understanding how selection imposed by drugs may alter transmission investment is critical, as any changes will have both clinical and epidemiological consequences.

Here, we predict the resource allocation patterns of malaria parasites that maximize fitness in drug-treated hosts. We extend a previously published mechanistic model of within-host malaria infection (Greischar et al., 2014; Greischar et al., 2016a) and use numerical optimization techniques to determine optimal conversion rates, that is, the proportion of infected host cells that produce transmission stages. Into this framework, we incorporate a simple model of drug action that was parameterized for the treatment of experimental rodent malaria infections with the antimalarial drug pyrimethamine (Huijben et al., 2013). By holding constant the duration and timing of drug treatment, but varying dose, this heuristic model allows us to explore the predicted impact of treatment of variable efficacy—from small to large reductions in parasite load—on parasite life history evolution. We explore optimal investment in transmission stages, first, by assuming parasites are constrained to a constant conversion rate throughout infections and, second, by permitting parasites to employ time-varying conversion rates. Finally, we quantify the extent to which altering life history according to these optimal patterns can buffer against the effects of drugs and we evaluate the consequences for host health and onward transmission.

## 2 | METHODS

### 2.1 | The model

Following Greischar et al. (2014, 2016a), we use delay-differential equations to model the within-host dynamics of a malaria infection, which tracks uninfected red blood cells ($R$), infected red blood cells ($I$), extracellular malaria parasites (merozoites, $M$) and gametocytes ($G$). The change in density of uninfected red blood cells (RBCs) over time, $t$, is given by

$$\frac{dR}{dt} = \lambda\left(\frac{1-R(t)}{K}\right) - \mu R(t) - pR(t)M(t). \quad (1)$$

The first term represents production of new RBCs by the host. Erythropoiesis is assumed to be a logistic function of current RBC density, where $\lambda$ is the maximum realized rate of replenishing depleted RBCs and $K$ determines the homeostatic equilibrium density. We assume that only uninfected RBCs count towards the homeostatic equilibrium because malaria parasites consume large amounts of haemoglobin during their development (e.g., Lew, 2003) and compromise the ability of infected RBCs to carry oxygen (Schmidt, Correa, Boning, Ehrich, & Kruger, 1994). We have found that including infected RBCs in this term makes little qualitative difference. In the absence of infection, RBC production balances natural death (which occurs at a rate, $\mu$), so

$K = \frac{\lambda R^*}{\lambda - \mu R^*}$, where $R^*$ represents the RBC density at homeostatic equilibrium. The final term represents a mass action infection process, and $p$ is the rate at which merozoites invade RBCs upon contact.

The dynamics of infected RBCs are given by

$$\frac{dI}{dt} = pR(t)M(t) - \mu I - pR(t-\alpha)M(t-\alpha)S \quad (2)$$

where $S$ indicates the proportion of infected RBCs surviving development, equal to $e^{-\mu\alpha}$ when $t > \alpha$ and in the absence of drugs. An infected cell is generated when a merozoite invades an uninfected RBC and can be lost via two different routes. First, infected RBCs can die at a background rate $\mu$. Second, infected RBCs burst to release merozoites after a period of $\alpha$ days (i.e., 1 day for the rodent malaria parasite, *P. chabaudi*). For simplicity, we omit immune responses that remove infected RBCs, although simulations of this model including a saturating immune response have delivered similar optimal conversion rate profiles (results not shown).

The dynamics of merozoites and gametocytes are described as

$$\frac{dM}{dt} = (1-c(t))\beta pR(t-\alpha)M(t-\alpha)S - pR(t)M(t) - \mu_M M(t) \quad (3)$$

$$\frac{dG}{dt} = c(t)pR(t-\alpha)M(t-\alpha)S - \mu_G G(t) \quad (4)$$

where $c(t)$ is the proportion of parasites in a given cohort of infected RBCs that become gametocytes after successful development (i.e., the conversion rate). We allow the conversion rate to vary over the course of infection, as has been observed in experimental data (Greischar et al., 2016b; Pollitt et al., 2011; Reece, Duncan, West, & Read, 2005). The burst size, $\beta$, is the number of merozoites released from each infected RBC surviving the developmental period. Merozoites die at a rate $\mu_M$, and gametocytes die at a rate $\mu_G$.

Equations 2–4 are defined for $t > \alpha$. The dynamics of the initial inoculum of parasites, $I_0$, are governed by

$$\frac{dI}{dt} = pR(t)M(t) - \frac{I_0 S}{\alpha} - \mu I \quad (5)$$

$$\frac{dM}{dt} = (1-c(t))\beta\frac{I_0 S}{\alpha} - pR(t)M(t) - \mu_M M(t) \quad (6)$$

$$\frac{dG}{dt} = c(t)\frac{I_0 S}{\alpha} - \mu_G G(t) \quad (7)$$

$$S = e^{-\mu t} \quad (8)$$

for $t \leq \alpha$.

## 2.2 | Drug action

We incorporate the model of drug action presented in Huijben et al. (2013), which was parameterized to describe the consequences of pyrimethamine for *Plasmodium chabaudi* parasites (Landau, 1965) in infections of female C57BL6 mice (Schneider et al., 2012). According to this model, as long as the drug is present at a sufficiently high concentration in the host, it kills a fixed proportion (94%) of parasites each day. The underlying within-host model assumed in Huijben et al. (2013) was in discrete-time and cohorts of infected cells burst synchronously. To approximate this drug action in our model, we apply an additional death rate, $\mu_d$, to infected cells. By setting $\mu_d = -\ln(1-0.94) = 2.81$, we ensure that ~94% of infected cells die within the 1 day parasite developmental cycle. Different drug doses, $d$, modify the length of drug action, $L$, beyond the days the drug was administered (see Figure A.1 in Appendix A, for how $L$ varies with dose):

$$L = 3.557 - \frac{2.586}{1+e^{-8.821+d}}. \quad (9)$$

Therefore, parasites are subject to a drug-induced mortality rate for each day that the drugs are administered, plus an additional $L$ days afterwards. To explore the consequences of different strengths of drug treatment on optimal patterns of conversion rates, we simulate several treatment regimes: drug doses of 0–15 mg/kg, each administered for two consecutive days (days 11 and 12 postinfection). Determining the survival of infected RBCs ($S$) requires integrating these mortality rates over the delay $\alpha$. For the case of drug-treated infections, that survival term is given by

$$S = \begin{cases} \exp(-\mu t), & t < \alpha \\ \exp\left(-\left(\int_{t-\alpha}^{11} \mu d\omega + \int_{11}^{t} \mu + \mu_d d\omega\right)\right), & 11 \leq t < \alpha + 11, \\ \exp\left(-\left(\int_{t-\alpha}^{t} \mu + \mu_d d\omega\right)\right), & \alpha + 11 \leq t < L + 12, \\ \exp\left(-\left(\int_{t-\alpha}^{12} \mu + \mu_d d\omega + \int_{12}^{t} \mu d\omega\right)\right), & L + 12 \leq t < L + 12 + \alpha, \\ \exp(-\mu\alpha), & \text{otherwise.} \end{cases} \quad (10)$$

Given our other model parameters, these treatment regimes encompass outcomes from a small, transient reductions in parasite loads, to a strong reduction in parasite load that would prevent further transmission on the timescale of our simulation. A schematic of the model of drug action is presented in Figure A.2 in Appendix A.

## 2.3 | Optimization

To find optimal patterns of transmission investment, we use the optim function in R version 3.0.2 and define the cumulative transmission potential as our measure of fitness. This metric translates daily estimates of gametocyte density into the probability of that density resulting in an infected mosquito, assuming mosquitoes are abundant and biting hosts on a regular basis. The relationship between gametocyte densities and transmission probability is assumed to be sigmoidal, as has been experimentally derived for *P. chabaudi* by Bell et al. (2012). Using their parametrization, our fitness function is calculated as

$$f(\eta) = \int_0^{\eta} \frac{e^{-12.69+3.6\log_{10}G(t)}}{1+e^{-12.69+3.6\log_{10}G(t)}} dt, \quad (11)$$

where $G(t)$ is the gametocyte density at time point $t$, and $\eta$ is the day postinfection at which our simulated infection ends. A sigmoidal relationship between gametocyte density and transmission success has also been reported for *P. falciparum* (Huijben et al., 2010) and gives similar results if used instead of the fitness function described here (see Figure A.3 in Appendix A). Our model describes early infection dynamics, before major adaptive immune responses develop. We therefore simulate a 20-day infection over which we calculate the cumulative transmission potential, as has been done previously (Greischar et al., 2016a).

In a first set of optimizations, we define transmission investment to be a constant ($c(t) = x$, for all $t$) and determine the optimal time-invariant conversion rate. Second, following Greischar et al. (2016a),

we use cubic splines for the optimization of time-varying conversion strategies, implemented in **R** with the "splines" package. Cubic splines require only four parameters to specify but allow considerable flexibility in the pattern of conversion over a 20-day infection, and more complicated splines yield minimal fitness gains (Greischar et al., 2016a). Conversion rates must be constrained to vary between zero and one, so we take the complimentary log-log of the value specified by the spline, that is, $c(t) = \exp(-\exp(\text{spline value at time } t))$. The starting values of the variables and the assumed value for each of the model parameters are given in Table 1, and each optimization is initiated by setting all spline parameters to an arbitrary starting guess of 0.5. Although no numerical optimization routine can guarantee finding a globally optimal solution, we sought to substantiate our findings by testing, for a given environment (i.e., drug dose), whether the putative optimal strategy for that environment outperformed the putative optimal strategies from other environments.

## 3 | RESULTS

### 3.1 | Constant conversion rates

Following previous work (Greischar et al., 2016a), we first constrained conversion rate in our within-host model to be constant, and determined which single rate, maintained throughout the whole infection, produced the highest estimate of our parasite fitness proxy (i.e., cumulative transmission potential). In the absence of drugs, we find a similar optimal level of transmission investment as predicted previously (Greischar et al., 2016a). Drug treatment reduces the optimal level of transmission investment, with the lowest conversion rate predicted for the highest drug dose simulated (Figure 1a). We found little variation in the optimal transmission investment over low and moderate drug doses, as would be expected given our assumption that the drug dose changes the number of days of drug action rather than the killing rate (Huijben et al., 2013). For doses below 6 mg/kg, this formulation predicts little difference in the duration of drug action (see Figure A.1 in appendix A) or consequences for parasite fitness, as can be seen in Figure 1b. We therefore focus on 5 mg/kg, 8 mg/kg and 15 mg/kg as representative low, medium and high drug doses, respectively, for the remainder of our analyses. The step-wise decrease in predicted conversion rates observed from a dose of 0 to 2 mg/kg and from a dose of 8 to 10 mg/kg closely follows the fitness effects that these increasing doses would have on parasites employing a non-drug-adapted conversion rate (Figure 1b, grey bars). Interestingly, we do not see a similar decrease in the predicted optimal conversion rate when the drug dose increases from 6 to 8 mg/kg, despite a substantial decrease in expected fitness for a non-drug-adapted strategy. An explanation for this may be found in the fact that a constant conversion rate represents a compromise, balancing the need to sustain a high enough asexual source population for conversion in the face of drug killing and having a sufficiently high conversion rate to successfully translate that asexual source population into onward transmission. Up to a dose of 8 mg/kg, slight increases in conversion rates can counteract lost fitness due to slight reductions in the asexual source population from higher doses. With a dose of 10 mg/kg or more, the asexual source population –and gametocytes– are reduced to such an extent that no more transmission is possible after the action of drugs. Therefore, the best option for a parasite is to restrain and increase the asexual source population that will be converted before the end of drug action.

We assume that all parasites within an infection are genetically identical; consequently, our fitness proxy is the cumulative probability of transmission over the course of infection. As our simulated infections run for 20 days, 20 represents the maximum cumulative transmission potential that would be achieved by a parasite genotype that sustained a sufficiently high gametocyte density to transmit to

**TABLE 1** Model parameters

| Parameter | Description | Value or range | References |
|---|---|---|---|
| $R^*$ | Red blood cell density of a healthy mouse | $8.5 \times 10^6$ cells/μl | Savill, Chadwick, and Reece (2009) |
| λ | Maximal red blood cell production rate | $3.7 \times 10^5$ RBCs/μl | Savill et al. (2009) |
| μ | Red blood cell death rate | 0.025/day | Miller, Råberg, Read, and Savill (2010) |
| $p$ | Maximal per merozoite invasion rate | $4 \times 10^{-6}$/day | Mideo et al. (2008) |
| α | Bursting delay | 1 day | Landau and Boulard (1978) |
| β | Burst size | 10 merozoites | Mideo et al. (2008) |
| $\mu_M$ | Merozoite death rate | 48/day | Mideo et al. (2008) |
| $\mu_G$ | Gametocyte death rate | 4/day | Gautret, Miltgen, Gantier, Chabaud, and Landau (1996) |
| $\mu_d$ | Drug-induced death rate of infected cells | 2.81/day | Adapted from Huijben et al. (2013) |
| $I_0$ | Initial dose of infected red blood cells | 43.85965/μl | ~$10^4$ per mouse |
| $d$ | Drug dose | 0–15 mg/kg | |

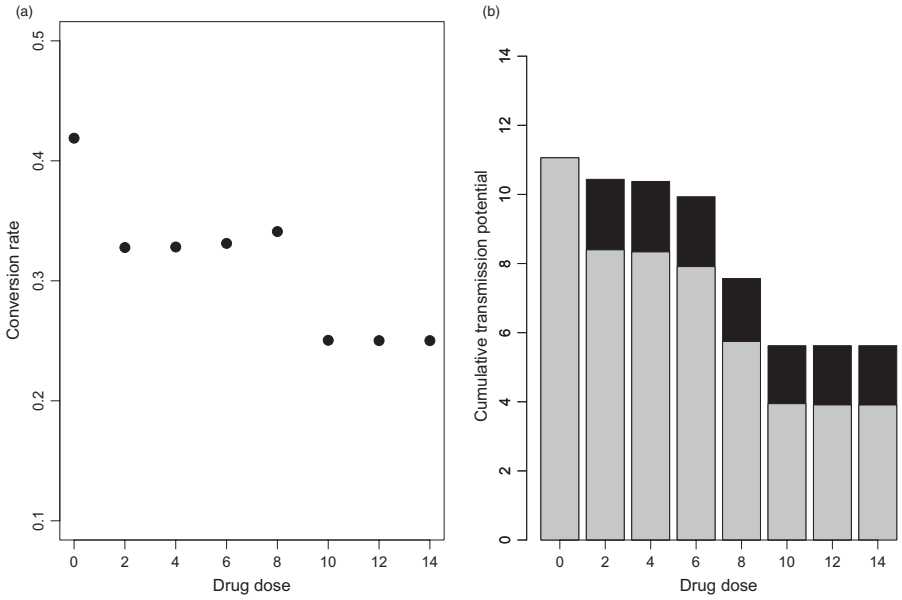


**FIGURE 1** Lower conversion rates can buffer the effects of drugs. (a) Optimal constant conversion rates in the face of drug treatment (labelled as doses in mg/kg) are lower than in the absence of drugs. (b) As expected, drug treatment reduces parasite fitness (i.e., cumulative transmission potential). Grey bars indicate fitness when parasites are constrained to the drug-free optimal conversion rate (~0.42). Black bars show the fitness gains achieved by adopting the dose-specific optimal conversion rate (from A). With lower conversion rates, parasites are able to recoup some of the fitness that is lost due to drugs

mosquitoes with 100% efficacy every day. Even in the absence of drugs, parasites cannot achieve 100% transmission efficacy at every point in the simulation, especially at the beginning of the infection when parasite numbers are low; hence, the maximum cumulative transmission potential is approximately 11 for the optimal level of fixed transmission investment of 0.42 in the absence of drugs (Figure 1b). The grey bars demonstrate the fitness achieved by parasites employing this same conversion rate (0.42) in the face of drug treatment. As expected, parasite fitness is lost as drug treatment reduces numbers. Some fitness can be recouped by adopting lower conversion rates (the drug dose-specific optima, black bars). Indeed, with low drug doses, reduced conversion rates allow parasites to maintain roughly 90% of the fitness achieved in the absence of drugs.

## 3.2 | Time-varying conversion rates

Next, we allowed the conversion rate to vary over the course of the infection and determined what pattern of transmission investment would maximize cumulative transmission potential (Equation 11). The work of Greischar et al. (2016a) suggests that, in the absence of drug treatment, optimal patterns of conversion rate comprise roughly four distinguishable phases: (i) an "initial replication" phase where parasites delay gametocyte production to increase their numbers; (ii) a "peak conversion" phase where parasites dramatically increase transmission investment to capitalize on their large numbers; (iii) a "trough" where parasites reduce transmission investment to compensate for declining numbers in the face of resource limitation; and finally, (iv) "terminal investment, " where parasites invest heavily into gametocyte production before the infection ends. We find qualitatively similar strategies (with the same four phases) in drug-treated infections (Figure 2). The corresponding dynamics of infected red blood cells and gametocytes are shown in Figure 3. A key difference in the predicted optimal patterns of conversion in drug-treated compared to untreated infections is an earlier and faster reduction in conversion rates (i.e., greater reproductive restraint) following the initial peak conversion (compare black to coloured lines in Figure 2). Comparing low and medium dose treatment regimes, we find that increasing dose is accompanied by greater reproductive restraint following treatment. The best response to a high drug dose is early terminal investment, which ultimately ends the infection (see infection dynamics in Figure 3c).

To identify the fitness consequences of these different strategies, we plot cumulative transmission potential over the course of infections. In Appendix A, we confirm that the putative optimal strategy against a given dose outperforms the putative optimal strategies from other doses (see Figure A.4). The optimal strategies—and the corresponding cumulative transmission potential—are similar prior to drug treatment (Figures 2 and 4, respectively). After drug treatment, the transmission investment strategies diverge, and there are clear costs to parasites that employ the incorrect strategy for the drug dose they encounter within the host (compare coloured to dashed grey curves in Figure 4). Specifically, in the absence of drug treatment, the optimal drug-free strategy accrues fitness at nearly the maximal rate, corresponding to an almost 100% chance of transmitting to mosquitoes each day (black lines, Figure 4). But, this strategy performs successively worse in the face of increasing drug doses (dashed grey lines Figure 4; see also Figure 3 for corresponding infection dynamics). The optimal strategies for low, medium and high drug doses allow parasites to recoup a substantial portion of these fitness losses (coloured lines

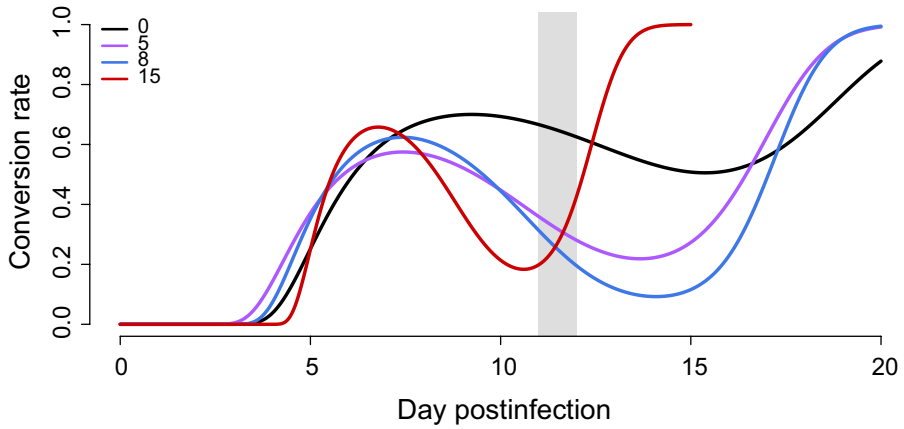


**FIGURE 2** The optimal pattern of conversion over the course of infections. The black line shows the predicted best response in an untreated infection. When infections are treated (coloured lines), regardless of dose, parasites do better by reducing conversion (purple: low dose, 5 mg/kg; blue: medium dose, 8 mg/kg; red: high dose, 15 mg/kg). Drugs are administered on the days denoted by the grey bar. If drug treatment reduces the infection to a degree where parasites cannot expect any future transmission, then the best response for parasites is to terminally invest (as suggested by the red line). Note that the patterns diverge before drug treatment due to the constraints of our fitting regime; however, early differences in investment patterns contribute little to fitness differences (see text)

in Figure 4), attributable to greater reproductive restraint immediately after drug treatment (Figure 2). Notice that in the face of a high drug dose, the drug-free strategy accrues no fitness following treatment (Figure 4c, dashed grey line), despite the fact that gametocytes are still circulating for days in those infections (Figure 3c, dashed grey line). This is because the densities are too low to achieve more than a negligible probability of transmission. In untreated infections, parasites that use reproductive restraint pay only a small fitness cost, whereas parasites employing strategies against high drug doses pay a more substantial fitness cost due to premature terminal investment (Figure 5a).

While reproductive restraint in response to treatment can, to some extent, buffer against the effects of drugs, our models predict that treatment still leads to reductions in parasite fitness and, importantly, reductions in transmission potential. As reproductive restraint necessarily means prioritization of asexual replication and it is these parasite stages that are most responsible for the virulence (harm) of a malaria infection, there may be consequences of shifting patterns of conversion at the host (or clinical) level. Drug treatment reduces infected RBC densities, even if parasites alter their conversion rates (Figure 3), but what if parasites employ drug-adapted strategies in an infection that remains untreated? Figure 5b shows that, in an untreated host, infections composed of parasites using a drug-adapted strategy (coloured lines) are predicted to result in much more rapid declines in uninfected RBC densities, and greater anaemia as measured by minimum RBC counts, compared to parasites using the best strategy in the absence of drugs (black line).

