THE UNIVERSITY *of* EDINBURGH

The Evolutionary Ecology of Reproductive Strategies in Malaria Parasites

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Lay Summary

Malaria parasites cause devastating disease of huge global medical and economic importance. In order to infect new hosts, malaria parasites need to produce specialised male and female stages, which are taken up by mosquitoes while blood-feeding. Mating then occurs within the mosquito and the resulting offspring are eventually transmitted to new hosts through infective bites. Despite several decades of biomedical research, the basic biology and behaviour of parasite reproduction remains unclear. This thesis investigates the complex strategies that malaria parasites employ to maximise their survival during an infection in the human host as well as during their subsequent transmission between hosts. Currently, the trigger for producing male and female stages is not known, neither is how many, or how often they are produced. Furthermore, how males find females within the hostile environment of the mosquito gut is not known. My approach covers two themes which bridge the scales between parasite strategies within the host and their mating tactics in the mosquito vector; both of which have important implications for the spread of disease. Using rodent malaria parasites as a model system, I conducted experiments to:

1. Identify factors that cause parasites to change their production of male and female stages.
2. Use holographic 3D microscopy (in collaboration with the Physics community) to identify the shape and swimming dynamics of male malaria flagella, and discuss the implications for mating success in the mosquito.
3. Show that high densities of red blood cells in the mosquito bloodmeal are likely to hinder successful mating.
4. Reveal that male gametes move non-randomly through the bloodmeal.
5. Demonstrate variation in the factors that initiate mating within the mosquito.

The data presented here advance our understanding of malaria parasite reproduction and could have important implications for disease control, by providing new avenues for the development of interventions, as well as maximising the effectiveness of existing strategies for control.

Abstract

For vector-borne parasites such as malaria, how within- and between-host processes interact to shape transmission is poorly understood. In the host, malaria parasites replicate asexually but for transmission via mosquitoes to occur, specialized sexual stages (gametocytes) must be produced. Once inside the mosquito vector, gametocytes immediately differentiate into male and female gametes, and motile male gametes must swim through the hostile environment of the bloodmeal to find and fertilise female gametes. Despite the central role that gametocytes play in disease transmission, explanations of why parasites adjust gametocyte production in response to in-host factors remain controversial. Furthermore, surprisingly little is known about the mating behaviour of malaria parasites once inside the mosquito. Developing drugs and/or vaccines that prevent transmission by disrupting sexual stages are major goals of biomedicine, but understanding variation in gametocyte investment and male gamete behaviour is key to the success of any intervention.

First, I propose that the evolutionary theory developed to explain variation in reproductive effort in multicellular organisms provides a framework to understand gametocyte investment strategies in malaria parasites. I then demonstrate that parasites appear to change their reproductive strategies in response to environmental cues and in a manner consistent with our predictions. Next, I show how digital holographic microscopy can be used to characterise the morphology and motility of male gametes. I then provide evidence for non-random movement of male gametes and that gamete interactions with red blood cells appear to hinder mating success in a bloodmeal. Finally, I discuss the variation in gametocyte differentiation and fertilisation success when exposed to a number of factors implicated in gametocyte activation. The data presented here provides important information on the basic biology of malaria parasite reproductive stages and demonstrates considerable variation in parasite traits and behaviours in response environmental changes; both in the host and in the mosquito vector.

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Signed Declaration

I confirm that I composed this thesis. The work submitted is my own, except where it has formed part of jointly-authored publications. My contribution to each chapter and that of other authors has been explicitly indicated below. I confirm that appropriate credit has been given within the thesis where reference has been made to the work of others.

Chapter 2

This chapter is published as: Carter LM, Kafsack BFC, Llinás M, Mideo N, Pollitt LC, Reece SE (2013) Stress and Sex in Malaria Parasites: Why Does Commitment Vary? *Evolution, Medicine, and Public Health*, 2013: 135–147, doi:10.1093/emph/eot011.