Of course, the likelihood of a drug-adapted strategy becoming fixed in the parasite population depends on the frequency that parasites encounter drug-treated hosts, the benefits of altered patterns of conversion in a drug-treated host, as well as the costs of that strategy in an untreated host. Using the fitness estimates for the different strategies in different environments (Table B.1 in Appendix B), we calculate the expected fitness for the drug-adapted and non-drug-adapted strategies in a host population where some proportion of hosts are treated (Figure B.1). If $b$ is the increase in fitness achieved by the drug-adapted strain in the presence of drugs (i.e., the benefit), $c$ is the reduced fitness of the drug-adapted strain in an untreated host (i.e., the cost), and $f$ is the proportion of infected hosts that are drug-treated, then it is trivial to show (see Appendix B) that the drug-adapted strategy has a higher fitness than the non-drug-adapted strategy when

$$f > \frac{c}{c+b}. \tag{12}$$

Put another way, the drug-adapted strategy will be favoured when the ratio of the benefits to costs of the strategy is greater than the relative frequency of encountering an untreated host:

$$\frac{b}{c} > \frac{1-f}{f}. \tag{13}$$

Given our estimated fitnesses for the different strategies in different host environments, the drug-adapted strategy will be favoured over the non-drug-adapted strategy when at least ~40% of infections are treated with a low or medium dose, or at least 86% of infections receive a high dose treatment. The early terminal investment strategy predicted to be optimal in the face of a high drug dose gains only a small fitness advantage in a treated host, while it suffers a large fitness cost in an untreated host (see also Table B.1), explaining why drug treatment would have to be very common to generate a sufficient selection pressure to favour that strategy.

## 4 | DISCUSSION

The evolution of drug-resistant parasites is a serious obstacle to the control of malaria (Dondorp et al., 2009; White, 2004). In addition to classical resistance mechanisms, we have shown that drug treatment can select for altered life history of malaria parasites and, specifically,

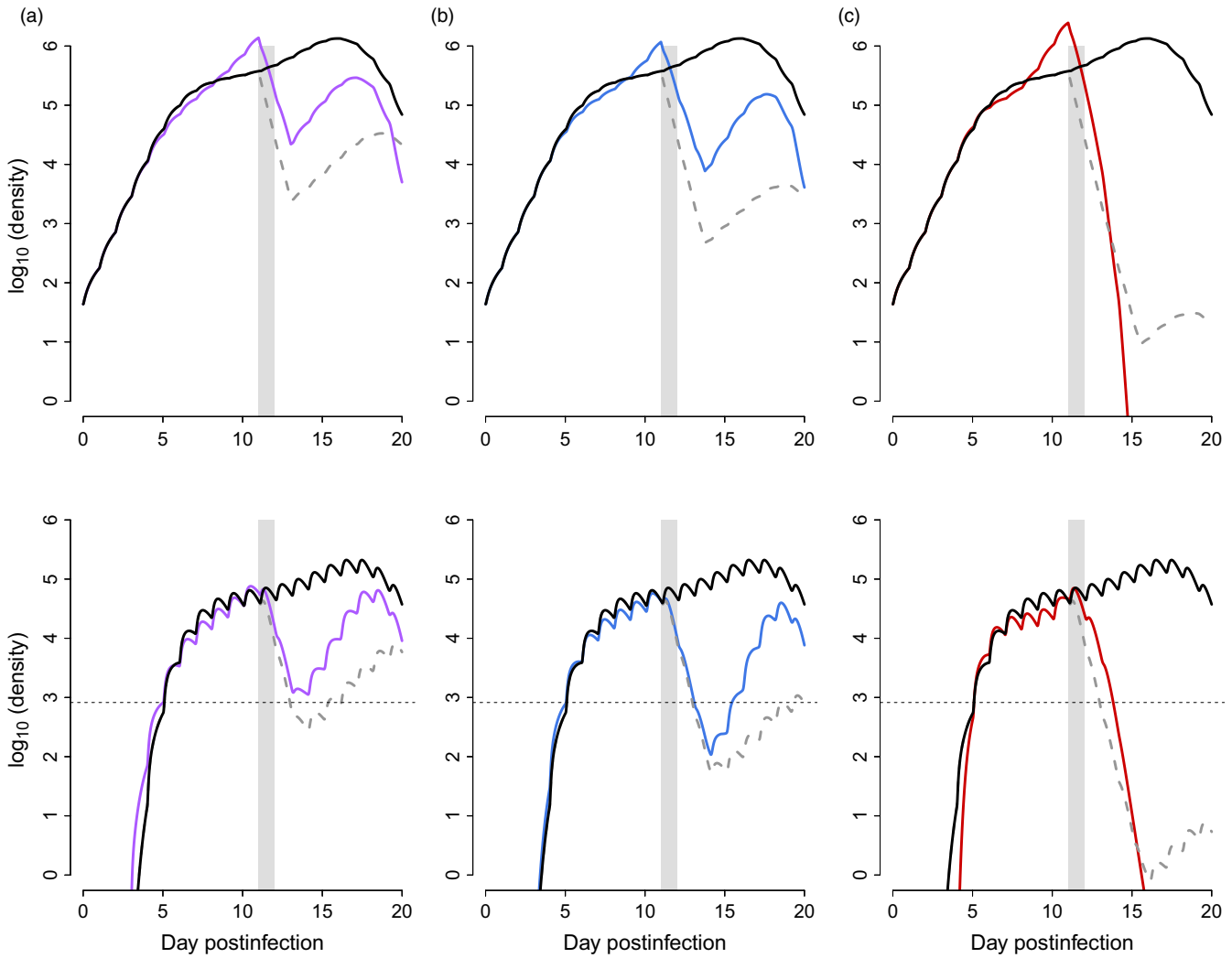


**FIGURE 3** The within-host dynamics of infected red blood cells (i.e., asexual parasites; top row) and gametocytes (bottom row). Coloured lines show dynamics when parasites are using the optimal conversion profiles for a given drug treatment (a: low dose, purple; b: medium dose, blue; c: high dose, red). The black lines show dynamics in the absence of treatment, for parasites using the optimal drug-free pattern of conversion, while the dashed grey lines show how the different drug treatment regimes impact these dynamics if parasite life history patterns are unchanged from the drug-free optimum. Grey bars denote the days of drug treatment and the horizontal lines in the bottom row indicate the gametocyte density at which there is a 10% probability of transmitting to a mosquito, according to Bell et al. (2012)

changing patterns of allocation to transmission versus asexual parasite stages. Our work predicts that reproductive restraint is adaptive in drug-treated infections, allowing parasites to partially compensate for the reductions in asexual densities caused by the drug. We also show that parasite adaptation to drug treatment could lead to worse outcomes for hosts that remain untreated, although as would be expected this outcome depends on the frequency with which parasites find themselves in treated hosts as well as the precise costs and benefits associated with different investment patterns in different environments.

Experimental evidence suggests that malaria parasites do alter their investment in transmission in response to drugs. Reece, Ali, Schneider, and Babiker (2010), for example, found a decrease in conversion in human malaria parasites exposed to low doses of drugs in vitro, as our model predicts, unless they were known to be "classically" drug-resistant parasites, which showed no change in investment (a result that highlights the multiple routes available for mitigating the effects of drugs). A similar study found no effect of drug dose on conversion rates (Peatey et al., 2009), and an in vivo rodent malaria experiment suggested that subcurative drug doses lead to increased conversion (Buckling, Taylor, Carlton, & Read, 1997). In contrast to the results of Reece et al. (2010), these latter two examples show parasite responses that appear maladaptive in the light of our model results, raising at least two further questions. First, have parasite strategies been accurately measured? Inferring conversion rates is fraught with difficulties that have only recently been resolved (Greischar et al., 2016b), and reanalysis of past data sets could reconcile the discrepancy between theoretical predictions and empirical estimates of transmission investment. Second, are parasites capable of evolving adaptive transmission strategies to the novel selection pressure of drug treatment? Addressing this question means evaluating whether the parasites in these experiments would have achieved greater fitness than ones with different responses, which necessitates tools for manipulating parasite

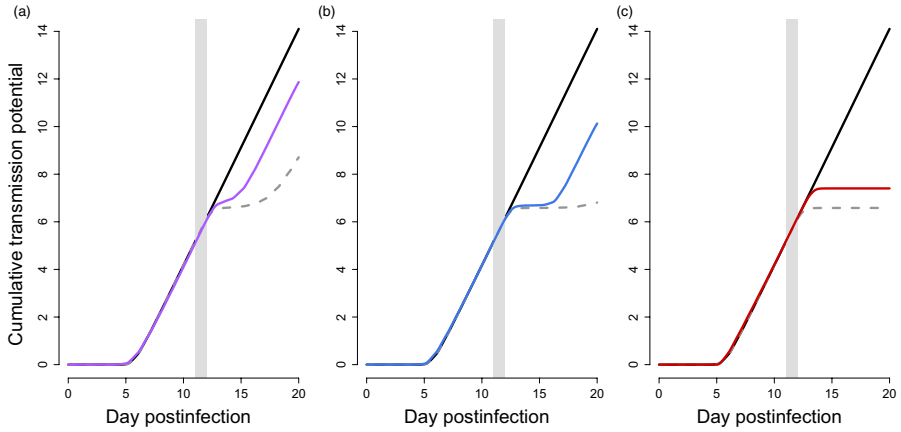


**FIGURE 4** Cumulative transmission potential (fitness) over the course of infections. Given our fitness function, a parasite can maximally transmit with a probability of 1 each day, reaching a cumulative transmission potential of 20 at the end of the simulated infection. Black lines show the fitness obtained by a parasite adopting the drug-free optimal pattern of conversion over the course of an untreated infection. Dashed grey lines show the consequences of drug treatment on parasites using that same strategy in the face of drug treatment: (a) low dose, 5 mg/kg; (b) medium dose, 8 mg/kg; (c) high dose, 15 mg/kg. Coloured lines show the fitness obtained by parasites using the drug dose-specific optimal patterns of conversion (from Figure 2) in the face of drug treatment and indicate that parasites can recover some of the fitness lost due to drug treatment by altering patterns of conversion. Grey bars denote the days of drug treatment

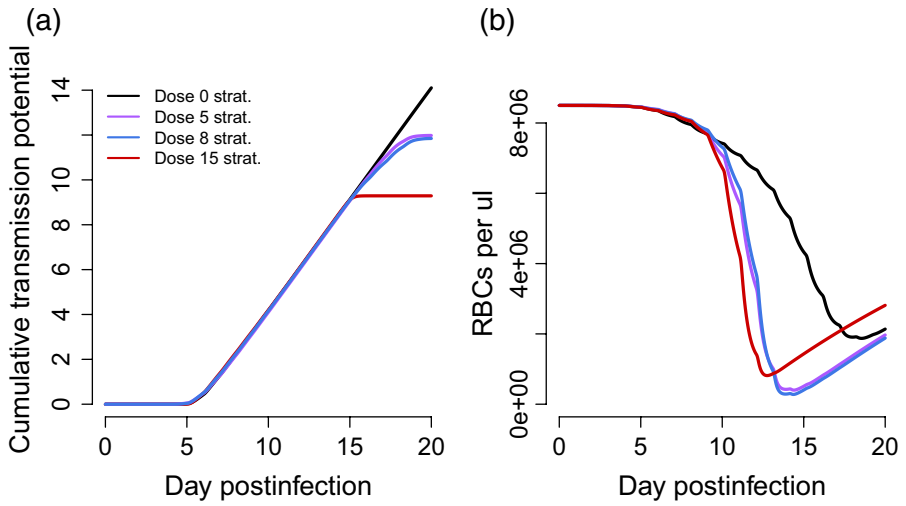


**FIGURE 5** Consequences of parasite adaptation to drug-treated infections. (a) The cumulative transmission potential in untreated infections where parasites employ different conversion rate strategies. Reproductive restraint in untreated infections produces only small transmission costs (purple and blue line) compared to strategies for untreated infections (black line), whereas terminating an infection early has bigger fitness consequences (red line). (b) The dynamics of uninfected red blood cells in those infections. Simulations assume optimal strategies for untreated infections (black), infections treated with a low dose (purple), medium dose (blue) and high dose (red). The reproductive restraint predicted for drug-adapted strategies leads to earlier declines in RBCs and lower minimum values (i.e., greater anaemia) when infections are not drug-treated

strategies. Advances in understanding the molecular pathways associated with commitment to gametocytogenesis (e.g., Brancucci, Goldowitz, Buchholz, Werling, & Marti, 2015) may bring such tools for experimental manipulation into reach.

Recent work has focused on dormancy as another nonclassical resistance mechanism thought to be employed by malaria parasites (e.g., Codd, Teuscher, Kyle, Cheng, & Gatton, 2011; Hott et al., 2015; Paloque, Ramadani, Mercereau-Puijalon, Augereau, & Benoit-Vical, 2016; Teuscher et al., 2010). This delayed development confers protection against the effects of fast-acting drugs that decay rapidly within a host, but whether such a strategy would be beneficial against drugs with longer half-lives is unclear. Parasites can stall their intra-erythrocytic development for many days, but only a small fraction—less than two per cent—appear to successfully recover and resume development even at low drug doses (Teuscher et al., 2010). It is not clear that such a low percentage of parasites entering dormancy can explain malaria dynamics in patients (Saralamba et al., 2011). Further, the fitness consequences of dormancy are not intuitive: surviving the effects of drugs is clearly good from the parasite's perspective, but stalling development means stalling production of transmission

stages and missing out on any transmission opportunities during the dormant phase. In contrast, parasites can recover substantially more than two per cent of their numbers by modifying transmission investment under some treatment regimes. Indeed, Figure 3 suggests that parasite densities can actually increase by an order of magnitude or more within less than 4 days and this modified life history translates to fitness gains (Figure 4). It is interesting to consider how these two mechanisms of nonclassical resistance would affect host health. At least in the short term, dormancy should reduce pathology associated with parasite replication as well as immunopathology, while reduced investment in transmission is likely to do the opposite.

We have shown that, in principle, altered life history can protect against the effects of drugs, and while we have used a model of drug action that was parameterized for a particular drug (pyrimethamine; Huijben et al., 2013), the phenomenological description we employ should capture the effects of many different drugs. Although there will be differences among individual hosts in drug metabolism that would affect, for example, the duration of drug action, our exploration of a range of drug doses should capture much of this variation. One exception to this generality is drugs that directly target gametocytes (e.g., primaquine, White, Ashley, et al., 2014). The relative susceptibility of asexuals and gametocytes to the drug will alter the costs and benefits of producing each stage, so different drugs may be expected to have different effects on optimal patterns of transmission investment. For example, a drug with a strong gametocidal effect may generate an advantage to reproductive restraint when drugs are present but promote the production of surplus gametocytes to compensate for those killed by drugs when drugs have cleared or may promote earlier production of gametocytes to compensate for lost transmission opportunities during drug treatment. Predicting evolutionary trajectories in response to such drugs will require precise calibration of the relative susceptibility of different parasite stages.

We have also ignored within-host competition and thus evolution operating at the within-host scale, but where malaria is endemic, multigenotype infections are the rule rather than the exception (e.g. Baruah, Lourembam, Sawian, Baruah, & Goswami, 2009; Juliano et al., 2010). Previous theoretical and experimental work shows that competition favours reproductive restraint (Greischar et al., 2016a; Greischar, Reece, et al., 2016; McKenzie & Bossert, 1998; Mideo & Day, 2008; Pollitt et al., 2011), so it is possible that our prediction of that same response in the face of drug treatment would remain unchanged. However, just as there is genetic variation for competitive ability (Bell, De Roode, Sim, Read, & Koella, 2006; de Roode, Helinski, Anwar, & Read, 2005; de Roode et al., 2005), there is likely to be genetic variation in sensitivity to drugs (and in *P. falciparum*, differentially sensitive genotypes may often share a host; e.g., Mideo et al. (2016)). If variation in drug sensitivity is unrelated to transmission investment, then it would alter the costs and benefits to different parasite genotypes of altering that investment. Modelling the dynamic consequences of competition and the interplay between different sources of resistance on the evolution of parasite life history would be an interesting route for future investigation. Importantly, there may also be genetic variation in the shape of the relationship between within-host gametocyte densities and the probability of transmission to mosquitos. As far as we are aware, this relationship has been quantified only a few times and only for a few distinct strains (Bell et al., 2012; Huijben et al., 2010; Paul, Bonnet, Boudin, Tchuinkam, & Robert, 2007). While the qualitative shapes of these relationships remain the same, there are quantitative differences in their parametrization. We found that these differences did not alter our predictions (see Figure A.3 in Appendix A), but further empirical exploration of this relationship is warranted, as is theoretical investigation of how any quantitative changes in this relationship alter evolutionary predictions.

While our model allows for variation across infections treated with different drug regimes and variation over time within infections, our heuristic analysis also constrains variation at both of these scales. First, to determine when evolution should favour a drug-adapted strategy, we assumed that there were only two strategies available to parasites: the pattern of transmission investment predicted to be best in an untreated host or the one predicted to be best in the presence of a particular drug dose. In a heterogeneous host population, some intermediate parasite investment strategy may perform better than either of these two "extremes". Second, our model does not allow for parasites to directly receive and respond to cues within infections; that is, it is not a model of plasticity. Put another way, the model implicitly assumes that parasites have perfect knowledge about the timing of drug treatment (which does not vary across treated hosts) and optimal patterns of investment may allow parasites to, in effect, prepare in advance for drug treatment. This scenario may not be too far from reality in some areas. Drug doses are standardized by WHO guidelines (WHO 2015b), and hosts likely seek treatment when symptoms appear, which generally correlates with peak parasite density (Kachur et al., 2006), although there will be variation across individual hosts in the timing of early dynamics. How much fitness could be gained by allowing parasites in our model to detect and respond to drug treatment more directly is unclear, as our results suggest that differences in investment early in infections (and, in particular, before drug treatment) have little effect on parasite fitness. Consistent with this, Greischar et al. (2016a) found that investing little in transmission at the beginning of infections is adaptive in untreated hosts, regardless of other changes to the within-host environment. Thus, it seems unlikely that allowing parasites more flexibility in pretreatment patterns of investment would result in different life history strategies than we have predicted. On the other hand, if parasites could respond plastically to the presence of drugs in the within-host environment (instead of through evolutionary change, as we have focused on), then this would avoid the negative consequences for host health we report.

The evolution of classical resistance is the expected result of using chemical interventions to kill parasites (or, in evolutionary terms, reduce their fitness), but, as we have shown, failing to consider the potential for nonclassical resistance, like life history evolution, can yield overly optimistic predictions about the epidemiological or clinical effects of those interventions. Similarly, Lynch, Grimm, and Read (2008) used models to investigate the influence of different antihelminth interventions on nematode life history, finding that disease control programs may frequently select for increasingly fecund worms, with ramifications for clinical outcomes and onward transmission. In an experimental system, filarial nematodes altered their reproductive schedules in the

presence of specialized immune cells, producing transmissible stages faster and in greater numbers (Babayan, Read, Lawrence, Bain, & Allen, 2010). As these are the same immune cells on which current experimental vaccines rely, this work suggests that nematodes could reduce the benefits of vaccination through plasticity in life history. Further, the mosquitoes that transmit malaria and other diseases can also respond to intervention efforts with nonclassical resistance, including, for example, changes in feeding behaviour or timing to avoid insecticide-treated bednets (Gatton et al., 2013; Sokhna, Ndiath, & Rogier, 2013).

An important question is how treatment recommendations would change in the light of our predictions about optimal malaria parasite life histories. Regardless of the life history shifts we predict here, parasite fitness and within-host densities are reduced by drug treatment. This suggests that despite the evolution of nonclassical resistance, drug treatment offers epidemiological and clinical benefits. Those benefits are not as great as they would be in the absence of life history evolution and, importantly, any hosts that remained untreated could be worse off if drug-adapted strategies became fixed in the parasite population. Further, as a result of altered patterns of transmission investment, parasites could maintain higher within-host densities in the face of drug treatment, potentially facilitating the evolution of classical resistance. The theory developed here provides a basis for assessing the constraints and limits on parasite life history evolution in response to human interventions.

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## APPENDIX A

### Supplementary figures

Huijben et al. (2013) parameterised a model for the action of pyrimethamine against *Plasmodium chabaudi* in mice, finding that the dose of drugs affected the duration of drug action. We show this relationship (i.e., solutions to Equation 9 of the main text) in Figure A.1. A schematic of the full drug action model is presented in Figure A.2. In Figure A.3, we explore the effects of using a different fitness function on the predicted optimal patterns of investment in the absence of drug treatment and with a medium dose drug treatment. Finally, Figure A.4 shows the fitnesses achieved by different strategies in different environments (i.e., untreated or treated hosts). In each case, the optimal strategy predicted for a given environment outperforms the predicted optimal strategies for other environments.

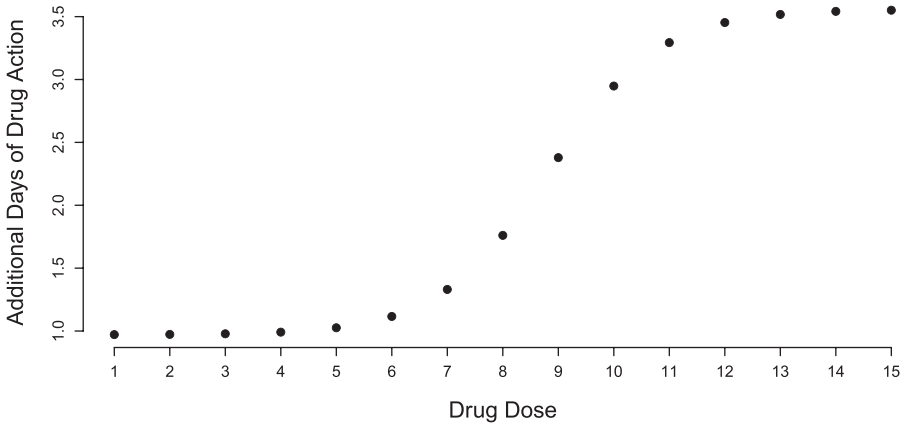


**FIGURE A.1.** Drug dose affects duration of drug-related parasite killing, but not the rate at which parasites are killed. Shown are the additional days of drug action, beyond the days when drugs are administered, when drugs are predicted to still be "active" (as defined in Huijben et al. 2013). Drug dose is expressed in mg/kg.

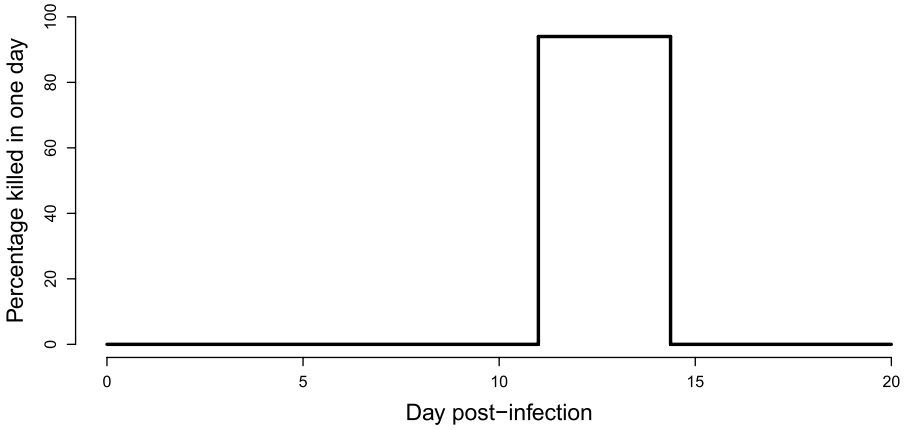


**FIGURE A.2.** Schematic of drug action in our model, a stylized version of how pyrimethamine acts against *P.chabaudi.* In this example, drug treatment is composed of two doses of 9 mg/kg, administered on day 11 and 12. The last dose determines how long the drugs will persist in the host after treatment, here an additional ~2.4 days of drug action. Before and after drug action, drug-related killing is zero.

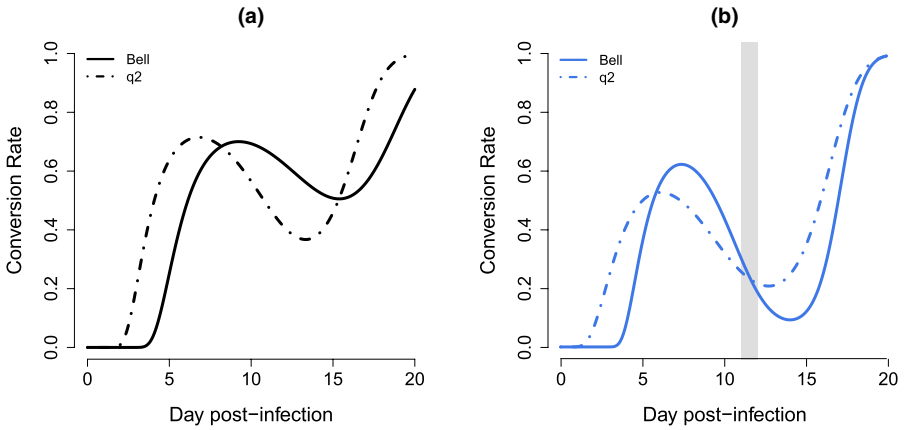


**FIGURE A.3.** The optimal pattern of conversion over the course of infections, using equation "q2" in Huijben et al. (2010), rather than equation 11 of the main text to define fitness. (a) The black line shows the predicted best response in an untreated infection for the q2 fitness equation and the fitness equation proposed by Bell et al. (2012), used in this paper and marked "Bell". (b) When infections are treated with a moderate drug dose (blue line, 8 mg/kg), parasites do better by reducing conversion, for both fitness functions. Drugs are administered on the days denoted by the grey bar.

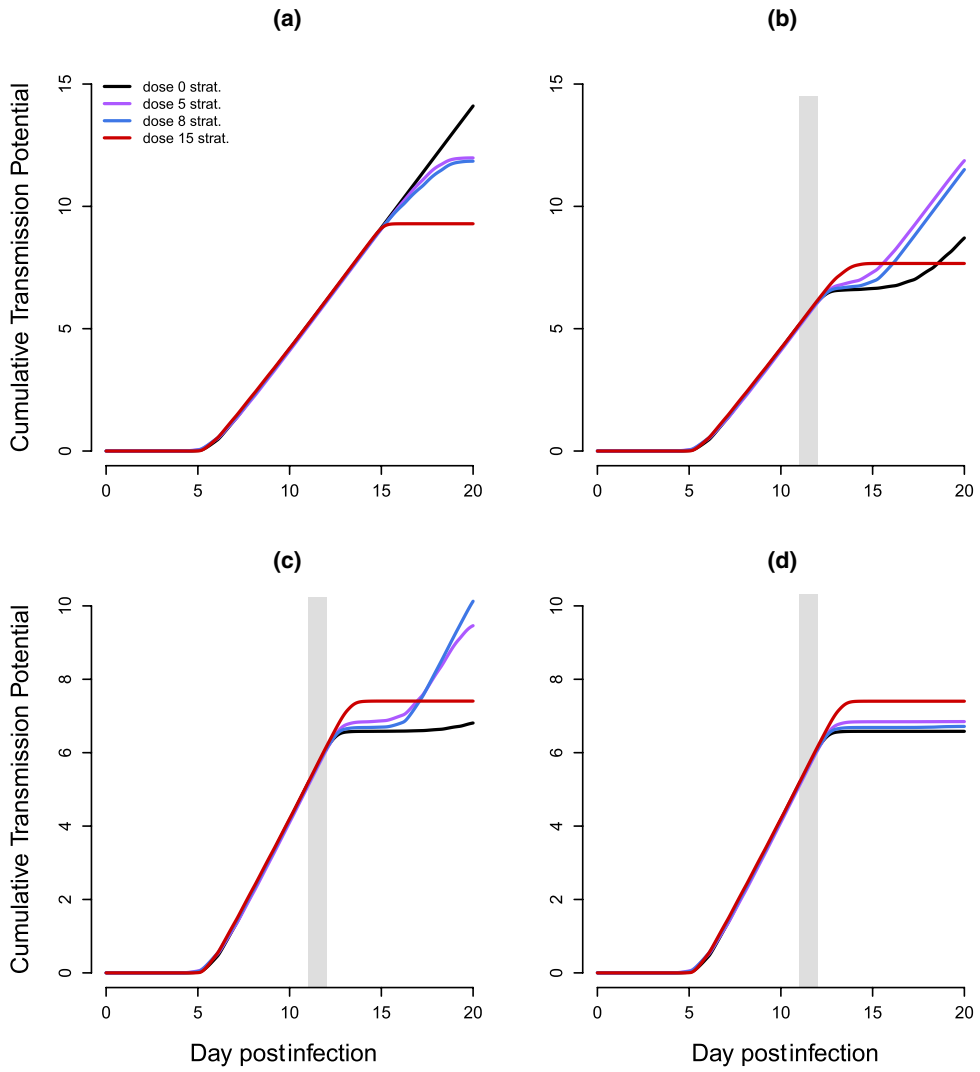


**FIGURE A.4.** The cumulative transmission potential of different drug-adapted strategies in untreated hosts (a), hosts treated with 5 mg/kg of drugs (b), 8 mg/kg (c), and 15 mg/kg (d). For each drug treatment, the putative optimal strategy against that dose outperforms the putative optimal strategies from other doses. Grey bars denote the days of drug treatment.

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## APPENDIX B

### Fitness calculations

Imagine the following set of fitnesses for a non-drug-adapted and a drug-adapted pattern of transmission investment (first subscript 0 or D, respectively) of malaria parasites in untreated and treated host (second subscript 0 or D, respectively).

$$\begin{aligned} w_{0,0} &= a \\ w_{0,D} &= a-d \\ w_{D,0} &= a-c \\ w_{D,D} &= a-d+b \end{aligned} \tag{B.1}$$

where d is the reduction in fitness of the non-drug-adapted strain due to drug treatment (i.e., the drug effect), c is the reduced fitness of the drug-adapted strain in an untreated host (i.e., cost to "resistance"), and b is the increase in fitness achieved by the drug-adapted strain in the presence of drugs (i.e., the benefit of "resistance").

We can write the expected fitness of the two different strategies in a host population, where a proportion, f, of hosts receive drug treatment:

$$\begin{aligned} E[w_0] &= fw_{0,D} + (1-f)w_{0,0} \\ E[w_D] &= fw_{0,D} + (1-f)w_{D,0} \end{aligned} \tag{B.2}$$

**TABLE B.1.** Estimated fitness values (i.e., cumulative transmission potential) for different transmission investment strategies in different host environments, as predicted by the model presented in the main text

| | Environment (drug dose) | | | |
|---|---|---|---|---|
| Strategy | 0 | 5 | 8 | 15 |
| 0 | 14.1 | 8.7 | 6.8 | 6.6 |
| 5 | 11.98 | 11.8 | | |
| 8 | 11.84 | | 10.1 | |
| 15 | 9.28 | | | 7.4 |

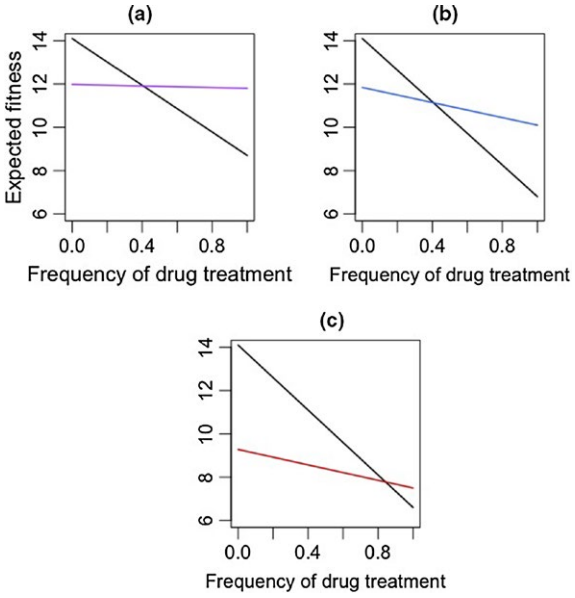


**FIGURE B.1.** Expected fitness for different transmission investment strategies in a host population treated with a particular drug dose (A: low; B: medium; C: high) at a given frequency. Lines show the weighted average of fitness achieved in untreated and treated infections (i.e., solutions to Equations B.2). Black lines represent the transmission investment strategy predicted to be best in the absence of drug treatment (the "non-drug adapted" strategy); coloured lines represent the transmission investment strategy predicted to be best in the face of a low drug dose (purple), medium drug dose (blue) or high drug dose (red).

**TABLE B.2.** Calculated benefits, b, and costs, c, of drug-adapted strategies

| | Effects of 'resistance' | |
|---|---|---|
| Strategy | b | c |
| 5 | 3.1 | 2.12 |
| 8 | 3.3 | 2.26 |
| 15 | 0.8 | 4.82 |

Substituting the fitness expressions from B.1 into B.2 and rearranging, we find that the drug-adapted strategy has a higher fitness when

$$f > \frac{c}{c+b} \tag{B.3}$$

Put another way, the drug-adapted strategy will be favoured when the ratio of the benefits to costs of the strategy is greater than the relative frequency of encountering an untreated host:

$$\frac{b}{c} > \frac{1-f}{f} \tag{B.4}$$

In Table B.1 we list the cumulative transmission potential (as predicted by our model), over a 20-day simulated infection, for each of the predicted drug-adapted strategies, in the presence and absence of drug treatment, as well as the non-drug-adapted strategy in each of these environments. From these values we can plot the expected fitness of different strategies (i.e., solutions to Equations B.2) over different values of f (Figure B.1). We see that over a range of f values, the non-drug adapted strategies performs better on average than the drug adapted strategy, for all drug doses, but above a given f value, the drug-adapted strategy will be favoured. From the fitness values, we can also calculate b and c for each of the drug-adapted strategies (Table B.2). Plugging these costs and benefits into Equation B.3, gives rise to the frequencies of drug treatment required to favour the drug-adapted over the non-drug adapted strategies reported in the main text (i.e., the intersection of the lines in Figure B.1).

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ORIGINAL RESEARCH ARTICLE


# A genetic model of the effects of insecticide-treated bed nets on the evolution of insecticide-resistance

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ABSTRACT

**Background and objectives**: The evolution of insecticide-resistance in malaria vectors is emerging as a serious challenge for the control of malaria. Modelling the spread of insecticide-resistance is an essential tool to understand the evolutionary pressures and dynamics caused by the application of insecticides.

**Methodology**: We developed a population-genetic model of the spread of insecticide-resistance in a population of *Anopheles* vectors in response to insecticides used either as adulticides (focussing on insecticide-treated bed nets (ITNs)) or as larvicides (either for the control of malaria or, as an inadvertent side-product, in agriculture).

**Results**: We show that indoor use of insecticides leads to considerably less selection pressure than their use as larvicides, supporting the idea that most resistance of malaria vectors is due to the agricultural use of the insecticides that are also used for malaria control. The reasons for the relatively low selection pressure posed by adulticides are (i) that males are not affected by the ITNs and, in particular, (ii) that the insecticides are also repellents, keeping mosquitoes at bay from contacting the insecticide but also driving them to bite either people who do not use the insecticide or alternative hosts.

**Conclusion**: We conclude by discussing the opposing public health benefits of high repellency at an epidemiological and an evolutionary timescale: whereas repellency is beneficial to delay the evolution of resistance, other models have shown that it decreases the population-level protection of the insecticide.

KEYWORDS: malaria control; insecticide-treated bed nets; repellency; insecticide-resistance

## INTRODUCTION

Long-lasting insecticidal nets and indoor residual spraying (IRS) have dramatically reduced malaria transmission, for they protect users from being bitten by the mosquito vectors of malaria [1–4] and, by decreasing the longevity of mosquitoes, offer additional protection at the level of the community [5, 6]. Unfortunately this success is being eroded by the evolution of various mechanisms of resistance, including behavioural resistance (e.g. failure to be repelled or shifting from indoor-biting to outdoor-biting) [7, 8] and the focus of this article: insecticide-resistance (IR) rendering mosquitoes less sensitive to the insecticide used on the insecticide-treated bed nets (ITNs) [9, 10].

It is clear that the extensive use of insecticides for the control of malaria will increase the selection pressure on mosquitoes to evolve resistance. The problem is exacerbated by the fact that, after the sharp drop of the use of dichlorodiphenyltrichloroethane, vector control has become very reliant on a single class of insecticides, pyrethroids, which are also extensively used in other contexts, in particular agriculture. Widespread exposure of mosquitoes to agriculturally used insecticides, rather than exposure to ITNs, is indeed thought to be one of the main driving factors for the evolution of resistance [11] and therefore helps to undermine the efficiency of insecticide-based control measures. However, there is only limited understanding of the contribution of epidemiological, ecological and behavioural forces to the evolutionary dynamics of IR of malaria vectors.

A number of models have been developed to consider the role of these and other factors on the evolution of resistance. Barbosa and Hastings [12], e.g. use a population-genetic model to describe the rate of evolution when coverage of the bed nets is patchy, and predict the effect of using a chemical synergist to delay resistance. Extensions of similar approaches include age-specific effects of the insecticide to compare the effects of insecticides that are late-acting with those that lead to immediate death and to predict which mosquito life stage should be targeted [13–15].

A critical feature of these models is the 'fitness' of sensitive and resistant mosquitoes, which is described in various ways. The simplest one, e.g. Gourley *et al*. [13] assume that the insecticide increases the death rate of sensitive mosquitoes, but not of resistant ones, by a constant factor. Barbosa and Hastings [12] use a more complex formulation by including the proportion of houses that are covered by the bed nets. However, models that make mosquito fitness dependent on its behaviour and life-history provide significant advantages over others as they allow integration of knowledge of medical entomologists with the population genetics of the model. This approach has been followed by a number of authors [14–15].

For example, Koella *et al*. [16] combined a population-genetic approach with aspects of the mosquito's feeding cycle to calculate 'effective coverage', the proportion of mosquitoes killed by the insecticide during a single gonotrophic cycle. We extended this approach by formulating a population-genetic model that calculates exposure rates from the mosquito's feeding cycle similarly to the model described by Le Menach *et al*. [17]. In doing so, we propose behaviourally and epidemiologically based fitness functions that help us to understand more fully the predictions of the genetic model.

Our aim was to predict at least qualitatively the rate of evolution of IR under different transmission settings and under different characteristics of insecticide deployment and ITN interventions. We are in particular interested in (i) the relative selection pressures imposed by agriculturally used insecticides and ITNs and (ii) the effects of repellency and the tendency of mosquitoes to feed on non-human animals on the evolution of resistance. This model could hence contribute to inform recent campaigns that rely on the mass deployment of ITNs like the Roll Back Malaria initiative [18].

## METHODS

We assumed that IR is determined by a single gene with two alleles *R* and *S*, giving rise to three different genotypes: homozygote resistant individuals *RR*, homozygote sensitives *SS* and heterozygotes *RS*. We calculated the fitness of each genotype as its lifetime reproductive success, and used these in a standard population-genetic approach to predict the rate of change of the allele frequencies. We further assumed that insecticides can be used as adulticides on insecticide-treated nets and as larvicides, either in a direct attempt to control mosquitoes or as an inadvertent consequence of their agricultural use.

### Larvae

The survival of larvae is determined by the presence of the larvicide in a proportion $\psi$ of the larval sites

and by their resistance to the insecticide. We assume that sensitive mosquitoes are invariably killed by the insecticide, that mortality is reduced by the resistance $\rho$ in *RR* individuals and that the resistance of heterozygous individuals is the product of $\rho$ and the level of dominance $h$. If we standardize the model by assuming that all larvae in unexposed sites survive, the survival of sensitive individuals is $1-\psi$, that of homozygously resistant individuals is $1-\psi(1-\rho)$ and that of heterozygous individuals is $1-\psi(1-h\rho)$.

## Males

We assume that males never encounter the ITNs, so their fitness is determined only by larval survival and by a potential cost of resistance in fertility, $Z$. One potential mechanism of a male fertility cost could be via decreased competitiveness of resistant males for access to females [19]. We assume that dominance affects the cost of resistance identically to survival, so that the cost in heterozygotes is $h\,Z$. Thus the fitness of sensitive males is $1-\psi$, that of resistant individuals is $(1-\psi(1-\rho))(1-Z)$ and that of heterozygous individuals is $(1-\psi(1-h\rho))(1-hZ)$. (Table 1 for a summary of fitness measures).

## Females

We start by considering insecticide-sensitive mosquitoes. Once females have survived the insecticides in the larval sites to emerge as adults, their reproductive success is determined by the likelihood that they contact the insecticide on ITNs during their feeding attempts. To estimate this exposure rate, we modelled a mosquito feeding cycle as described in detail in [17] and reiterated in Fig. 1. Note that in this article, we are not interested in behavioural resistance, so that, in contrast to earlier articles, we ignore the possibility of outdoor feeding on humans. Although outdoor feeding would of course reduce the selection pressure for resistance it will not affect the qualitative conclusions of our model, providing that outdoor feeding has not directly evolved in response to the presence of ITNs.

We assume that at each feeding attempt, a proportion $1-Q$ of the mosquitoes feeds on an animal. A proportion $Q$ of the mosquitoes attempt to enter a house to feed on a human. If the mosquito encounters a protected house (with probability $\phi$), it is repelled (or mechanically blocked by the net) and starts a new host search with probability $r$. If it is not repelled (with probability $1-r$), it survives the exposure to the insecticide with probability $s$ and feeds successfully on the human host. We assume that each bite, be it on humans or animals, carries some risk of feeding-associated death, which the mosquito survives with a probability $\sigma$.