I wrote this manuscript which reviews the literature and interprets it in a new way, and Laura Pollitt, Nicole Mideo, Björn Kafsack, Manuel Llinas and Sarah Reece contributed data and ideas.

Chapter 3

This chapter is published as: Carter LM, Schneider P, Reece SE (2014) Information use and plasticity in the reproductive decisions of malaria parasites. *Malaria Journal* 13:115, doi:10.1186/1475-2875-13-115.

The experiments were designed by me with help from Petra Schneider and Sarah Reece. Aidan O'Donnell and two honours students: Michael Lazaris and Caroline

Hosking assisted with data collection. I analysed the data and wrote the manuscript with contributions from Petra Schneider and Sarah Reece.

Chapter 4

The experiments were designed and the data analysed by Laurence Wilson, Sarah Reece and myself. Roni Mooney assisted with data collection. Sarah Reece and I wrote section 4.3, which formed the large supplementary information section (designed to explain and interpret the results of a biophysics study for parasitologists) for the following publication: Wilson LG, Carter LM, Reece SE (2013) High-speed holographic microscopy of malaria parasites reveals ambidextrous flagellar waveforms. *Proceedings of the National Academy of Sciences*, doi: 10.1073/pnas.1309934110.

I analysed and wrote sections 4.4 and 4.5 with the help of Sarah Reece and Laura Pollitt.

Chapter 5

The experiments were designed by Sarah Reece and myself. Roni Mooney assisted with data collection. I analysed the data and wrote the chapter with the help of Sarah Reece and Joanne Thompson.

This work has not been submitted for any other degree or professional qualification.

Signed: Date:

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1 General Introduction

1.1 Disease Ecology

The evolutionary ecology of infectious diseases has attracted increasing interest over the past 30 years (Williams and Nesse, 1991, Read and Taylor, 2001, Poulin, 2007, Harrison, 2007, Stearns and Koella, 2008, Leggett et al., 2014, Lively et al., 2014) (figure 1.1). Pathogens (broadly defined as viruses, bacteria and eukaryotic micro- and macro parasites) are responsible for huge morbidity and mortality of humans, livestock and crops worldwide, and are ubiquitous across a wide range of ecosystems. Despite several decades of interventions, evolution has continually eroded efforts to control their spread. For example, drug resistance in pathogens and insecticide resistance in their vectors threaten to reverse many of the advances achieved in the last century (French, 2005, Klein et al., 2012). Therefore, integrating evolutionary theory with mechanistic approaches is key to understanding and predicting the spread of diseases through populations over space and time (Foster, 2005, Restif, 2009, Mideo and Reece, 2011). Furthermore, being able to predict how virulence and transmission varies according to a range of possible interventions could enable an informed choice of the most "evolution-proof" strategy (Williams, 2010). However, this ambition is precluded by a poor understanding of how ecological interactions affect parasite life history traits and behaviour.