Overall, the probability that the mosquito obtains a blood meal and survives (i.e. is successful) during a single feeding attempt is

$$\begin{aligned}&\sigma(1-Q)+\sigma Q(1-\phi)\\&+\sigma Q\phi(1-r)s=\sigma(1-Q\phi(1-(1-r)s))\end{aligned}\tag{1}$$

Let us call this term $\Xi$. If the mosquito does not obtain a blood meal, it will start a new feeding attempt and repeat this until it is successful or dies. The mosquito is therefore successful, if it succeeds on its first attempt, or it is repelled once and succeeds on its second attempt, or it is repelled twice and then succeeds on its third attempt, etc. We assume that each time the mosquito is repelled and attempts to feed again, it will encounter an additional risk of death $\mu_r$. We can then calculate the probability of success as the geometric series:

$$\begin{aligned}&\Xi+Q\phi r(1-\mu_r)\Xi+(Q\phi r(1-\mu_r))^2\Xi+\ldots\\&\quad+(Q\phi r(1-\mu_r))^n\Xi+\ldots\\&\quad=\sigma(1-Q\phi(1-(1-r)s))\sum_{n=0}^{\infty}(Q\phi r(1-\mu_r))^n\\&\quad=\frac{\sigma(1-Q\phi(1-(1-r)s))}{1-Q\phi r(1-\mu_r)}\end{aligned}\tag{2}$$

Once fed, the mosquito must survive through the duration of its gonotrophic cycle (i.e. the time it takes to develop and lay its eggs) before it starts a new feeding attempt. The probability of feeding-independent mortality during the gonotrophic cycle is $\mu_{gt}=1-(1-\mu)^{gt}$, where $\mu$ is the daily mortality and $gt$ is the length of the gonotrophic cycle. Note that, in contrast to [17], we assume that the length of the gonotrophic cycle is not modified by repeated host searches. This is a good approximation unless each feeding attempt lasts a long time. The latter could happen, e.g. if a mosquito has to travel large distances between potential hosts or if ITN coverage is close to 100%. This can be understood by considering a situation of high host density; here, the search for a new host may last only for a few minutes. Then, even if the mosquito is repelled 10 times (e.g. under conditions of say 95% coverage and 9% repellency), the gonotrophic cycle length will at most

**Table 1.** Fitnesses of male and female genotypes

| Genotype | Males | Females |
|---|---|---|
| RR | $W_{m,RR} = (1-\psi(1-\rho))(1-Z)$ | $W_{f,RR} = \frac{(1-\psi(1-\rho))\kappa(1-Z)}{1-(1-\mu_{gt})\frac{\sigma(1-Q\phi(1-(1-r)(s+\rho(1-s))))}{1-Q\phi r(1-\mu_r)}}$ |
| RS | $W_{m,RS} = (1-\psi(1-h\rho))(1-hZ)$ | $W_{f,RS} = \frac{(1-\psi(1-h\rho))\kappa(1-hZ)}{1-(1-\mu_{gt})\frac{\sigma(1-Q\phi(1-(1-r)(s+h\rho(1-s))))}{1-Q\phi r(1-\mu_r)}}$ |
| SS | $W_{m,SS} = (1-\ \psi)$ | $W_{f,SS} = \frac{(1-\psi)\kappa}{1-(1-\mu_{gt})\frac{\sigma(1-Q\phi(1-(1-r)s))}{1-Q\phi r(1-\mu_r)}}$ |

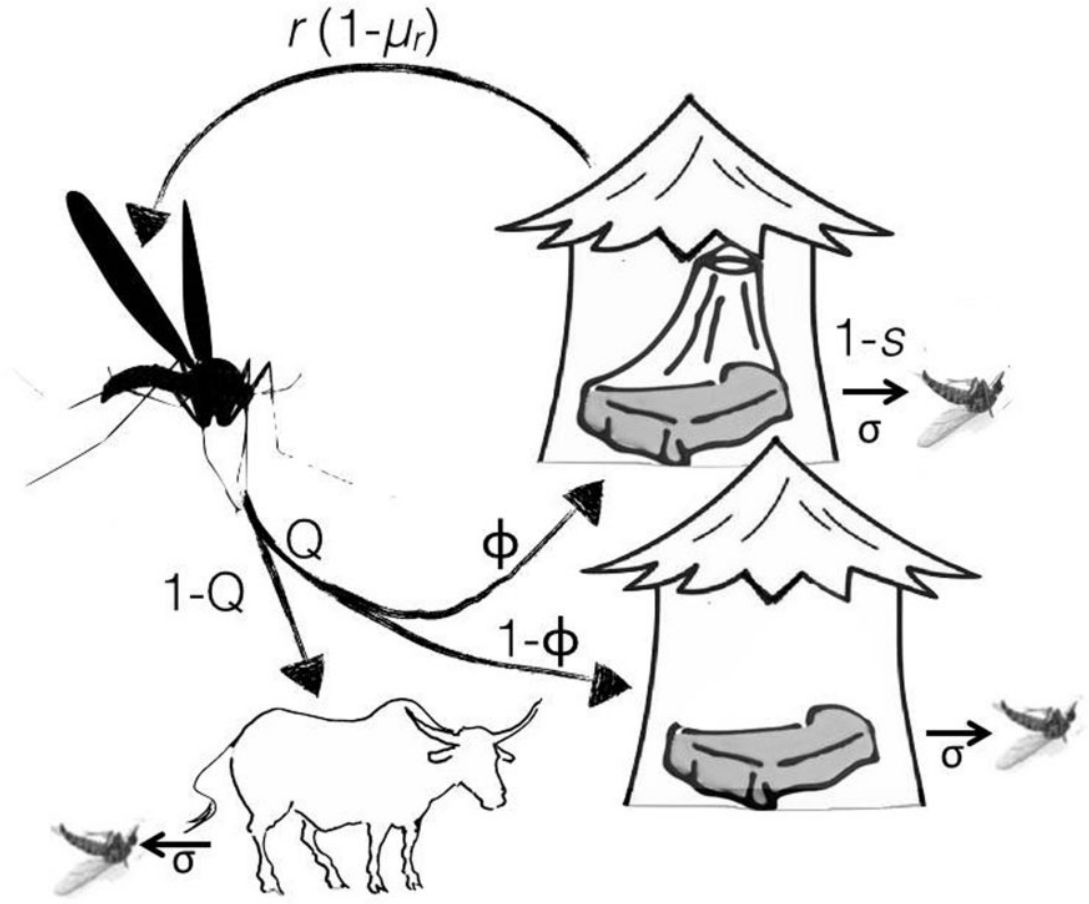


**Figure 1.** Host searching cycle of a mosquito: A mosquito bites an animal with probability $1-Q$, while a proportion $Q$ of the mosquitoes attempts to bite a human host inside houses, of which a proportion $\phi$ is protected by ITNs. We assume that mosquitoes survive feeding-associated death, same in humans and animals, with a probability $\sigma$. If mosquitoes target a protected house, there are three possible outcomes: the mosquito is repelled by the insecticide (or mechanically blocked by the net) with a probability $r$, or, if not repelled, it feeds and then escapes the risks of insecticide-associated death with probability $s$

increase by a few percent. The probability of surviving a gonotrophic cycle (the combination of feeding-related and feeding-independent mortality) is hence:

$$(1-\mu_{gt})\frac{\sigma(1-Q\phi(1-(1-r)s))}{1-Q\phi r(1-\mu_r)} \qquad (3)$$

giving an average lifespan (in multiples of the gonotrophic cycle) of:

$$\frac{1}{1-(1-\mu_{gt})\frac{\sigma(1-Q\phi(1-(1-r)s))}{1-Q\phi r(1-\mu_r)}} \qquad (4)$$

IR affects the probability that a mosquito survives blood feeding once it has entered a house. The

parameter $s$ in equation 4 is the probability that sensitive mosquitoes survive exposure to the insecticide. In homozygously resistant mosquitoes, the sensitivity to the insecticide is reduced by the parameter $\rho$, so that the probability of being killed by the insecticide is reduced to $(1-s)(1-\rho)$ and the probability of success inside an ITN-home is $(s+\rho(1-s))$; the probability that heterozygous mosquitoes succeed is $(s+h\rho(1-s))$.

Using these survival terms in equation 2, we obtained the average longevity of each genotype of adult female mosquitoes:

$$\text{Lifespan } (SS) = \frac{1}{1-(1-\mu_{gt})^{\frac{\sigma(1-Q\phi(1-(1-r)s))}{1-Q\phi r(1-\mu_r)}}} \quad (5)$$

$$\text{Lifespan } (RS) = \frac{1}{1-(1-\mu_{gt})^{\frac{\sigma(1-Q\phi(1-(1-r)(s+h\rho(1-s))))}{1-Q\phi r(1-\mu_r)}}} \quad (6)$$

$$\text{Lifespan } (RR) = \frac{1}{1-(1-\mu_{gt})^{\frac{\sigma(1-Q\phi(1-(1-r)(s+\rho(1-s))))}{1-Q\phi r(1-\mu_r)}}} \quad (7)$$

The fitness of each genotype is obtained by multiplying this quantity by a typical value of female mosquito fertility, $\kappa$, and by the probability that larvae survive the insecticide applied to larval sites, $1-\psi$. Larval mortality is affected by resistance according to the equations given earlier for the mortality of males. We finally assume that resistance is costly in that the fecundity of homozygous resistant mosquitoes is reduced by the factor $Z$ and that of heterozygotes is reduced by $hZ$ (note that for simplicity, we assume that the cost of resistance is equal for males and for females). This gives the fitness values of males and females, shown in Table 1.

### Evolution

Designating the frequencies of the resistance allele in males and in females by $p_m$ and $p_f$, respectively, and the frequencies of the susceptibility allele by $q_m = 1-p_m$ and $q_f = 1-p_f$, the genotype frequencies in males and females after selection are given by the following equations [20]:

$$SS_m = \frac{W_{m,SS}q_m q_f}{\overline{W}_m}$$

$$RS_m = \frac{W_{m,RS}(p_m q_f + p_f q_m)}{\overline{W}_m}$$

$$RR_m = \frac{W_{m,RR}p_m p_f}{\overline{W}_m} \quad (8)$$

$$SS_f = \frac{W_{f,SS}q_m q_f}{\overline{W}_f}$$

$$RS_f = \frac{W_{f,RS}(p_m q_f + p_f q_m)}{\overline{W}_f}$$

$$RR_f = \frac{W_{f,RR}p_m p_f}{\overline{W}_f}$$

where $\overline{W}_M$ and $\overline{W}_F$ are the mean fitnesses of males and females in the population and are given by:

$$\overline{W}_m = W_{m,RR}p_m p_f + W_{m,RS}(p_m q_f + p_f q_m) + W_{m,SS}q_m q_f \quad (9)$$

$$\overline{W}_f = W_{f,RR}p_m p_f + W_{f,RS}(p_m q_f + p_f q_m) + W_{f,SS}q_m q_f \quad (10)$$

We assume discrete and non-overlapping mosquito generations. Consequently, the frequencies of the resistance allele in males and females from one mosquito (parental) generation, $(t)$, to the next (offspring) generation, $(t+1)$, are:

$$p_m(t+1) = \frac{W_{m,RR}p_m(t)p_f(t) + 0.5W_{m,RS}(p_m(t)q_f(t) + p_f(t)q_m(t))}{\overline{W}_m} \quad (11)$$

$$p_f(t+1) = \frac{W_{f,RR}p_f(t)p_m(t) + 0.5W_{f,RS}(p_f(t)q_m(t) + p_m(t)q_f(t))}{\overline{W}_f} \quad (12)$$

The parameters of our model, together with their typical values, are listed in Table 2.

## RESULTS

Our model always leads to either fixation or elimination of the resistance allele. We therefore show two types of results, obtained from simulations: (i) the conditions that lead to fixation of the allele (Fig. 2) and (ii), for conditions that enable fixation of resistance, the number of generations it takes for the allele to reach a frequency of 50% (Fig. 3).

We considered two pressures selecting for IR: ITNs, to which only adult females are exposed, and larvicides, which affect larvae of both sexes. Figure 2 shows that the selection pressure imposed by ITNs is considerably weaker than that imposed by the

**Table 2.** Parameters and their typical values

| Parameter | Explanation | Typical value | Reference |
|---|---|---|---|
| $\phi$ | ITN coverage | | |
| $\Psi$ | proportion of mosquitoes exposed to agriculturally used insecticide | | |
| $Q$ | feeding rate on humans | 0.7 | [21] |
| $r$ | repellency rate | 0.7 | [22] |
| $s$ | probability of surviving ITN insecticide exposure | 0.16 | [22] |
| $\Sigma$ | survival of risk of feeding-induced death | 0.9 | |
| $gt$ | length of gonotrophic cycle (days) | 3 | [23] |
| $M$ | daily mortality rate of vector | 0.1 | [24] |
| $\mu_{gt}$ | mortality in one gonotrophic cycle | 0.27 | [24] |
| $\mu_r$ | additional mortality if repelled once | 0.03 | |
| $h$ | dominance of IR allele | 0.25 | [25] |
| $\rho$ | level of resistance conferred by IR allele | 0.95 | |
| $Z$ | cost of resistance | 0.10 | [26] |
| $\kappa$ | female fecundity | 100 | [27] |

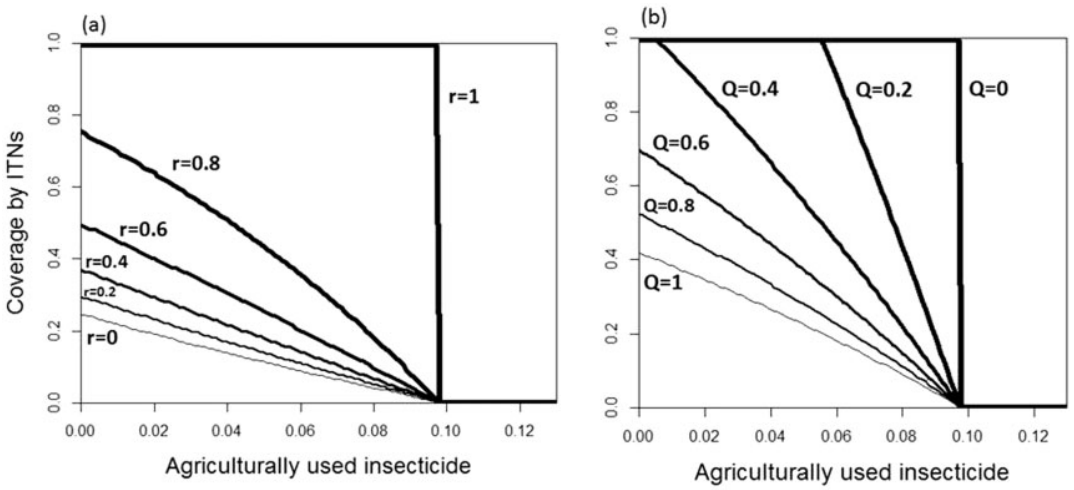


**Figure 2.** The combination of coverage by ITNs and by larvicides that enable resistance to be fixed (lines) or eliminated (below lines) for (**a**) repellency, $r$ ranging from 0 along the thin line to 1 along the thick line with an interval of 0.2 between adjacent lines and for (**b**) human feeding, $Q$, ranging from 1 along the thin line to 0 along the thick line. Other parameter values are given in Table 2

larvicides. Indeed, with the typical parameters given in Table 2, resistance is fixed as a response to only larvicides if more than 10% of the larval sites are treated with a lethal concentration of the insecticide, whereas if mosquitoes are exposed to ITNs only, resistance is fixed only if at least ~20% of the houses are treated with ITNs even in the extreme case of no repellency (Fig. 2a) and no animal-feeding (Fig. 2b). The selection pressure due to ITNs depends strongly on the repellency of the insecticide and the extent of animal-feeding by the mosquitoes. As repellency increases, more mosquitoes are diverted from the insecticide, so that it becomes less likely that resistance is fixed; if all mosquitoes are repelled, the insecticide kills no mosquitoes, so the ITNs impose no selection for resistance (Fig. 2a). Similarly, if mosquitoes are more likely to feed on animals, they are less exposed to the insecticide, so that the selection pressure decreases (Fig. 2b).

These results are reflected in simulations giving the time it takes for the resistance allele to reach a frequency of 50% (Fig. 3), starting at an initial gene

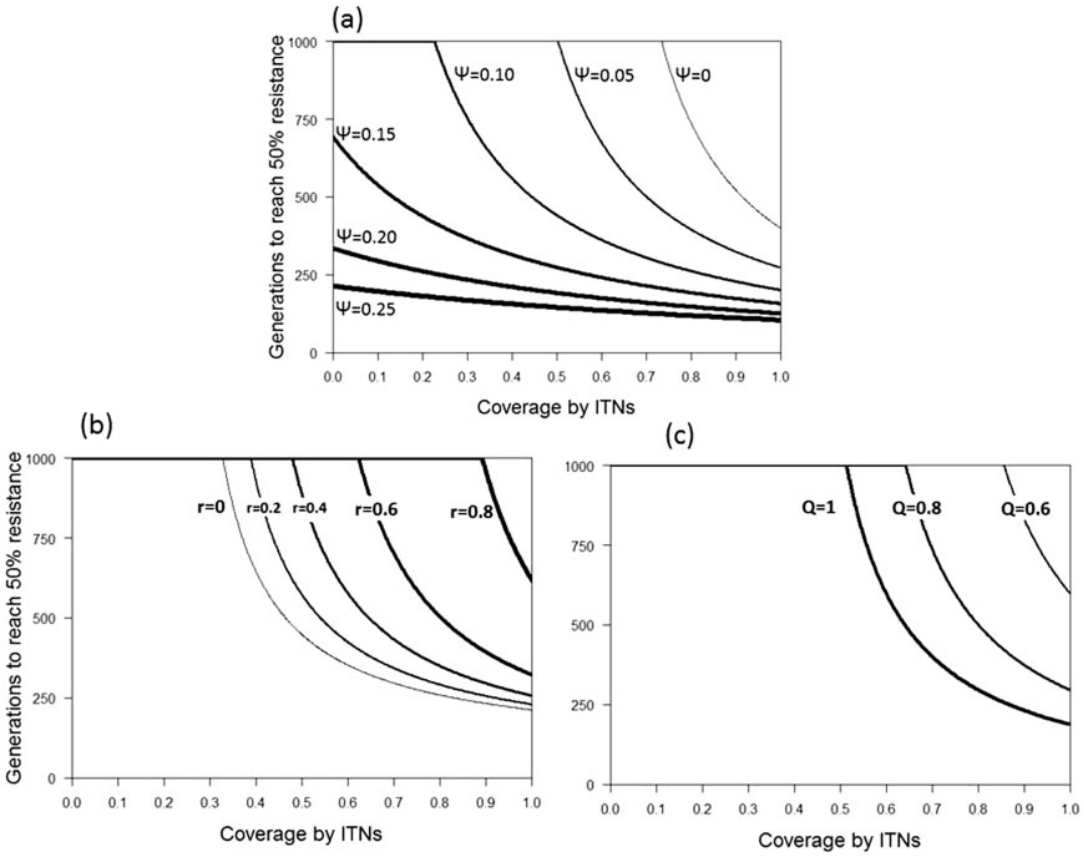


**Figure 3.** The rate of evolution of resistance against ITNs as a function of their coverage and for several parameter values. The rate of evolution is given as the time (in number of mosquito generations) it takes for a resistance allele to reach a frequency of 50%, starting with a frequency of 1/100000, (**a**) The role of a larvicide used simultaneously at a coverage $\psi$ ranging from 0 (thin line) to 25% (thick line). (**b**) The role of the repellency of the ITN, with repellency $r$ ranging from 0 (thin line) to 0.8 (thick line). (**c**) The role of the likelihood that mosquitoes feed on animals, with indoor human-feeding $Q$ ranging from 1 (thick line) to 0.6 (thin line). Other parameter values are given in Table 2

frequency of $p_f = p_m = 0.00001$. In the absence of larvicides, the time to evolve resistance decreases strongly with increasing coverage by ITNs (Fig. 3a). However, as the coverage of larvicides increases, the effect of coverage by ITNs on the time to evolve resistance diminishes. Indeed, at high coverage by the larvicides, the effect of ITNs is almost negligible, whereas even at complete coverage by ITNs increasing the use of larvicides substantially decreases the time to evolve resistance (Fig. 3a). The time to evolve resistance is also strongly increased by the repellency of the insecticide (Fig. 3b) and the likelihood that mosquitoes feed on animals (Fig. 3c).

The difference between the selection pressures posed by larvicides and by ITNs is seen most clearly in Fig. 4, which shows the ratio of the time it takes for the resistance allele to reach a frequency of 50% in situations where an insecticide is used only on an ITN or as a larvicide with the same level of coverage. Larvicides have the strongest effect on driving the evolution of IR with speeding up the evolution at least 8–10 times compared with a same coverage of ITNs alone. This effect however also depends strongly on the repellency of the net: higher levels of repellency slow significantly the evolution of resistance.

## DISCUSSION

Our model, which adds the behaviour of mosquitoes to population-genetic theory, shows that ITNs can lead to a substantial selection pressure for the evolution of IR. Yet, this selection pressure is weakened considerably by the repellent effects of the insecticide and, in some ecological settings, by the propensity of mosquitoes to feed on animals other than humans. Furthermore, the selection pressure

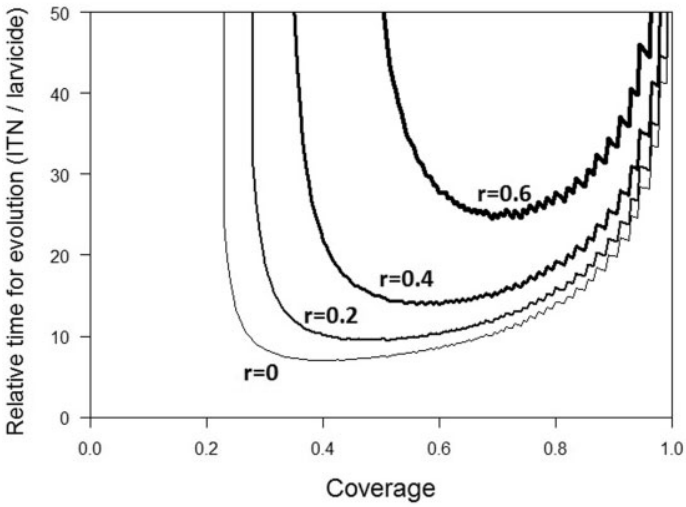


**Figure 4.** The comparison of the rate of evolution in situations where the insecticide is used only on ITNs or only as larvicides. The y-axis shows the ratio of the two times (in number of mosquito generations) it takes for a resistance allele to reach a frequency of 50%, starting with a frequency of 0.00001 in males and females; the x-axis shows the coverage by the insecticide in either situation. The lines show different levels of repellency, *r*, ranging from 0 (thin line) to 0.6 (thick line). Other parameter values are given in Table 2

imposed by larvicides is considerably stronger than that imposed by ITNs.

With the typical parameters (Table 2), we found in reviews of field studies (e.g. 70% repellency and 70% human-feeding), our model predicts that for intermediate to high coverage by ITNs it takes ~200–300 mosquito generations for the frequency of a resistance allele to reach 50%. If we assume a year-round transmission setting with the mosquito's generation time of ~20 days, that would translate into a time of between 10 and 15 years. Allowing for a considerable variation in the values of transmission parameters, this timescale is roughly similar to what is observed in reality. There is much evidence that the deployment of ITN or IRS fuels the rapid spread of resistance alleles like the kdr allele [28–31]. In a controlled field trial in Mexico, e.g. *Anopheles* populations went from 0 to 20% resistance in 3 years of IRS [32], and once close to complete coverage by ITNs was started in Western Kenya most mosquitoes were resistant within 10 years [33].

Nevertheless, it appears that it is often agricultural use of larvicides rather than malaria control that underlies the evolution of resistance in *Anopheline* mosquitoes [11, 34–36]. Our model gives a theoretical backing to this observation. Indeed, our model predicts that for a wide range of parameter values it takes at least 20 times longer for resistance to evolve if ITNs are the sole selection pressure than if larvicides are (Fig. 4). This should come as no surprise: larvicides impose stronger pressure than ITNs, for they target all individuals, whereas ITNs target only females. Rather than comparing 'coverage' of both intervention strategies as defined in this article, it would be a fruitful effort to compare the effect on resistance evolution of those interventions employing different bases of comparison, e.g. comparing the effect of a certain quantity of insecticide used either as a larvicide or an ITN. This could for example take the shape of a cost-effectiveness analyses (cost-'resistance' analysis), similar to efficiency analyses run for antimalarial intervention methods [37, 38]. Finally, it has to be recognized however that the importance of the larvicides, whether deliberately deployed for mosquito control (larval source management) or as an agrochemical by-product, is highly dependent on mosquito control or agricultural activity in the considered region [39, 40] and that both repellency and animal-feeding keep mosquitoes away from the ITNs and therefore reduce their exposure to the lethal effects of the insecticide.

Naturally, the quantitative predictions of our model depend strongly on its assumptions. Several of these are reasonable. We assume, e.g. that the resistance allele gives a similar level of resistance to larvae and adults and that the level of dominance is similar in the two life stages, as observed in insecticide bioassays conducted with larval and adult mosquitoes of various genotypes [41, 42]. In our model, we talk about a lethal concentration but in the natural setting, this will also depend on the

exposure length (compare a short contact with an ITN to a more prolonged contact in the larval environment) as well as the concentration of the insecticide in a given environment (potentially a stronger concentration on an ITN).

Other assumptions make little difference to the conclusion. Thus, we assume that the cost of resistance is paid through reduced fecundity rather than through reduced longevity, for which there is some experimental evidence [16, 43]. We avoided doing so in order not to further complicate the expression for longevity. We also assume that males experience a similar cost of resistance that affects fertility. This could for example happen via reduced competitive success for females compared with susceptible males, reduced sperm viability or female preference for susceptible males (either via standard or cryptic sexual selection). Some evidence for a male fertility cost of resistance, if in competition with susceptible males, has been provided by [19] for *Culex pipiens*, but we are unaware of any investigation that has looked for a male cost in *Anopheles* mosquitoes. A main assumption is that the behaviour of the mosquitoes—the likelihoods that they bite animals and that are repelled by insecticides—does not evolve as a response to insecticide pressure. Any genetic variation would of course lead to selection pressure, as the mosquitoes would thereby be less likely to be killed [8]. The qualitative consequences of selection for behavioural resistance for the evolution of IR seem clear. In the simplest case, when behaviour is not linked to resistance, selection would reduce contact with the insecticide, thus weakening the selection pressure for true resistance and strengthening our conclusion that larvicides impose stronger selection for resistance than ITNs. Things become more complicated if behaviour and resistance are genetically linked. In this case the evolutionary dynamics will depend critically on the sign of the genetic correlation between behaviour and resistance—a positive correlation would enforce selection of resistance; a negative one would constrain it. As we have no evidence of such a correlation and can therefore not make more quantitative predictions, we ignore behavioural resistance in our model.

An important feature of our model is that it uses the mosquitoes' behaviour to estimate their fitness, and thus combines an ecological approach with population genetics. The importance of the behaviour linked to the repellency of the ITNs is clearly seen in Figs. 2a and 3b. Most other models describing the evolution of IR, whether discussing the mosquitoes that transmit malaria [12, 44] or other insects [45] ignore the behavioural response of the insects to the insecticide. On the other hand, several epidemiological models have profited from incorporating the mosquitoes' behaviour, thus emphasizing the importance of linking behavioural ecology with the epidemiology and evolution of resistance [15, 16] ([46] for an application to behavioural resistance).

In summary, we described a scenario in which IR could evolve in response to a given coverage by ITNs. First, we showed that, while ITNs can lead to the rapid evolution of resistance, larvicides—whether they are used for malaria control or kill mosquitoes as a by-product of agricultural use—are likely to impose a much stronger selection pressure. This gives the theoretical basis for the claim that it is the agricultural use of insecticides rather than ITNs that has driven the evolution of insecticide-resistant malaria vectors in many parts of Africa. Second, we showed that the repellent property of ITNs has a strong effect on the evolution of IR, so that the strong repellency can help to maintain the efficacy of insecticides in the long-term. This benefit to the community complicates the conflicting effects of repellency, which on the one hand offers personal protection to their users [47] but on the other hand may have little impact [48] or can even have detrimental effects on the community as a whole by keeping mosquitoes from being killed and therefore increasing prevalence [49] (P. L. G. Birget and J. C. Koella, submitted for publication). Overall, thus, attempts to slow the evolution of resistance against insecticides must take into account the complexity of the evolutionary process, which is substantially influenced by details of the use of insecticides and of the mosquitoes' behavioural response to the insecticide.

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PLOS ONE

RESEARCH ARTICLE

# An Epidemiological Model of the Effects of Insecticide-Treated Bed Nets on Malaria Transmission

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## Abstract

Insecticide-treated bed nets (ITNs) have become a central tool for malaria control because they provide personal and community-wide protection through their repellent and insecticidal properties. Here we propose a model that allows to assess the relative importance of those two effects in different epidemiological contexts and we show that these two levels of protection may oppose each other. On the one hand, repellency offers personal protection to the users of ITNs. The repellent action, however, is a two-edged sword, for it diverts infectious mosquitoes to non-users, thereby increasing their risk. Furthermore, with increasing ITN coverage, the personal protection effect of repellency decreases as mosquitoes are forced to perform multiple feeding attempts even on ITN users. On the other hand, the insecticidal property, which offers community-wide protection by killing mosquitoes, requires that mosquitoes contact the insecticide on the ITN and is thus counteracted by the repellency. Our model confirms that ITNs are an effective intervention method by reducing total malaria prevalence in the population, but that there is a conflict between personal protection, offered by repellency, and community-wide protection, which relies on the ITN's insecticidal properties. Crucially, the model suggests that weak repellency allows disease elimination at lower ITN coverage levels.

## Introduction

Insecticide-treated bed nets (ITNs) are among the most important and cost-effective intervention measures against malaria relying on three main mechanisms: 1) the nets create a physical barrier between the human and the mosquito vector, 2) the insecticide used to treat the bed net repels mosquitoes ("excito-repellency" or "deterrence", simply referred to as repellency in this paper), thus increasing the personal protection offered by the net, and 3) if a mosquito fails to be repelled, it will often rest on the bed net after biting, and may then be killed by contacting the insecticide. For mosquitoes with some degree of zoophily (also known as zoophagy or

animal feeding), ITNs also provide protection by diverting mosquitoes to non-human hosts [1–3]. In addition, ITNs may increase the host searching time of a mosquito, which increases the duration of the gonotrophic cycle and thus the risk that the mosquito dies before obtaining its blood-meal [4]. ITNs thus offer a mix of personal protection—blocking the bites of mosquitoes, thereby reducing the transmission from mosquitoes to humans—and community protection—reducing the longevity of mosquitoes and therefore the prevalence of sporozoites, the infectious stage of malaria, in mosquitoes.

The personal protection offered by insecticide-treated bed nets has been documented in many studies e.g [5–8]. In a large randomised control trial, for example, Gimnig et al [5] found up to 95% lower resting densities of mosquitoes in houses with nets than in houses without nets, and mosquitoes were less likely to carry sporozoites. Comparisons before and after extensive bed net coverage showed similar results [9–11]. ITNs also have a good record of reducing the intensity of transmission within the whole community i.e. not only among ITN-users but also among non-users. Hawley et al [12], for example, found reduced disease incidence up to 300m around a house where ITNs are used, and [13] report a 4.2 fold reduction of the entomological inoculation rate (EIR) experienced by unprotected people with a coverage of 75% of untreated nets and an 18-fold reduction if those nets are treated with insecticides.

Although these field studies provide valuable information about the success of ITNs for malaria control, it is not clear which aspect of the ITN—its physical barrier, its insecticidal effect or its excito-repellency—is the most important characteristic in reducing malaria transmission. Though many field studies show a positive effect of ITN coverage even to non-users, the possibility remains that, at some levels of coverage and some epidemiological settings, ITNs divert mosquitoes to unprotected people in a way that increases their risk, making ITNs an ethically challenging intervention [1, 14–17].

Several studies have attempted to fill that gap by proposing models that predict the impact of ITNs on disease transmission. Chitnis et al [2] developed a mathematical formalisation of malaria model where mosquitoes are allowed to obtain blood from a diverse host population, which is essential if we want to model the effect of ITNs as we need at least two categories of humans hosts, ITN-users and non-ITN users. In a numerical simulation applied to ITNs, they find that ITNs have a community-wide positive effect. Similarly, Killeen and colleagues used a description of the mosquito's feeding behaviour to calculate the relative exposure of protected compared to unprotected hosts and the effect of ITNs on the EIR [3, 18–20]. An extension of these models allowed to disentangle the protective effects of the various properties of the ITN, namely their insecticidal effect (toxicity) and repellency [17]. The authors showed that repellency may indeed erode community-wide protection offered by high ITN coverage. This finding is confirmed by Gu et al. [16], who developed an individual-based model of mosquitoes feeding in a village surrounded by breeding areas and calculated the effect of various coverage by ITNs on the mosquito dynamics and human prevalence. The conclusions of the paper were that the effectiveness of the nets was most sensitive to the insecticidal effect of the insecticide and that a strong repellent effect of impregnated nets can lead to a greater risk for people who do not use bed nets. Finally, LeMenach et al [4], who described the feeding success and survival of mosquitoes in a gonotrophic cycle by a mathematical dissection of their feeding behaviour, make the point that one of the reasons for the effectiveness of ITNs is that zoophilic mosquitoes are diverted to non-human hosts. Thus, whether repellency offers community-wide protection or not crucially depends on the feeding preferences of the vector (see [1] for a detailed review).

While these studies confirm that ITNs generally have a protective effect and, indeed, that repellency can increase the risk among unprotected individuals, they generally lack a solid integration of the epidemiological dynamics of infections in humans and mosquitoes by assuming fixed values of infectivity from humans to mosquitoes. Most of the models described above

calculate the effect of ITNs on the entomological inoculation rate (EIR), a measure of the intensity of transmission. Though there are models of how to deduce the actual prevalence of malaria in the human population from the EIR [21], it would be desirable to directly model prevalence (also called parasite rate) in infected and uninfected people, especially since the latter has been an important quantity for intervention method decision making [21, 22]. The coupling of malaria infection dynamics of humans with those of mosquitoes also allows more precise modelling of herd effects, which is a crucial component of the ITN intervention. In this study we therefore extend the classical Ross-Macdonald model for malaria transmission [23–25], see [26] for a recent review) to describe malaria transmission and its prevalence in ITN-protected and unprotected people, and combine the model with the vector's behavioural parameters that are derived from a mosquito feeding cycle.

## Materials and Methods

Our epidemiological model combines epidemiological theory [25] with equations describing the mosquito's feeding cycle and its behavioural response to ITNs (Fig 1).

### Feeding cycle

We extended the approach described by LeMenach et al [4] to calculate the proportion of mosquitoes that bite ITN-users (a proportion $\phi$ of the population) and non-users (proportion $1-\phi$). We distinguish two stages of the biting attempts: the probability of initiating a bite and probing on a human (which suffices for the transmission of malaria from mosquitoes to humans) and the probability of completing the bite and surviving possible contact with the insecticide (which is required for the transmission from human to mosquitoes). To calculate these probabilities, we assumed that host-seeking mosquitoes target humans with a probability $Q$ and other animals with probability $1-Q$, and that of the mosquitoes targeting humans, a proportion $\epsilon$ are endophilic, i.e. bite at a time when humans are sleeping indoors, while a proportion $1-\epsilon$ are exophilic (note that in our model endophily is irrelevant for mosquitoes targeting animals). If the indoor-host is protected by an ITN, the mosquito is repelled and starts a new host search with probability $r$. Note that the repellency parameter includes both, repellency caused by volatiles of the insecticide as well as repellency due to the sheer physical feature of the net. If it is not repelled (probability $1-r$), it overcomes the mechanical protection offered by the net to blood-feeds, but is killed by the insecticide with probability $1-s$. Thus, a mosquito can initiate a bite on an ITN-user in two ways. First, it can target ITN-users during the time when they are still outdoors. The probability of this event is

$$H_{p,o} = Q(1-\epsilon)\phi \tag{1}$$

Second, if it bites indoors (at a time when people are sleeping), it can target an ITN-user during its first biting attempt, during its second attempt (having been repelled once), during its third attempt (having been repelled twice), etc. The probability of biting an ITN-user after a single attempt is $Q\phi(1-r)$; if each additional search of a host brings with it the risk $\mu_r$ of dying, the probability of having been repelled $n$ times is $(Q\phi r(1-\mu_r))^n$. Thus, the probability that a mosquito initiates a bite on an ITN-user sleeping indoors is

$$\begin{aligned} H_{p,i} &= Q\epsilon\phi(1-r)\sum_{n=0}^{\infty}[Q\phi r(1-\mu_r)]^n \\ &= \frac{Q\epsilon\phi(1-r)}{1-Q\phi r(1-\mu_r)} \end{aligned} \tag{2}$$

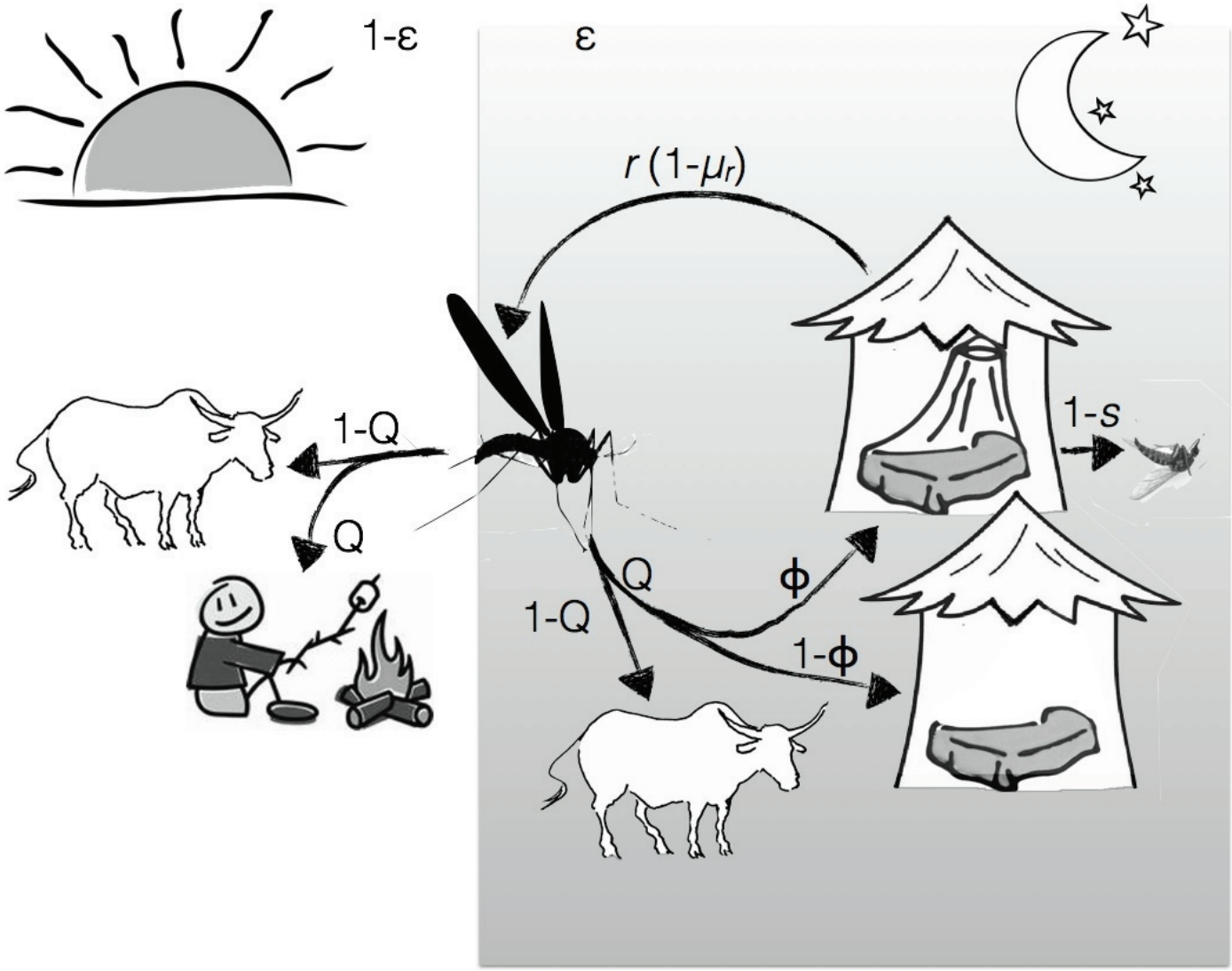


**Fig 1. Host searching cycle of a mosquito.** A mosquito bites indoors with probability $\epsilon$ (for night-active and highly anthropophilic mosquitoes, this happens mainly at night) and takes a bite outdoors with probability $1 - \epsilon$. A mosquito then bites humans with a probability $Q$. If biting indoors, it will enter a house where a person sleeps under a bed net with a probability $\phi$ (the ITN coverage) or a house with an unprotected person with a probability $1 - \phi$. If the person is protected, the mosquito is repelled by the insecticide (or mechanically blocked by the net) with a probability $r$; if it is not repelled, it takes its bite and escapes with probability $s$ or it is killed by the insecticide on the net with probability $(1 - s)$. If a mosquito is repelled by a bed net, it leaves the house and continues to search for a host. There is a mortality cost $\mu_r$ associated with each repellency event. We assume that a mosquito will always land a successful bite on unprotected people and and on animals, whereas the feeding success on protected people depends on $r$ and $s$. The host search happens once per mosquito gonotrophic cycle, i.e. once every three days (see Table 1).

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The probability that a mosquito bites an ITN-user (indoors or outdoors), $H_p$, is the sum of Eqs (1) and (2):

$$H_p = Q\left[1 - \left(1 - \frac{(1-r)}{1 - Q\phi r(1-\mu_r)}\right)\epsilon\right]\phi \tag{3}$$

Similarly, the probability that a mosquito bites an unprotected person (indoors or outdoors) is

$$H_u = Q\left[1 - \left(1 - \frac{1}{1 - Q\phi r(1-\mu_r)}\right)\epsilon\right](1-\phi) \tag{4}$$

These ideas can be extended to calculate the probabilities that mosquitoes survive their biting attempts on ITN-users, $P_p$, and non-users, $P_u$. As we standardized the equations by letting all mosquitoes survive their biting attempts on unprotected hosts, $P_u = H_u$; the probability of surviving a biting attempt on a protected host is

$$P_p = Q\left[1 - \left(1 - \frac{(1-r)s}{1 - Q\phi r(1-\mu_r)}\right)\epsilon\right]\phi \tag{5}$$

## Human dynamics

We modified the system of differential equations describing the epidemiology of malaria [25] by writing separate equations for the prevalence of disease in protected people, $y_p$, and in unprotected people, $y_u$:

$$\dot{y}_p = m\ w\ a\ \frac{H_p}{\phi}(1 - y_p) - \rho\ y_p \tag{6}$$

$$\dot{y}_u = m\ w\ a\ \frac{H_u}{1-\phi}(1 - y_u) - \rho\ y_u \tag{7}$$

where $m$ is the the number of mosquitoes per person, $w$ is the proportion of mosquitoes that are infectious (i.e. that carry sporozoites in their salivary glands), $a$ is the biting rate of the mosquitoes on humans, and $\rho$ is the recovery rate from malaria. (Note that $H_p$ and $H_u$ are the probabilities within the total human population that a mosquito bites a protected or an unprotected person, respectively; to get the probabilities within the protected or unprotected sub-populations, we must divide the former probabilities by $\phi$ or $1 - \phi$.)