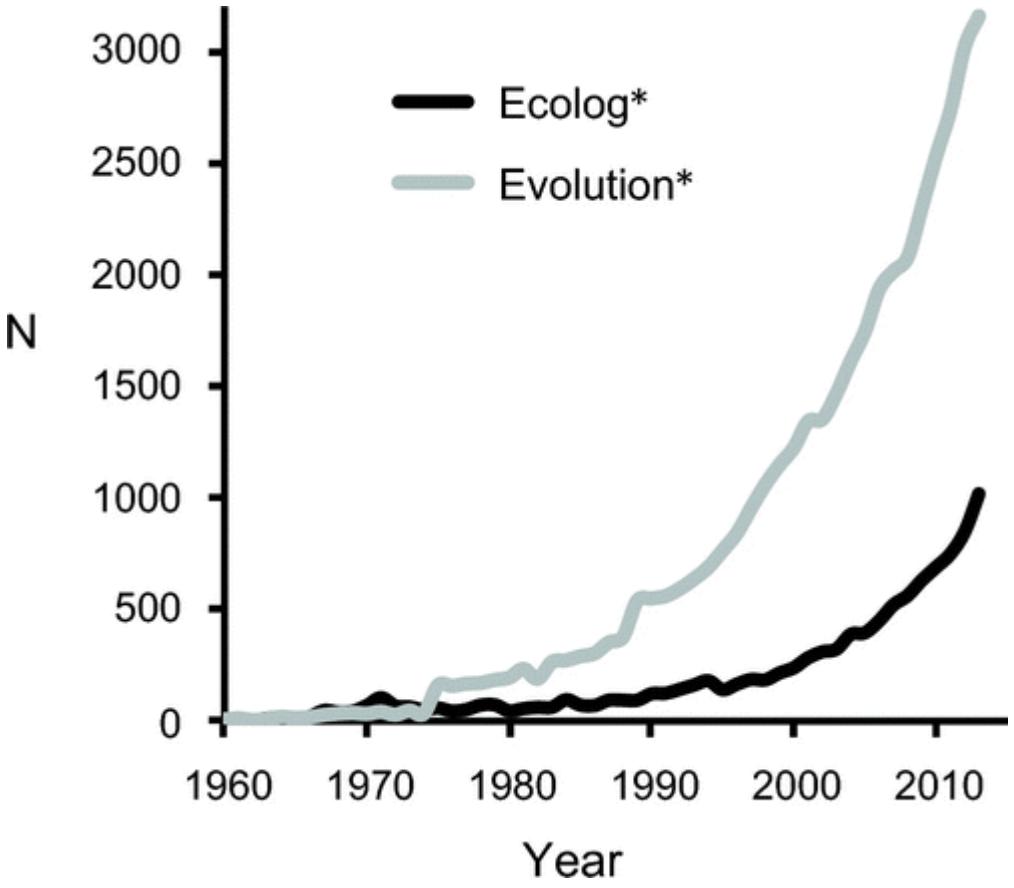

Figure 1.1. Increasing research effort on the evolution and ecology of disease. Taken from (Lively et al., 2014). N: Number of publications per year, as revealed by a PubMed advanced search with the term "disease" and with either the term "ecolog*" or "evolution*," where the asterisks indicate the inclusion of any alternate endings to the words.

Historically, research has focussed on the mechanistic aspects of within-host traits of pathogens (using imaging, cell and molecular biology approaches), but evolutionary ecology is increasingly being integrated into the study of within-host traits and behaviours (Harrison, 2007, Graham, 2008, Reece et al., 2009) (chapter 2). Evolutionary theory can provide a framework for understanding variation in the parasite traits that underlie disease virulence and transmission (Paul et al*.,* 2003, Poulin, 2007, Reece et al*.,* 2009). So far, this interdisciplinary effort has revealed a range of interesting interactions between pathogens and with facets of their within-

host environment (e.g. resource availability, presence of competitors, host immune responses and anti-parasite drugs). These interactions are known to have a significant impact on the growth, virulence and transmission of pathogens throughout populations (Levin et al., 1999, Read and Taylor, 2001, Lloyd-Smith et al., 2005, Harrison, 2007, Graham, 2008, Reece et al., 2009, Matthews, 2011, Lopez et al., 2011, Mideo and Reece, 2011, Stearns, 2012).

However, two factors that are essential for determining patterns of virulence and transmission have been overlooked: i) the extent of plasticity in these traits and behaviours in variable environments and ii) the relevance of between–host interactions. Regarding the first point, theoretical approaches have provided a predictive framework to understand plasticity in parasite strategies (Alizon and van Baalen, 2008, Mideo et al., 2008), but explicit experimental tests of these are lacking. In chapters 2 and 3, I address this issue and test the effect of environmental manipulation on parasite reproductive strategies. Regarding point ii), between host factors are many and varied; they include the availability of susceptible hosts, and the mode (i.e. horizontal vs vertical) and route (e.g. vector vs direct) of transmission (Poulin, 2007, Boots and Mealor, 2007). Many medically important parasites are transmitted between their hosts via vectors (e.g., *Plasmodium* (Baton and Ranford-Cartwright, 2005), *Trypanosoma* (Krafsur, 2009), *Leishmania* (Killick-Kendrick, 1990), *Brugia*, *Onchocerca*, and *Schistosoma* (Matthews, 2011)). I address the lack of data on within-vector ecology by characterising the biology and behaviour of sexual reproduction within the vector (chapters 4 and 5). Detailed introductions are

provided for each chapter, and the evolutionary concepts underlying my research questions and the relevant biology of my study system are outlined below.