## Mosquito dynamics

To simplify, we assumed that each infected person is infectious to mosquitoes. We calculated the inoculation rate of mosquitoes by averaging the probabilities that a mosquito successfully feeds on a protected person ($P_p$) or on an unprotected person ($P_p$): $A = P_p\ y_p + P_u\ y_u$

We calculated the mosquito's mortality from the feeding cycle. According to our assumptions, mosquitoes die if their attempt at blood-feeding is not successful. The probability of completing a blood-meal—whether on a protected human, an unprotected humans or an animal—is the sum of the probabilities of success during a single attempt, accounted by the probability that the mosquito could have landed a successful bite after $n$ repellency events:

$$\begin{aligned} S_b &= ((1-Q) + Q(1-\epsilon) + Q\epsilon(1-\phi) + Q\epsilon\phi(1-r)s)\sum_{n=0}^{\infty}[Q\epsilon\phi r(1-\mu_r)]^n \\ &= \frac{1 - Q\epsilon\phi(1-(1-r)s)}{1 - Q\epsilon\phi r(1-\mu_r)} \end{aligned} \tag{8}$$

Once fed, the mosquito must survive through the duration of its gonotrophic cycle (i.e. the time it takes to develop and lay its eggs) before it starts a new feeding attempt. The probability of feeding-independent mortality during the gonotrophic cycle is $\mu_\tau = 1 - (1-\mu_0)^\tau$, where $\mu_0$ is the feeding-independent daily mortality and $\tau$ is the duration of the gonotrophic cycle (note

that, in contrast to [4], we assume that the gonotrophic cycle is not prolonged by repeated host searches. This is a good approximation unless each search for a host lasts a long time or coverage is close to 100% so many searches are necessary). The probability of surviving a gonotrophic cycle is the combination of feeding-related and feeding-independent mortality:

$$S = (1-\mu_\tau)\frac{1-Q\epsilon\phi(1-(1-r)s)}{1-Q\phi r(1-\mu_r)} \tag{9}$$

giving the daily mortality rate

$$\mu = -ln(1-(1-S)^{1/\tau} \tag{10}$$

Following earlier approaches [25], we can then describe the dynamics of the proportion of latent ($v$) and infectious ($w$) mosquitoes as:

$$\dot{v} = a(1-v-w)A - a\hat{A}(1-\hat{v}-\hat{w})S^{T/\tau} - \mu v \tag{11}$$

$$\dot{w} = a\hat{A}(1-\hat{v}-\hat{w})S^{T/\tau} - \mu w \tag{12}$$

where the incubation period of malaria in mosquitoes is $T$ days and where $\hat{v}$, $\hat{w}$ are the number of latent and infectious mosquitoes and $\hat{A}$ the infectious reservoir $T$ days earlier. As the epidemiological dynamics in the mosquitoes are much more rapid than those of the humans, we considered them to be at equilibrium relative to the humans and therefore set $\dot{v} = 0$ and $\dot{w} = 0$. Thus we obtained an expression for $w$ as a function of the prevalences of protected and unprotected people in $A$:

$$w = \frac{aAS^{T/\tau}}{aA+\mu} \tag{13}$$

We found the equilibrium prevalences by calculating the equilibria of Eqs (6) and (7) with the function `stode` of the R-package rootSolve [27]. Parameter values were obtained from published studies of the highly anthropophilic *Anopheles gambiae* species complex and *Plasmodium falciparum* (Table 1). Note that the parameter "density of mosquitoes" includes parameters that are not explicitly given in the equations, e.g. the probabilities of infection and variabilities of parameters; its value was therefore chosen to give a reasonable description of the epidemiology rather than to reflect observed densities of mosquitoes.

## Results

We find that increased coverage of ITNs decreases malaria prevalence through a combination of the personal protection given by the repellency of the insecticide and the community protection given by its insecticidal action (Fig 2). Whether it is personal protection or community protection that is more relevant depends on the context defined by the details of the parameters.

If repellency is weak, personal protection against mosquito bites is low, so that most of the impact on prevalence is due to the insecticidal action of the bed nets. As coverage increases, so does the number of mosquitoes killed by the insecticide, thus decreasing transmission and prevalence in protected and, through a herd effect, unprotected people (Fig 3a). If repellency is stronger, the bed nets provide more personal protection but fewer mosquitoes contact the insecticide and die. This leads to a greater difference in prevalence between protected and unprotected people who are infected. Furthermore, as coverage increases, more mosquitoes are diverted to unprotected people, which increases the risk of the unprotected people (Fig 3b).

**Table 1. Parameters and variables.** All parameters were set to their typical values unless explicitly mentioned.

| Parameter | Explanation | Typical value | Reference |
|---|---|---|---|
| $\phi$ | ITN coverage | 0.5 | |
| $m$ | mosquitoes per person | 1 | |
| $a$ | biting rate (per day) | 0.33 | [28] |
| $\rho$ | recovery rate from malaria (per day) | 0.01 | [24] |
| $Q$ | probability of feeding on humans | 0.95 | |
| $\epsilon$ | probability of indoor feeding | 0.9 | [29] |
| $r$ | probability of repellency | 0.6-0.9 | [30] |
| $s$ | survival after feeding | 0.16 | [30] |
| $\mu_0$ | background mortality of mosquitoes (per day) | 0.1 | [31] |
| $\mu_r$ | mortality during host searching (per search) | 0.03 | |
| $T$ | time for sporozoite development (days) | 10.3 | [32] |
| $\tau$ | duration of gonotrophic cycle | 3 days | |
| **Variables** | | | |
| $y_p$ | prevalence of malaria in protected individuals | | |
| $y_u$ | prevalence of malaria in unprotected individuals | | |
| $v$ | number of latently infected mosquitoes | | |
| $w$ | number of infectious mosquitoes | | |

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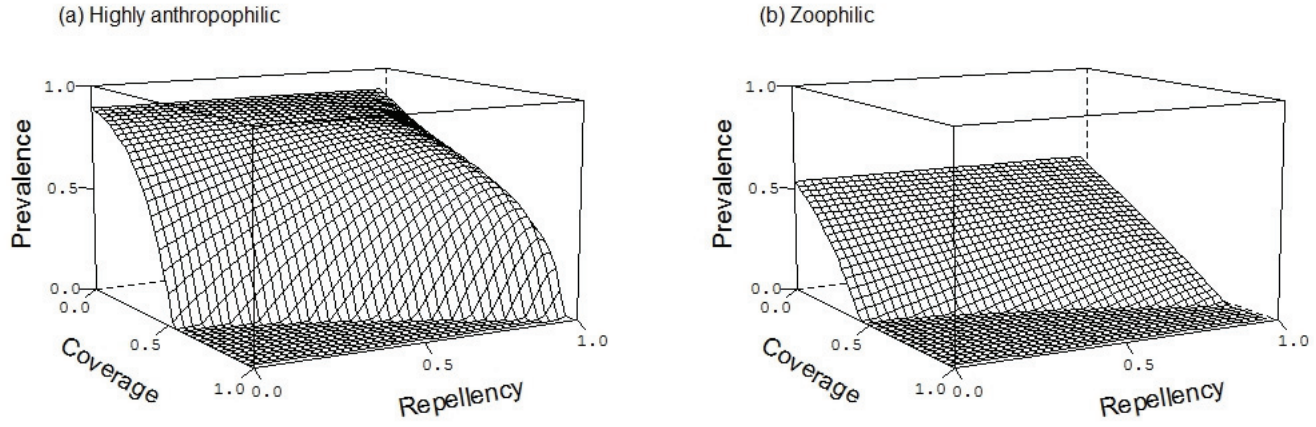


**Fig 2. The effect of bed net coverage ($\phi$) and repellency ($r$) on malaria prevalence.** Panel (a) shows a situation with highly anthropophilic mosquitoes ($Q = 0.95$; panel (b) with zoophilic mosquitoes ($Q = 0.3$). Other parameters are given in Table 1.

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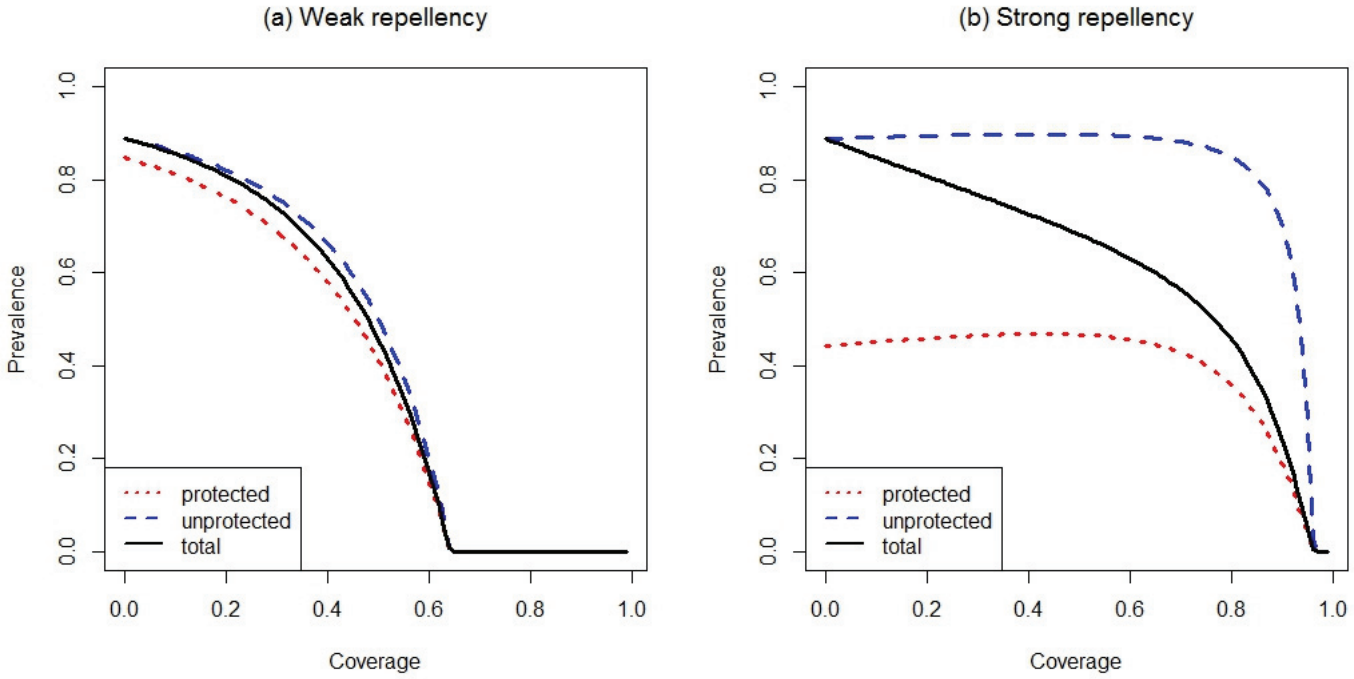


**Fig 3. The effect of coverage on malaria prevalence at the epidemiological equilibrium.** Prevalence in unprotected people is shown by the dashed line, in protected people by the dotted line, and the population as a whole is represented by the solid line. In panel (a) repellency is $r = 0.3$, in panel (b) $r = 0.9$. Other parameters are given in Table 1.

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More surprisingly, as coverage of strongly repellent nets increases, so does the prevalence in *protected* people (Fig 3b). The reason is that repelled mosquitoes are not only diverted to unprotected humans and animals as some of the repelled mosquitoes will attempt to bite protected individuals. Some of these attempts will also be successful if repellency is not complete. Thus, although people using an ITN obtain personal protection, the fact that their neighbors also use ITNs makes this protection less effective.

However, it is important to bear in mind that, although prevalence of protected and unprotected may increase with ITN coverage, total prevalence still decreases because increasing coverage, by definition, means moving people from the unprotected to the protected category with the latter facing a substantially smaller risk of receiving an infectious bite than the former.

Similar arguments explain why increasing repellency *increases* prevalence and increases the coverage required to eliminate the parasite (Fig 2). As the insecticidal impact of the nets becomes more important with increasing coverage, lower levels of repellency enable the parasite to be eliminated from the population at lower coverages (Fig 2). For more repellent nets, ITNs achieve their main impact by diverting mosquitoes from the protected individuals to unprotected ones which consequently receive more infectious bites (Fig 4). Increased repellency offers more personal protection, so that the difference in prevalence between protected and unprotected individuals increases. Neverthless, at sufficiently high coverage increasing repellency also increases prevalence on *protected* individuals (unless repellency is close to perfect) (Fig 4b). The personal protection effect offered by repellent ITN will therefore be most

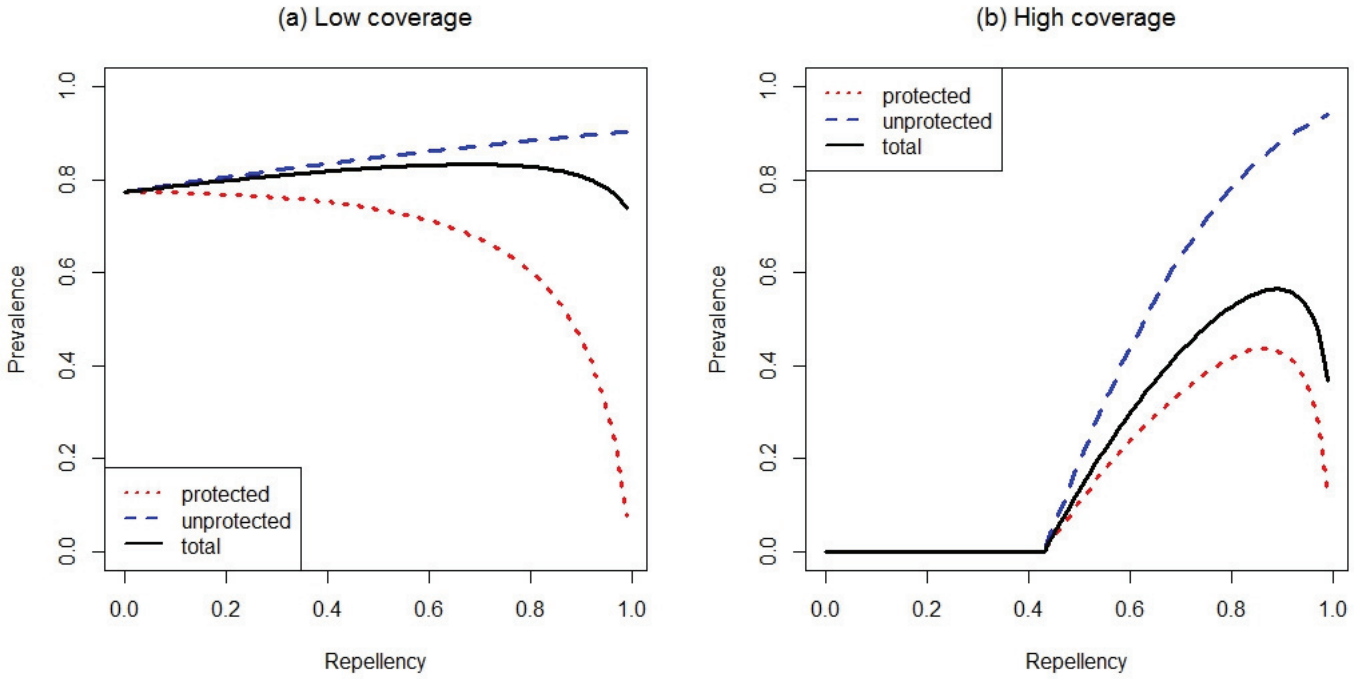


**Fig 4. The effect of repellency on malaria prevalence at the epidemiological equilibrium.** Unprotected people are represented by the dashed line, protected people by the dotted line, and the population as a whole by the solid line. In panel (a) coverage is $\phi = 0.2$, in panel (b) $\phi = 0.7$. Other parameters are given in Table 1.

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important in a context of low ITN coverage, where diverted mosquitoes find enough alternative hosts and are therefore unlikely to find their way back to the ITN user. Many of the other conclusions are intuitively more obvious. For example, when mosquitoes are zoophilic (Fig 2b), repellency can eliminate mosquitoes at lower coverage than when mosquitoes are strongly anthropophilic because infectious mosquitoes may be diverted to animals (Fig 2a). These patterns are qualitatively similar for highly anthropophilic mosquitoes (Fig 2a) and for mosquitoes that bite humans only rarely (Fig 2b), although of course prevalence is lower for the latter.

The impact of the insecticidal action reflects the role of repellency in being coverage-dependent. At weak repellency and high coverage, increasing insecticidal action (i.e. reducing the probability that mosquitoes survive their bite) strongly reduces prevalence in protected and unprotected individuals (Fig 5b). As survival increases, so does prevalence. With strong repellency, however, the insectidal action of the ITN almost disappears, as most mosquitoes do not contact the ITN. As shown in Fig 2, high repellency also leads to high prevalence because the mosquitoes are diverted to unprotected (and also to protected) individuals. At low coverage the community-wide insecticidal benefit of ITNs is low because few mosquitoes encounter the insecticide, i.e. neither the insecticidal nor the repellenct action has a large impact on total malaria prevalence in the population (Fig 5a).

## Discussion

Insecticide-treated bed nets protect individuals against malaria by blocking and repelling mosquitoes, and they protect the community by killing mosquitoes. The repellent and the

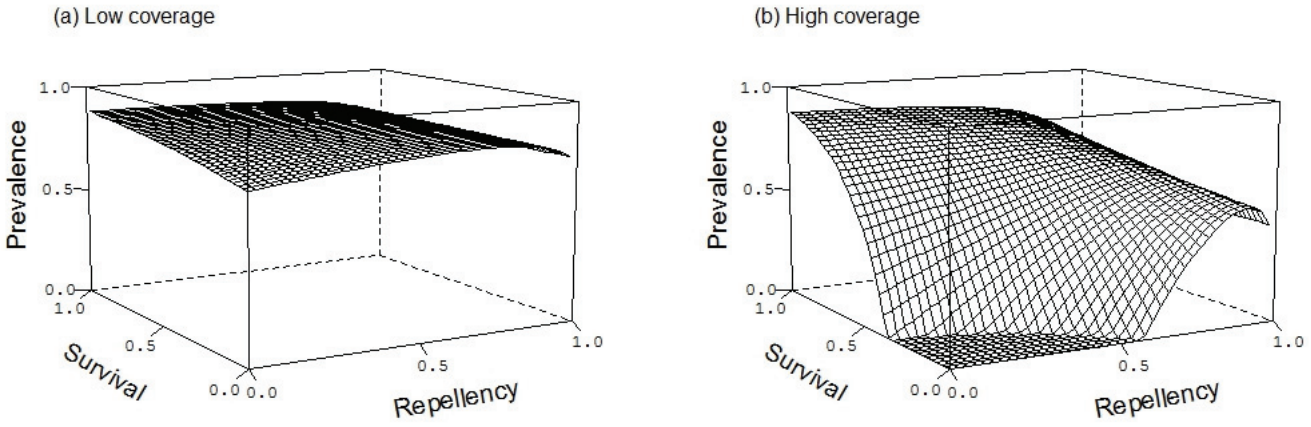


**Fig 5. The effects of repellency and probability of surviving the exposure to the insecticide on malaria prevalence.** The epidemiological equilibrium prevalence is shown for low ITN coverage ($\phi = 0.2$) and high ITN coverage ($\phi = 0.7$). Other parameters are given in Table 1.

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insecticidal impacts of the insecticide on control, however, oppose each other: nets that are more repellent reduce the number of mosquitoes that are exposed to the insecticide and therefore kill fewer mosquitoes. As a consequence, our model, which combines the two impacts by merging the biting behaviour of mosquitoes with the epidemiology of malaria, predicts a conflict between individual and community effects: although increasing repellency provides better personal protection, it reduces the community-wide benefit of insecticides and increases the prevalence in the community above the level that could be achieved with non-repellent insecticides. Therefore, at the community level, repellency may be detrimental for the control of malaria.

Specifically, the opposing forces of the repellent and the insecticidal actions lead to three other important predictions: (i)Higher levels of repellency offer better protection to bed net users, but divert more mosquitoes to unprotected people, and thus increase their risk of infection. Stronger repellency therefore leads to greater difference in prevalence between protected and unprotected people. More surprisingly, at a given coverage, stronger repellency also increases the prevalence in *protected* people. The reason is that repelled mosquitoes are diverted not only to animals and unprotected individuals, but also to other ITN-users. Unless repellency is perfect, some of these will penetrate the defence of the net and bite the person sleeping under it. If coverage is sufficient (i.e. if a sufficient number of mosquitoes are diverted from all the ITNs), the repellency of a given net will be outweighed by the increased number of mosquitoes attempting to bite. Exactly the same increase of infection risk in ITN users was observed in the model proposed by Killeen et al. [17].

(ii) If repellency is weak, increasing the coverage of ITNs kills more mosquitoes, thereby decreasing transmission and offering community-wide protection of people with and without a net. As most of the protection is due to the insecticidal action of the insecticide, the community effect dominates so that there is little difference of the prevalence between the two groups. If, however, repellency is strong, the effect of the ITNs is dominated by personal protection. Unprotected individuals therefore are at risk from the diverted mosquitoes, and this risk increases with coverage unless coverage is so high that the unprotected individuals benefit from a herd-effect. (iii) At low coverage or high repellency, few mosquitoes encounter the insecticide. Therefore, in these situations losing the insecticidal action by the evolution of resistance has less impact. Rather, we would expect that evolution would favor behavioural changes of the mosquito, for example by biting at a time when people are still outdoors, as a response to the use of ITNs. Only when coverage is high and repellency low will the evolution of resistance substantially increase the prevalence of disease. In this situation, we would expect strong evolutionary pressure for resistance. Thus, repellency underlies a second public health conflict: between short-term and long-term success. Although stronger repellency may increase prevalence, it will delay the evolution of insecticide resistance, as we show in a different paper [33]. There is however a distinct possibility that genetic resistance to insecticides is genetically linked to the behavioural trait of failing to be repelled by ITNs, as potentially observed in *A. gambiae* [34]. If mosquitoes fail to be repelled and killed by the insecticide, ITNs are reduced to their protective feature of establishing a physical obstacle between human and mosquito and, though repellency will no longer cause a conflict between users and non-users, ITNs will also lose any community-protective effect.