1.2 Life history theory and phenotypic plasticity

Life history theory provides a framework to explain the complex traits of growth, reproduction and survival observed for a variety of different taxa (Stearns, 1992). Natural selection acts to maximise the fitness of an organism through the expression of advantageous traits. However, due to the numerous costs and constraints that organisms face, there are limits to the possible combination of traits that can be expressed (Roff, 1992, Stearns, 1992). This is in large part thought to be due to resource allocation trade-offs: where resources are invested in one trait at the expense of other traits. The high cost of investing resources into reproduction at the expense of growth (and vice-versa) is the driving force behind one of the most commonly studied trade-offs: between survival and reproduction (Williams, 1966, Stearns, 1992).

Further complications in adopting the best life-history strategy for a given organism arise because the optimal solution is most likely to be dependent on the external environment, and how this varies, both temporally and spatially. Phenotypic plasticity: a process where an organism can produce a range of phenotypic responses under different environmental conditions (i.e., genotypes alter their phenotype in response to environmental cues) is central to understanding the impact of the

environment on trait evolution (Scheiner, 1993). In its broadest form, plasticity ranges from aspects of physiology (including homeostasis and consequences of developmental constraints) (Pigliucci et al., 2006), to quantifiable changes in behaviours, morphologies and phenotypes. Plastic changes can have a wide range of effects on fitness; they can be adaptive, maladaptive or neutral (Pigliucci, 2001, Ghalambor et al., 2007). Whilst there are plenty of examples where plasticity appears to be non-adaptive (with the majority of studies in plants; reviewed in Ghalambor et al. (2007), this thesis and the discussion herein refers to adaptive plasticity; i.e., a mechanism that enables an organism to maintain fitness across a range of environments (Pigliucci, 2001). Plasticity can facilitate the evolution of fixed traits by enabling organisms to quickly adopt the best phenotype in a novel environment (Pigliucci, 2001, Schlichting and Pigliucci, 1998). This can either occur via the assimilation of a plastic trait, or through promoting the survival of an organism, allowing more time for the evolution of a novel trait (Chevin et al., 2010). A simple example of adaptive phenotypic plasticity is when plants avoid shade by elongating their stems in the direction of light (Schmitt et al., 2003). Furthermore, when exposed to predator cues, *Daphnia pulex* (a freshwater crustacean) produces morphological defences (neck spines) (Tollrian, 1995). Adaptive phenotypic plasticity has also been observed in mammals; for example red deer (*Cervus elaphus*) adjust their sex ratio according to population density (Kruuk et al., 1999).

Whilst phenotypic plasticity allows genotypes to rapidly respond to environmental changes, it is also thought to be subject to various costs and constraints (McNamara and Houston, 2009, Reece et al., 2009). For example, the maintenance of

mechanisms required to detect and respond to environmental change are predicted to be energetically costly, exemplified by the negative correlation between plasticity in the shell morphology of the freshwater snail and its growth rate (deWitt et al., 1998). However, broader evidence of the costs of plasticity remains scarce (Auld et al., 2009). Furthermore, genetic variation for life history traits is commonly observed, and differences between genotypes can interact with plastic responses to environmental factors in complex ways (gene- by-environment interactions (G x E)) (Stearns and Koella, 2008). This can generate considerable variation in the phenotypic range of different genotypes and thus, shape evolutionary outcomes. Figure 1.2 demonstrates the reaction norm concept, which provides a framework to understand plasticity and the evolutionary consequences of G x E interactions. Whilst the majority of data and theory on phenotypic plasticity is centred on multicellular organisms, investigating plasticity in parasite phenotypes has largely been neglected. However, parasites share many of the same traits, behaviours, trade-offs and selective forces of multicellular organisms, but how much this matters for survival and disease transmission remains unknown. Understanding when plasticity will facilitate or constrain evolutionary change could have important implications for disease control (chapter 2, Carter et al., 2013).

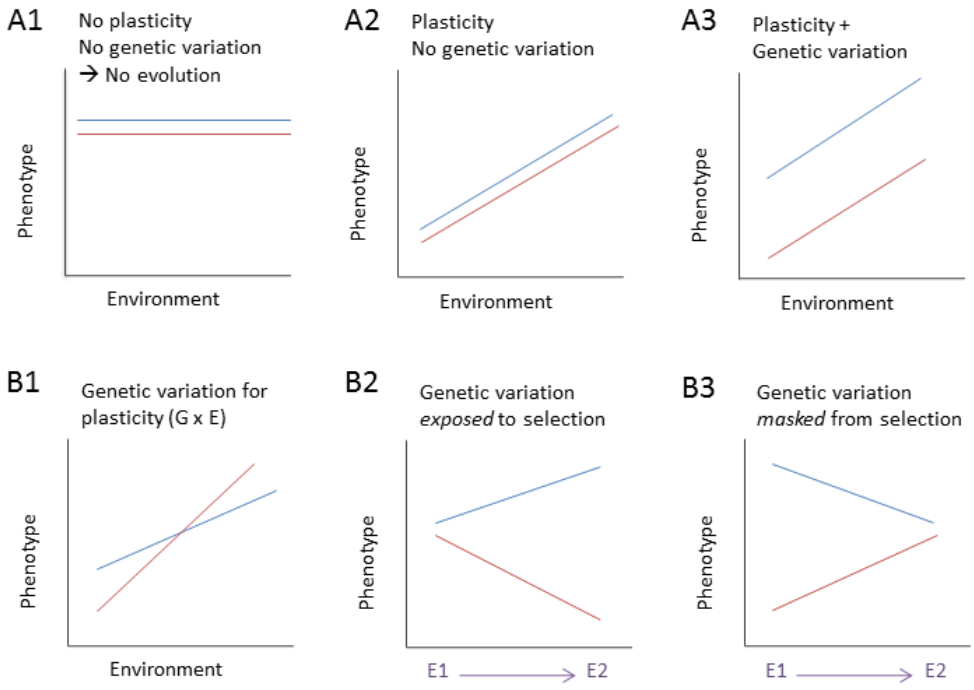

Figure 1.2 Demonstration of the reaction norm concept and phenotypic plasticity. Red and blue lines represent different genotypes. **A**: How the expected phenotype of a given genotype changes as a function of environmental change (Scheiner, 1993). This is used when modelling plasticity for continuous environmental variables. **B**: Examples of the evolutionary consequences of when there is genetic variation for reaction norms. "E1" and "E2" represent two distinct environments. In B2, the shift from environment 1 (E1) to environment 2 (E2) facilitates evolution because the phenotypes of the two genotypes diverge, and selection will act to favour the most appropriate (fittest). In B3, the shift between environments results in the phenotypes of the two genotypes converging and therefore the genetic variation is masked from selection (Leggett et al., 2014).

1.3 Parasites as a model system for testing life history theories

Whilst it is important to understand variation in the traits, behaviours and trade-offs of malaria parasites in their own right - as a deadly parasite - parasites also provide a useful model system for testing the predictions of life history theory. Despite being a central concept to evolutionary ecology, experimental manipulations to explicitly test

the fitness consequences of reproductive investment decisions are scarce, and theory is ahead of the data (but see (Creighton et al., 2009, Cotter et al., 2011)). In multicellular organisms, measuring reproductive strategies is challenging and consistently confounded by environmental variation, numerous aspects of reproductive investment (e.g. parental care) and age related senescence or improvement due to experience (Clutton-Brock, 1984). Parasites offer a simple model system to experimentally test evolutionary theories, as they lack most of these confounding effects (Poulin, 2007). Furthermore, parasites have well-defined and quantifiable traits, which provide an ideal basis for testing the underlying evolutionary theory as well as the mechanisms underpinning both reproductive decisions (addressed in chapters 2 and 3) and mating behaviour (addressed in chapters 4 and 5).