(iv) The general patterns are only slightly affected by the level of zoophily, although, of course, zoophily decreases the overall risk of infection. While repellency diverts many mosquitoes to animals, some will be diverted to humans (whether protected or not). Therefore the predictions are qualitatively similar, though the effects are less strong for zoophilic than for anthropophilic mosquitoes. Similarly, as long as vectors have some degree of zoophily (which may also be determined by the host composition in a given transmission setting), repelled mosquitoes may be diverted to animals. Hence the more zoophilic a vector is, the less conflict is introduced by a repellency (graphs not shown).

It has been suggested that the impacts of ITNs and of vaccines are comparable, for they both lead to a herd effect, where protecting some individuals can protect non-users by reducing the rate of transmission [19]. Our model suggests that this can, indeed, be the case if the ITN is only weakly repellent. There is, however, a crucial way of how ITNs differ from vaccines: vaccinated hosts do not divert pathogens, whereas hosts sleeping under an ITN divert mosquitoes to unprotected individuals. Our model shows that this difference has important consequences: if repellency is strong, personal protection can lead to higher prevalence in unprotected individuals. In this context, the function of ITNs act differently to vaccines: whereas a vaccine provides personal protection and protects surrounding unvaccinated people, ITNs provide personal protection but could expose surrounding unprotected people at a higher risk. Thus, ITNs can lead to a clear conflict between individual and community effects.

Our results corroborate several other models that predict that the repellent action of ITNs can increase the prevalence in unprotected individuals e.g. [3, 4, 16, 17]. We agree with Killeen et al [19] who found that for the highly anthropophilic mosquito *Anopheles gambiae* relative exposure to non-users stays high across the whole range of repellency for a given coverage. Our results, however, differ in several important respects, partly because we considered a greater range of parameter values (e.g. coverage levels), and partly because we allowed an epidemiological feedback between the mosquitoes' behaviour and the risk that they become infected.

Whereas Killeen et al [19] report an increase in personal protection with repellency while keeping the coverage constant at 75%, we found that repellency only improved personal protection at low coverage levels, as discussed in point (ii). Though, the model of [17] also shows that personal protection degrades at high repellency levels, their model does not highlight the effect of coverage and how it interacts with repellency as they examine their model only at a fixed coverage of 80%. Like [16, 17, 19], we find that the insecticidal property of ITNs is the most important determinant of the community effect of ITNs. In contrast to the earlier studies, however, we suggest that this is the case only when repellency is low and coverage is high, i.e. where the impact of the ITNs is dominated by the insecticidal action rather than the repellency. Most field studies suggest a positive community-wide effect of ITNs [12, 13, 35–37]. Most of these have been conducted in communities with a very high coverage of bed nets (ranging from 70% to near complete coverage), i.e. in conditions where our models also predict a strong community effect. Even in such conditions, bed net users typically have prevalences which are around 30%-40% lower than in non-users [6, 38, 39].

In field settings it is difficult to test which feature of the net is responsible for decreased prevalence in the population. Studies that compare communities using treated and untreated nets could provide some proxy for the effect of the insecticide. While there is some support for the superiority of ITN over untreated bed nets e.g. [11, 13, 35, 40]—and thus for the superiority of the combined insecticidal and repellent actions of the ITNs—research on the effect of ITN repellency alone has given mixed results. Repellency is still widely seen as a desirable feature of vector control; its use in clothing, topical repellents, ITNs and area repellents is a well-established protective measure. The evidence that repellents provide efficient personal protection is compelling [41, 42]. Following this trend, there is a body of research that considers the application of additional repellents to ITNs but so far it remains unclear whether it offers any benefits for malaria control. A model proposed by Kiszewski et al. [43], for example, predicts that an efficient repellent would reduce malaria infection to a level lower to that achieved by ITNs. They assume, however, that the biting rate per untreated person stays constant, which (as we argue here) is unlikely to be the case in particular in areas where mosquitoes are highly anthropophilic. In contrast, a recent field study has shown that using topical repellents are probably overpowered by the much stronger repellent effect of the ITN, therefore making them superfluous [44]. The idea that repellency increases the mosquito biting rate on non-users has received mixed support from field studies. Hewitt et al. [45] finds that ITN-applied repellent is strong enough to protect nearby unprotected people in a house. In contrast, Moore et al [15] find that unprotected people sitting one meter away from people wearing topical repellent experience up to 36% more mosquito landings. It is therefore unclear at which spatial scale repellency operates and it seems to depend strongly on the type of intervention with freshly impregnated or new ITNs offering a larger repellency radius than ITNs whose impregnation has worn off or topical repellents of their own. Repellency has also received attention under "push-pull" approaches of malaria control, which have are claimed to offer strong potential as an intervention if deployed over a wider area [46]. Our model suggests that an ineffective push-pull system, i.e. where the pushing component is more important than the trapping component, could potentially put at risk unprotected people, but the latter depends a lot on the vector species and therefore on its feeding preferences [47, 48]. However, the possibility remains that the push-pull approach could be used to partly offset the "excess" mosquitoes that are repelled by ITNs, especially when coverage is high. Regarding the potential negative effect of repellency on community-protection level we argue that it should be the subject of more extensive field research to find out first whether the phenomenon does take place in real transmission settings and second, if so, how to off-set it in, a push-pull system only being one example. It is also important to keep track of the actual transmission context, especially about the mosquito community

composition and the feeding habits of the different species because these are paramount to choosing the optimal intervention strategy [1]. The latter becomes clear in the model proposed by [49] who focus on more zoophilic mosquitoes, which are the most important malaria vectors outside Africa. If transmission is dominated by zoophilic mosquitoes it becomes irrelevant if mosquitoes are targeted by purely toxic or purely repellent compounds, but our model suggests that repellency is still counter-productive at the community level.

Another aspect not occurring in our model but potentially being important in real life transmission settings is the development of adaptive immunity to malaria, which is reliant on repeated exposure to infectious mosquito bites [50, 51]. As ITNs precisely prevent infectious bites, concerns have been raised that this may result in the delayed acquisition of natural protective immunity and thereby lead to an increase of infection in the long term [52, 53]. Temporarily acquired immunity has been integrated in a number of other mathematical models of malaria e.g. [2, 54, 55] but has not been considered under the original formulation of the Ross-Macdonald model, on which our model is based. However, acquired immunity is loosely defined in malaria and most often designates the situation where the a person has developed some resistance against symptoms but still sustains and transmits parasites [50, 56, 57]. Thus, this subpopulation is still captured here by modelling malaria prevalence rather than disease episodes. Finally, it is important to recognize that the personal protection provided by ITN repellency in case of high indoor feeding may be a significant motivation factor for using it, hence leading to higher coverage rates, which in turn have much a greater effect on prevalence than repellency. Thus, although repellency may be detrimental for the control of malaria, its impact on coverage is likely to be beneficial. We argue by no means against ITNs as an intervention strategy: indeed our model shows that whatever the coverage level, the total prevalence of malaria is always reduced. Our model makes formal observations of how the speed at which prevalence is reduced depends on the ITNs properties and how those properties may have opposing effects at different coverage levels. The finding that malaria elimination is more easily achieved with low repellency levels provide a potential tool to the design an "end-game strategy", a commonly discussed theme in infectious disease control [58, 59]. In summary, our paper highlights that repellent insecticide-treated bed nets introduce a conflict between personal and community protection for malaria control for areas where the main vector is strongly endophilic. Indeed, despite the personal protection offered by repellency, protecting the community would benefit from finding and using insecticides with less repellent action. However, the interactions between personal, epidemiological, evolutionary and social impact of using ITNs are complex, making predictions about the long-term benefits of repellency difficult.

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## Author Contributions

Conceived and designed the experiments: PLGB JCK. Performed the experiments: PLGB JCK. Analyzed the data: PLGB JCK. Wrote the paper: PLGB JCK.

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RESEARCH

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# Maternal effects, malaria infections and the badge size of the house sparrow

Philip L. G. Birget[1,2*†] and Stephen D. Larcombe[3,4,5,6†]

## Abstract

**Background:** The evolution of sexual signals is not only determined by immediate sexual selection but also by selection arising from the environment and the interaction with developmental effects. In this study we aimed to investigate how the badge size of male house sparrows (*Passer domesticus*) is correlated to avian malaria infections as well as to prenatal testosterone exposure, measured as the 2D:4D digit ratio. The rationale behind this study is that the immunosuppressive effect of maternal testosterone deposition combined with the fitness cost imposed by parasites may cause important trade-offs to the development of secondary sexual signals.

**Methods:** Assuming that vector abundance is a key variable associated with infection risk by avian malaria, we caught adult male sparrows from eight different populations in the Camargue, France, four of which were located in a vector-controlled area, and the other four in an untreated area. For each bird we measured its badge size, digit ratio and took blood to determine its infections status. We used PCR to identify the malaria parasite species and strain that caused the infection.

**Results:** Contrary to our expectation, prevalence of disease did not differ across the vector-treatment regions, with around 80 % of birds being infected in both areas, and those infections were caused largely by a single strain, *Plasmodium relictum SGS1*. Although infected birds had a badge size not significantly different from uninfected males, there was a condition-dependent association between badge size, infection status and maternal testosterone deposition.

**Conclusions:** This study illustrates that the complexity of temporal and ecological dimensions makes the relationships between disease, testosterone-related traits and secondary sexual signals that have been previously reported less general than thought.

**Keywords:** Avian malaria, Digit ratio, *Passer domesticus*, Sexual signals

## Background

The optimal allocation of limited resources is a fundamental selection pressure faced by any individual (Stearns 1992). Diseases pose a particular problem to an organism, since trade-offs can occur at two levels: (1) the parasite uses up host energy for its own growth and transmission and (2) the host invests energy to fight the parasite with costly immune responses (Hamilton and Zuk 1982; Sheldon and Verhulst 1996). Hamilton and Zuk first highlighted the potential importance of parasites in maintaining genetic variation involved in sexual selection, mediated by trade-offs affecting the development of costly secondary sexual signals (Hamilton and Zuk 1982). It is the costliness of male secondary sexual signals that offers the basis for female choice (Zahavi 1975). Foldstad and Karter's (1992) immunocompetence hypothesis (ICH) proposed a proximate mechanism for a trade-off between elaboration of sexual signals and disease susceptibility involving testosterone. This hormone is involved in the development of many secondary sexual signals (Andersson 1994) and also has a well-documented immunosuppressive effect (Saino et al. 1995; Verhulst et al. 1999; Duffy et al. 2000), which may lead to a situation where individuals cannot qualitatively increase their sexual ornamentation without increasing

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their susceptibility to disease. Hence, only individuals with an inherent "quality" such as an ability to acquire large energy reserves, or possession of parasite-resistance genes, can afford higher testosterone production. However the importance of these hypotheses in natural populations remains controversial as many studies fail to consider the intricate natural context in which sexual signals evolve. Sexual signals are influenced by natural selection, early development, as well as phenotypic plasticity; all of which are known to vary widely across environments (Cornwallis and Uller 2010). Varying parasite abundance constitutes a particularly important mediator of environmental heterogeneity and hence may differentially affect the evolutionary ecology of sexual signals in hosts. The early developmental environment deserves special consideration in the development of sexual signals due to its susceptibility to parental effects—a theory now widely accepted by evolutionary biologists (Danchin et al. 2011). "Parental effects" can be defined as any process mediated by the parent(s) that has an impact on the developing phenotype of the offspring. In order to gain a more complete picture of the evolutionary context of secondary sexual traits, parental effects should be considered as they may have important impacts on the ontogeny and evolution of these traits (Badyaev et al. 2009). One important parental effect in birds is the amount of testosterone deposited into the egg by the female (Schwabl 1993). Embryonic development in eggs with variable testosterone concentration has been shown to predict such important phenotypic traits as begging behaviour (Müller et al. 2007), posthatching growth (Schwabl 1996), immunocompetence (Groothuis et al. 2005; Tschirren 2005; Sandell et al. 2009), aggression (Partecke and Schwabl 2008), and secondary sexual signals (Galván and Alonso-Alvarez 2010). Many immunological and ecological studies show that testosterone has a distinct immunosuppressive effect (Zuk 1996; Roberts et al. 2004). A well-established correlate of embryonic and foetal testosterone exposure across many vertebrates is the 2D:4D digit ratio (Manning 2002). In birds, high androgen deposition results in elevated digit ratios which have been shown to correlate with morphological, physiological and behavioural traits (Burley and Foster 2004; Navarro et al. 2007; Cain et al. 2013). The House Sparrow (*Passer domesticus*) is a promising model species for investigating secondary sexual signals in their evolutionary context for several reasons: it has a well-studied sexual signal (the male badge), it occurs along different environmental matrices, it has low rates and distances of dispersal (Anderson 2006) and offers scope for studying adaptation to local selection pressures (Loiseau et al. 2009). The highly variable badge size of male house sparrows has been the target of a number of investigations over the last three decades, e.g., (Møller 1987; Griffith et al. 1999; Laucht and Dale 2012), though the function and condition-dependence of this melanin-based signal remain controversial: One meta-analysis (Nakagawa et al. 2007) confirmed its importance in social signaling to establish hierarchies, as reported by a number of studies Møller (1987); Poiani et al. 2000; Buchanan et al. 2010), but found weak evidence that it is actually a sexually-selected signal. Although the ability to mount an immune response has been linked to badge size on several occasions (Navarro et al. 2004; Buchanan et al. 2003), remarkably few studies have established correlations between badge size and parasite load. Indeed there are two mechanisms by which parasites could influence badge size in natural populations: (1) direct physiological trade-offs, or (2) alteration of dominance hierarchies, which has been shown to impact badge size at the following moult (Dolnik et al. 2010).

In this study, avian malaria was our disease of choice as it is one of the most prevalent and best investigated avian vector-transmitted diseases. Avian malaria infections can be caused by by three genuses of apicomplexans: *Haemoproteus*, *Plasmodium* and *Leucocytozoon* which are transmitted by hippoboscid flies, *Culex*-mosquitoes and blackflies respectively. The considerable heterogeneity in transmission of vector-transmitted diseases across time and space in conjunction with the high prevalence in affected bird populations across the world make these infections a major source of divergent selection pressures to host populations that drive genetic evolution (Bonneaud et al. 2006; Randolph and Rogers 2010; Marzal et al. 2011). To explore the effect of malaria and developmental testosterone exposure on badge size we needed sufficient sample sizes of birds that differ in their infection status but should otherwise experience a similar environment. The Camargue, a region in the Rhône delta of southern France known for its high mosquito prevalence during the summer months (Ponçon et al. 2007) provides an ideal environment for investigating such effects because some areas have been subjected to mosquito control for several decades, whereas other areas have been protected from these control programs (Poulin 2012). We reasoned that since regular large-scale insecticide treatment alters vector densities (Ponçon et al. 2007), this should be reflected in the avian malaria prevalence of house sparrows. We attempted to use this environmental difference to set the male badge into its ecological context, and address the links between risk of malaria infection, male badge size, and foetal testosterone-exposure (see Fig. 1 for a schematic representation).

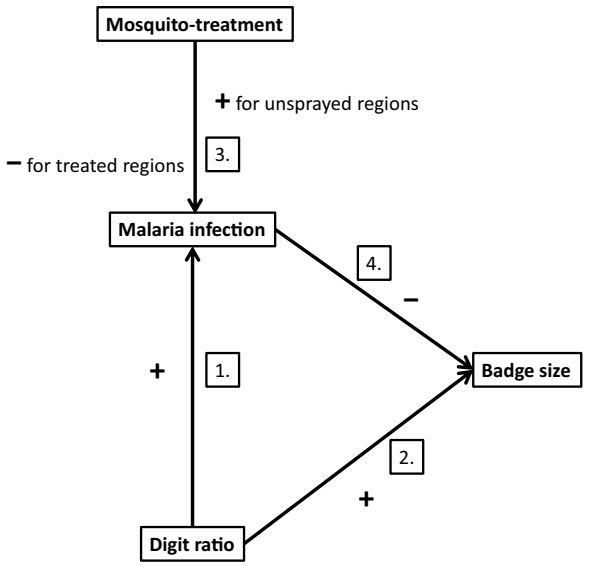


**Fig. 1** Model describing the hypotheses investigated in this study. Numbers in the figures correspond to processes hypothesized to take place: *1.* High prenatal testosterone exposure, which increases digit ratio has been shown to reduce immunocompetence and hence increases the risk of being infected by malaria, *2.* at the same time birds with higher digit ratios have been shown to have a larger badge size, *3.* spraying insecticide reduces vector abundance which in turn has a negative impact on malaria prevalence in a population. The opposite is true in unsprayed, natural areas, and *4.* malaria in turn may affect badge size through trade-offs due to the effects of the parasite or by provoking an immune response

## Methods

### Study site

The Camargue is characterized by a Mediterranean climate with dry and warm summers and mild winters. For this study, two regions, about 20 km apart, were selected based on habitat similarity but differing in their mosquito control treatment (Fig. 2). The mosquito-controlled region was situated south-east of the protected area Etang de Scamandre and is characterised by a large abundance of rice fields, cattle and horse meadows and water channels. Mosquito control has occurred in this region at regular intervals since the 1970s and although the insecticide campaign targets the vector of human malaria and West-Nile virus, there is some evidence that the two local *Culex* species, the vectors of *Plasmodium* in birds, are equally affected by the treatment (Ponçon et al. 2007). The unsprayed region we studied was located east and north of the Etang de Vaccarès and has never experienced mosquito control as it is under regional protection. The land surface cover of the mosquito-treated region is very similar, although agricultural activities are more limited. The landscape in both regions is characterized by large isolated agricultural exploitation units ("mas" and "domaines") many of which sustain substantial sparrow colonies.

In each region four sparrow populations were chosen on basis of similarity of the environment, and of sufficient size of the sparrow colony to allow easy capture. In the controlled region, these sites were: the Centre de découverte Scamandre; Scamandre-Alentours; Domaine de Canavère; and Domaine de de la Fosse. In the natural region we caught sparrows at Tour du Valat; Mas du Pont de Rousty; Armellière; and Romieu. Fieldwork was carried out daily from the 8th of July to 14th of August 2011, a period which encompassed much of the breeding season of the sparrows in the Camargue and in which birds might be particularly susceptible to infections (Sheldon and Verhulst 1996). In total we caught 163 adult males, 78 in the mosquito-treated region and 85 in the natural zone.

### Morphological measures and blood sampling

When adult male house sparrows were captured we immediately took a small blood sample (<50 μl) via the brachial vein, and stored it in 90 % ethanol. Following the blood sampling we weighed each bird and measured three aspects of their badge with a digital caliper (to the nearest 0.01 mm). These three different measures were picked to account for the dichotomy of visible and total badge size, and were as follows: (1) the maximum length of the badge from the base of the bill to the maximal extent of the black feather on the breast, (Griffith 2000), (2) the directly visible width of the badge allowing us to calculate visible badge size (VBS) and (3) the total width of the badge (also defined by feathers with a dark base but clear tips at the edge of the badge; Møller and Erritzøe 1992) for calculating total badge size (TBS). We used the formula given by (Møller 1987) to calculate the actual size of the badge. We measured the digit ratio following the method of (Navarro et al. 2007): the extended right foot of the bird was gently pressed against a piece of cardboard and both ends of the toe were marked with holes punched into the cardboard with a teasing needle. The distance between the holes was then recorded with a digital caliper.