Rodent malaria (*Plasmodium)* parasites provide a particularly good model system in which some of these questions can be investigated. The physiology and immune system of rodents is very well characterised, malaria parasites can be genetically modified and there are numerous imaging, cellular, molecular, and immunological tools and techniques available to test and manipulate parasite traits and in-host environmental variables (Babiker et al., 2008, Wykes and Good, 2009, Guttery et al., 2012, Menard et al., 2013). Results from studying malaria parasite traits can also be applied more broadly to other parasites that reproduce sexually (e.g., trypanosomes) (Pollitt et al., 2011a).

1.3.1 Lifecycle of *Plasmodium*

The complex two-host lifecycle of *Plasmodium* consists of asexual proliferation in the vertebrate host and sexual reproduction in the dipteran mosquito vector (figure 1.3). Infected female *Anopheles* mosquitoes inject *Plasmodium* sporozoites into the skin of the host, from which they enter the bloodstream, travel to the liver and invade hepatocytes (Menard et al., 2013). Sporozoites multiply and differentiate into thousands of haploid merozoites which re-enter the bloodstream and initiate asexual replication within red blood cells (RBCs) (although some species lay dormant in the liver for months before they re-enter the bloodstream) (Markus, 2012).

Within every asexual cycle, a small proportion of asexual parasites commit to developing into developmentally arrested male and female sexual precursor cells, termed gametocytes, which circulate in the peripheral bloodstream of the vertebrate host. When female mosquitoes feed on the host (generally at dawn and dusk) they ingest gametocytes (along with asexual stage parasites and other components of the host infected blood) (Baton and Ranford-Cartwright, 2005). Whilst the 'Hawking hypothesis' suggests that reproductive effort is coordinated so that gametocytes reach maximum infectiousness when mosquitoes feed (Hawking, 1966), at present, there is a lack of data to directly link circadian rhythms in gametocyte and vector biology (Mideo et al 2012). Moreover, differences in cell cycle duration (from 24-72 hours) and gametocyte development times (2 – 14 days) between species (for the rodent malaria parasite *P. berghei* and the human malaria parasite *P. falciparum* respectively) could have a significant impact on the patterns of transmission

observed (Mideo et al 2012), which is further discussed in chapter 2. Immediately upon uptake by the mosquito, gametocytes are triggered to undergo gametogenesis (gamete differentiation); producing haploid gametes (Baton and Ranford-Cartwright, 2005). Female gametogenesis results in a single female gamete emerging from the residual gametocyte-infected RBC, but male gametocytes undergo a process termed exflagellation; producing up to eight flagellated and motile male gametes (microgametes) from each gametocyte (MacCallum, 1897). Microgametes must locate and fertilise the non-motile female gametes within one hour (Carter and Nijhout, 1977, Sinden et al., 2010), forming diploid zygotes. Over the next 18-20 hours, each fertilised zygote differentiates into a motile ookinete, which traverses the midgut wall and transforms into an oocyst. Within each oocyst, the parasites replicate asexually to produce haploid sporozoites, which are released into the haemocoel when the oocyst ruptures. Sporozoites then migrate to the salivary glands and are injected into a new host during the next blood meal the mosquito takes (Baton and Ranford-Cartwright, 2005).