### PCR parasite screening

In the laboratory we thoroughly dried a small fragment of the ethanol preserved blood before extracting the DNA using DNeasy blood and tissue kits (Qiagen). We encountered some problems with clotted and dried blood failing to lyse properly in the initial step of DNA extraction, so each dried blood pellet was homogenized for 30s in 200 μl of PBS using a Tissuelyser (Qiagen) prior to extraction. The DNA was then extracted according to the manufacturer's guidelines. For each extracted DNA sample

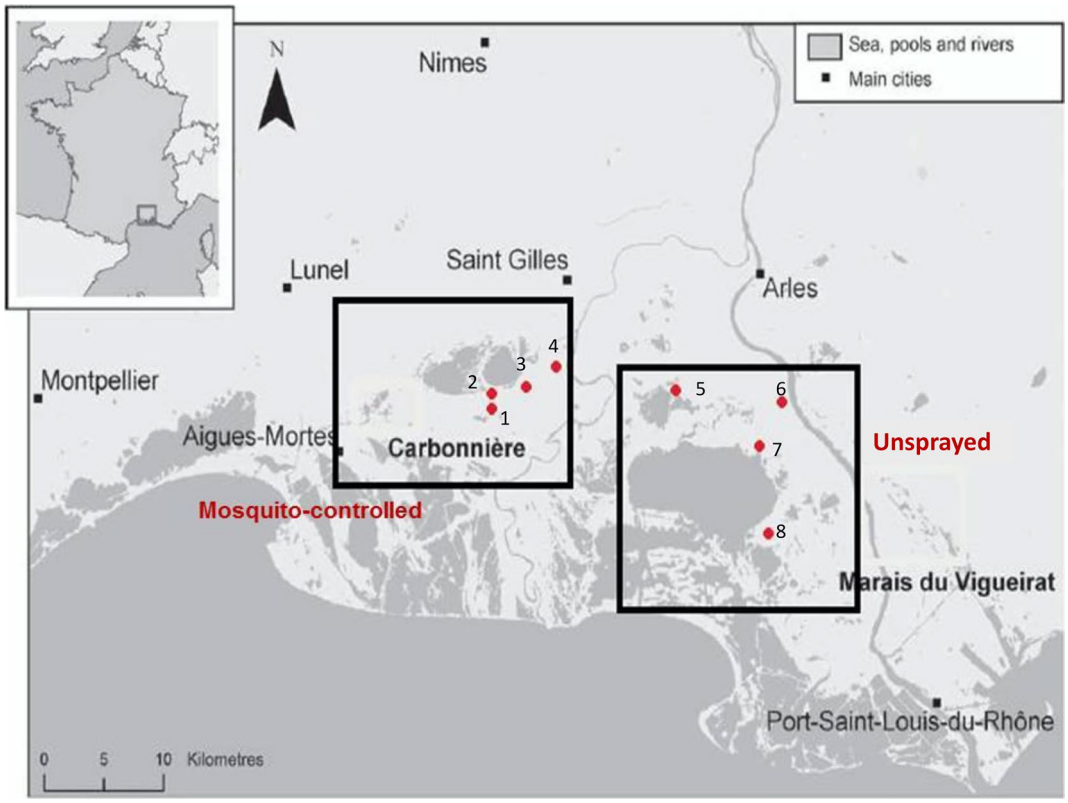


**Fig. 2** Map of the study area. The *left square* is roughly encompassing the mosquito-controlled and the *right square* is highlighting the natural, uncontrolled zone. The *numbered dots* within each square are the location of the different sparrow colonies in this study: *1* Scamandre-Alentours, *2* Centre de découverte du Scamandre, *3* Domaine de Canavère, *4* Domaine de la Fosse, *5* Mas du Pont de Rousty, *6* Château Amellière, *7* Romieu, *8* Tour du Valat (map adapted from Ponçon et al. 2007)

we used a nested PCR approach developed by (Waldenström et al. 2004) to identify *Plasmodium*/*Haemoproteus* infections. We did not test for *Leucocytozoon* infection as previously we found no evidence of *Leucocytozoon* in Camargue sparrows (unpublished data). Both reactions were performed in 25 µl volumes. The first reaction contained 2 µl of genomic DNA, 0.2 µM of each primer (HaemNF [5′-CATATATTAAGAGAATTATGGAG-3′] and HaemNR2 [5′-AGAGGTGTAGCATATCTATCTA C-3′]) and 12.5 µl MangoMix (Bioline) PCR mastermix. The second reaction contained 1 µl of product from reaction 1 with 0.2 µM of each primer (HaemF [5′-ATG GTGCTTTCGATATATGCATG-3′] and HaemR2 [5′-GCATTATCTGGATGTGATAATGGT-3′]) and 12.5 µl MangoMix PCR mastermix (Bioline). All PCR runs contained several known positive controls (from blue tits) and negative controls (water). Infections were identified by the presence or absence of bands following the PCR. In order to check that absence of bands was definitely a lack of infection, we used another PCR with universal primers to test that the avian DNA in each sample successfully amplified and any negative samples for malaria infection were repeated to confirm the diagnosis. All positive samples were sent for sequencing (LGC Genomics, Berlin, Germany), and sequences were then edited using Genious (v5.5.6, Biomatters Ltd). All edited sequences were compared to known avian malaria sequences in GENbank and the MalAVi database. We also examined all of the sequences for signs of double peaks associated with mixed infections (Wood et al. 2007).

### Statistical analysis

All statistical analysis was performed in R v.2.15.1. and in SAS (9.1.3 SAS Institute, Cary, USA). Sample size differs for the different tests performed due to missing values. Differences in infection status between treatment regions and sites were tested using a generalised linear model (GLM) defining a binomial error structure. Badge size and digit ratio between infected and uninfected birds was compared with a two-sample t test. We assessed the relationship between digit ratio and badge size using linear regression. Differences in badge size between sites were compared using analysis of variance. Because we found significant differences in badge sizes between sites, we

included capture site as a random effect in models where badge size was the dependent variable. To do so, we used generalised linear mixed models (GLMMs) with the lme function of the lme4 package in R v.2.15.1. The most complex model included digit ratio, infection status and bird condition (tarsus and weight) and their interaction as fixed effects and capture site as a random effect. We then performed sequential model simplification, dropping the least significant term until the minimal suitable model was reached (Crawley 2007).

## Results

### Malaria prevalence

Contrary to our expectations, there was no significant difference in the prevalence of infections between regions or sites experiencing different insecticide treatments (treatment regions: $z = 0.449$, $p = 0.653$; sites: $z$ min $= 0.131$, $z$ max $= 1.072$, $p$ values $min = 0.28$, $max = 0.89$). Prevalence was high in both regions, with 78.4 % of birds infected in the sprayed zone and 81.3 % infected in the unsprayed zone, and within-site prevalence ranged from 70 to 90 % in both zones. The most prevalent strain at each site was *Plasmodium relictum* SGS1 (82.3 % of all infections), followed by *Plasmodium relictum* GRW11 (5.8 % of all infections), PADOM5 (0.025 %), COLL1 (0.017 %) and P5 (0.0084 %). The absence of a difference in prevalence between regions is an indication that large-scale mosquito control has no significant effect on malaria occurrence in sparrow populations. For the purposes of this study we assume that pathogen-imposed selection pressures in both habitat types are very similar, contrary to our initial prediction.

### Badge size

The range of badge sizes observed in males was substantial (276.2–980.2 $cm^2$) for both visible and total badge size. With a repeatability of 0.936, badge measures were highly repeatable ($F = 30.45$, $p < 0.001$, $n = 163$). Different sites comprised males that differed significantly in badge size, highlighting the importance of local effects due to genetic or environmental differences (VBS: $F = 3.485$, $p = 0.0172$, $n = 158$, TBS: $F = 3.391$, $p = 0.00217$, $n = 158$). However that difference was not directly due to the infection status of birds because infected and non-infected birds did not differ in badge size even when the site of capture was controlled for as a random effect (VBS: $t = 0.57$, $p = 0.56$, $n = 147$, TBS: $t = 0.21$, $p = 0.83$, $n = 147$, Fig. 3). Since the infections were so strongly composed of one parasite strain we could not test for differences in badge size mediated by different strains. To test whether differences in badge size between infected and non-infected birds are condition-dependent, we constructed a GLMM including weight, tarsus length, digit ratio and infection status as well as all their interaction as fixed effects and capture site as a random effect. After sequential simplification we found that visible badge size varied with the interaction between infection status and tarsus ($t = 2.10$, $p = 0.0379$, $n = 124$) and the interaction between digit ratio and weight ($t = 2.12$, $p = 0.0364$). Total badge size changed solely by the interaction between infection status and tarsus ($t = 2.34$, $p = 0.0206$, $n = 145$). Interaction plots revealed that visible and total badge size declined with tarsus but less so if birds were infected. Visible badge size also changed with weight depending on the size of

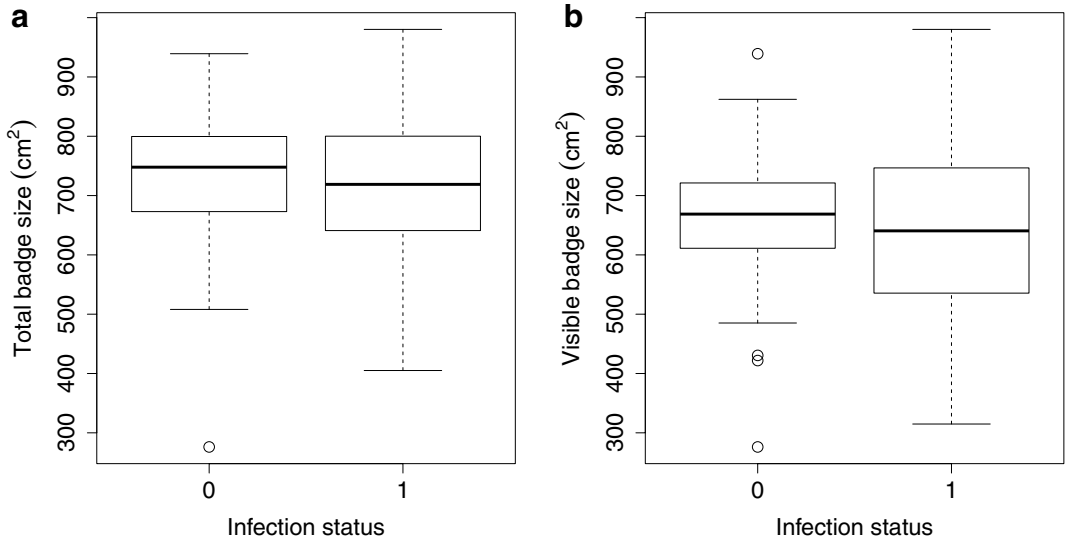


**Fig. 3** Variation of total badge size (**a**) and visible badge size (**b**) with infection status (*1* means infected, *0* means uninfected). *Horizontal line* represents median, *top of box* 75th percentile, *bottom of box* 25th percentile, *whiskers* 1.5 times interquartile range, *dots* outliers

digit ratio (Fig. 4). In contrast to findings by others, we could not find any direct correlation between digit ratio and badge size, even when sites were controlled for as a random effect (VBS: $t = 0.545003$, $p = 0.5867$, $n = 140$; $TBS : t = 0.599513$, $p = 0.5499$, $n = 140$) (Fig. 5). Infected and non-infected birds also had a similar digit ratio, suggesting that maternal testosterone deposition may not affect malaria susceptibility by reduced immunocompetence ($t = 0.38$, $p = 0.702$, $n = 131$, digit ratio measures were square-root-transformed). Including sites as a random effect did not change the relationship.

## Discussion

In this study we tested if a secondary sexual signal of male house sparrows (badge size) is connected to avian malaria infections and to a trait related to maternal testosterone deposition (digit ratio). Although there were significant differences in badge size between sites,

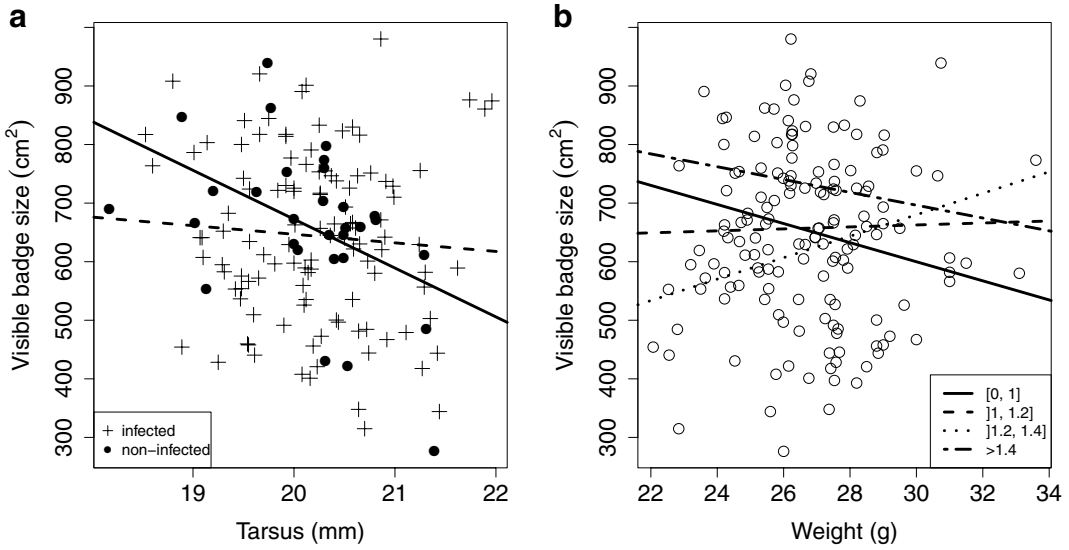


**Fig. 4** **a** Interaction plot of visible badge size against infection status and tarsus, infected birds being marked with a *cross* and *dashed line*, uninfected ones with *solid dot* and *solid line*, **b** plot illustrating the interaction between digit ratio and weight on visible badge size with different sizes of digit ratio marked with *different lines* (see legend)

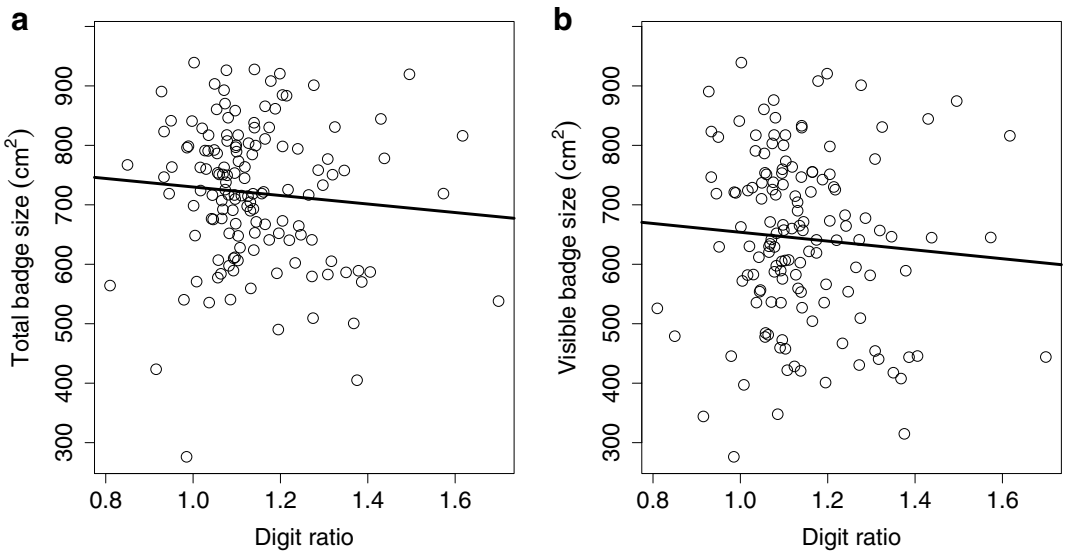


**Fig. 5** Variation of total badge size (**a**) and visible badge size (**b**) with digit ratio. The *lines* represent the lines of best fit, assuming a linear correlation

infected and non-infected birds had no significant difference in badge size and digit ratio. Furthermore there was no direct relationship between digit ratio and badge size, which stands in contrast to other studies that looked at the same traits. Our results offer rather a more complex picture where both visible and total badge size correlate with the interaction between infection status and condition of the bird (tarsus and/or weight) and where the latter may also interact with digit ratio. This observation supports results by Laucht and Dale (2012) who find that badge size is dependent on tarsus and weight but we add the additional insight that those two variables may also interact with malaria infections and maternal testosterone deposition. We also found that the interaction between digit ratio and weight correlates with visible badge size. Visible badge size is achieved by wearing-off of the white feather tips located at the edge of the badge, which is a function of bird activity (Anderson 2006). The maternal testosterone environment has been reported to have an effect on bird aggression (Partecke and Schwabl 2008) and weight may be correlated with fighting ability (Liker and Barta 2001), all-in-all having an effect on the abrasion of feathers.

In contrast to our expectation that reduced vector abundance in mosquito-controlled regions would lead to a lower prevalence of malaria, we found no differences in malaria prevalence between any sites. The high prevalence of *Plasmodium* in both treatment zones is very similar to that reported from two other studies in this area (Loiseau et al. 2011; Bichet et al. 2014) and seems to be among the highest prevalences observed for house sparrows, at least across France (Bichet et al. 2014). The fact that large-scale vector treatment has no effect on the prevalence of a vector-transmitted disease in this wild population may have several explanations. First, based on previous evidence in the Camargue (Ponçon et al. 2007), we assumed that insecticide spraying predominantly directed against *Aedes caspius* has an equal effect on *Culex* mosquitoes. However, we did not directly assess the abundance of different vector species in this study to confirm the effects of insecticide treatments on different mosquito species at each study site and insecticide resistance is known to occur in *Culex pipiens* near our study area (Lenormand et al. 1999; Labbé et al. 2005). Assessment of location-specific abundance of mosquitoes in relation to mosquito-control programmes is an ongoing project for our research group. Secondly, even assuming that there was a difference in *Culex* abundance between sites, it is possible that there is an asymptotic relationship between malaria prevalence and vector abundance. In this case, despite reduced numbers of vectors prevalence of disease in the host population would remain unaffected, a possibility already recognized by Ross (1910). Addressing the links between malaria prevalence and mosquito abundance at the sites we used in this study remains a topic for further investigation. Finally, other variables associated with the vector-controlled zone, most notably the insecticide itself or agriculture-associated pollutants that region which also sustains higher agricultural use, could weaken the sparrows' immune system and increase malaria susceptibility, as previously shown by Bichet et al. (2013), though that effect was most evident in sparrows of highly urbanised areas.

The findings of our work must be seen in the light of the complexity revealed by previous work on sparrow badge size development and avian malaria. First, there is a clear possibility for a temporal mismatch between badge development and timing of infection. Badge development probably depends on the condition of the bird at moult (Moreno-Rueda 2010) whereas infection may have occurred at any time until two weeks before the bird was caught (the limit of detection of malaria blood stages is generally two weeks post-infection; Valkiunas 2004). Some evidence suggests that wild birds are able to clear malaria infections (Knowles et al. 2011), so if there really is a trade-off between badge size and malaria infection, it would be valuable to investigate the infection status at moult (i.e. in the late summer/autumn) and assess badge size the following year. However, data from other birds in the same population suggests that the males in this study were probably carrying chronic infections which they were likely to have maintained for a long time (Larcombe, unpublished data). This temporal mismatch can also occur over longer periods: a "snapshot" where a correlation is established between badge size and other factors at a distinct point in time inevitably ignores some crucial information. This picture is complicated by the fact that badge size can change substantially from one breeding season to another according to factors like paternal investment (Griffith et al. 1999) and diet (Veiga and Puerta 1996) or due to factors that influence the whole cohort like population size and weather (Jensen et al. 2006). It is imaginable that males that experience multiple malaria infections during their lifetime would trade-off badge size development in time rather than through direct physiological trade-offs. The exact age of the birds in this study was not known, although age has been shown to correlate with badge size (Nakagawa et al. 2007; Roberts et al. 2012) and susceptibility to infection (Mackinnon and Marsh 2010; Bichet et al. 2014). Measuring both badge size and infection status over the course of a life-time may account for these differences, however, in wild populations this is technically extremely difficult to accomplish.

Badge size has been shown to be sensitive to many environmental effects, as suggested in this study by the significant differences in badge size between capture

sites. Jensen et al. (2006) showed the existence of strong cohort effects on badge size, determined by weather and density-dependence during the early development. The sparrow populations in this study may have differed in e.g. food availability, population size or age structure, which could influence the observed inter-site differences. As badge size has a hereditary component and as there is only little dispersal between sparrow colonies (Griffith et al. 1999; Anderson 2006), genetic drift may play some role in badge size, though Loiseau et al. (2009) reckon that genetic differences at such small scales should be unimportant. There are many other potentially important variables we did not investigate in our study, for example current plasma testosterone levels in the blood (Gonzalez et al. 2001; Buchanan et al. 2010; Laucht et al. 2011), nutritional status (Veiga and Puerta 1996) and social environment (Laucht and Dale 2012), all of which could interact with infection status to affect badge size. Furthermore, an avian malaria infection is not just characterised by the presence of the parasite in the blood: males with higher levels of parasitemia may face bigger trade-offs, a fact we could not control for by using absence/presence data for infections. However, since parasitemia is not constant during the course of an infection but is characterised by an initial acute phase before dropping to low levels with occasional relapses, it remains unclear how informative single measures in time of parasitemia taken from adult birds would be Cornet et al. (2014). Co-infections by other parasites may also play an important role: if badge size represents an integrative signal of the overall long-term parasite burden of an individual, as has been shown for bill colour in blackbirds (Biard et al. 2010), malaria status on its own may be insufficient in influencing badge size significantly. Finally, there is a clear possibility that the chronic nature of many malarial infections don't pose enough of a challenge to the bird for that trade-off to be visible in sexual signals, especially since there is some evidence that avian malaria prevalence is not directly associated with the elaboration of sexual signals in birds (Garamszegi and Møller 2012).

Egg-testosterone deposition, measured by the digit ratio, has been reported to correlate with numerous advantages like increased growth rate, accelerated embryonic development and higher social rank (Mazuc et al. 2003). A potential cost of testosterone deposition may be decreased immunocompetence (Navarro et al. 2007; Sandell et al. 2009). We found no evidence for this in this study, as there was no difference in digit ratio between infected and non-infected birds. Explanations similar to those provided above for badge size may also account for the lack of effect of digit ratio on infection. However, badge size did not correlate directly with digit ratio in this study, which contradicts the results of Navarro et al. (2007) who found a positive correlation between visible badge size and digit ratio. The absence of a direct correlation in this study with a larger sample of males than (Navarro et al. 2007) shows that a positive relationship between digit ratio and badge size is far from universal and may be season or population-specific and more importantly, that digit ratio may interact with bird weight as found in this study. Seasonal effects may be especially relevant for visible badge size, the size of which increases with increasing time from moult (Anderson 2006). This study highlights one of the problems in finding evidence for the hypotheses explaining maintenance of sexual signals: though trade-offs between disease and sexual signals are probably present, they may be very hard to observe due to the many confounding variables and varying timescales at which they operate. This study makes this case for avian malaria in house sparrows. We showed that infection status and maternal effects may have a condition-dependent effect on the house sparrow badge.

**Authors' contributions**

PLGB did most of the fieldwork but with considerable support from SDL, who also performed most of the labwork. Both authors read and approved the final manuscript.

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**Competing interests**

Both authors declare that they have no competing interests.

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