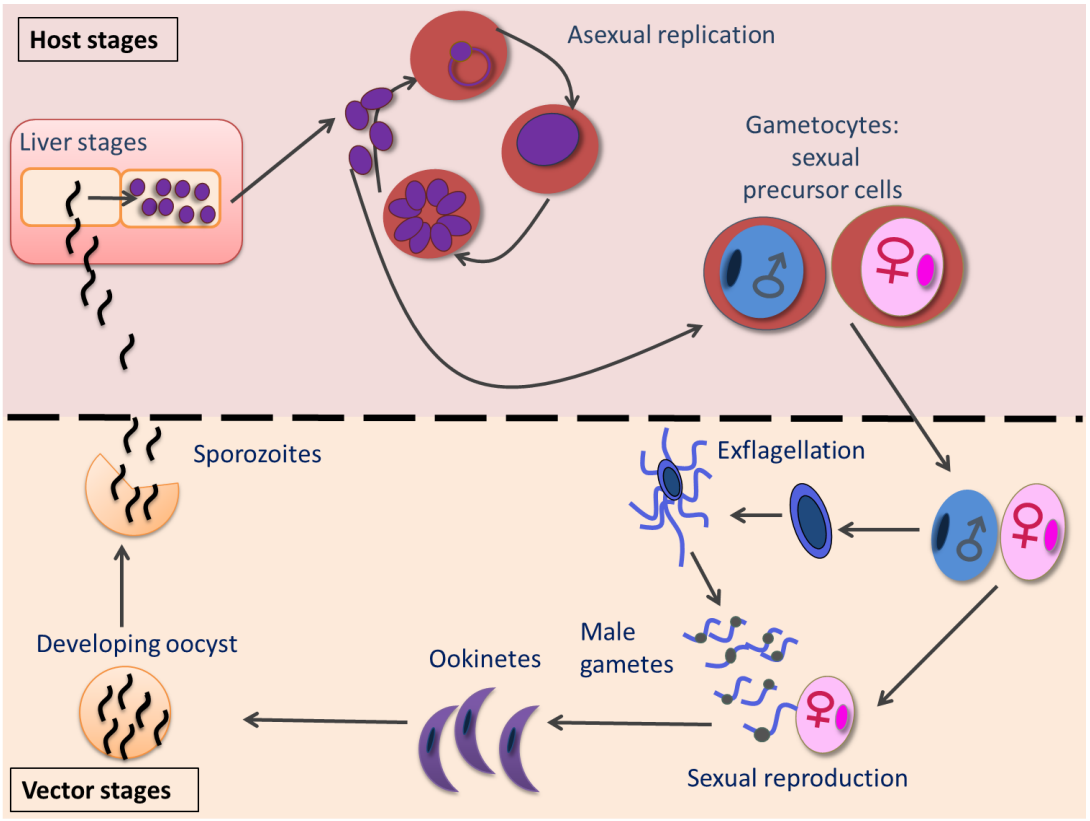

Figure 1.3 The lifecycle of *Plasmodium* parasites consists of asexual replication in the host (upper panel) and sexual reproduction (followed by asexual replication) in the mosquito vector (lower panel). When taking a blood meal from an infected host, mosquitoes ingest gametocytes. After sexual reproduction in the midgut, oocysts form and asexual replication results in large numbers of sporozoites which are injected into the skin of the next host the mosquito bites.

1.4 Ecology of *Plasmodium* reproductive decisions

Whilst life history trade-offs are generally resolved at the level of single organisms, when applied to *Plasmodium* infection dynamics, a single genotype within an infection is analogous to a whole organism, as the target of natural selection (Gardner and Grafen, 2009). As such, the in-host asexual replication stages are representative of growth and survival, whilst the sexual stage gametocytes represent reproduction (Reece et al., 2009) (see figure 1.3 for details of the lifecycle). For malaria parasites, key trade-offs experienced at every cell cycle include the division

of resources between 1) the production of asexually replicating stages (required for in-host survival) *vs.* sexual stage gametocytes (required for between-host transmission), termed reproductive effort; and 2) the production of male vs. female gametocytes. Together, these two trade-offs are referred to as "reproductive strategies". Malaria parasites have been shown to plastically alter their reproductive strategies in response to variation in resource availability, (Drakeley et al., 1999, Paul et al., 2000, Reece et al., 2005, Robert et al., 2003), competition (Dyer and Day, 2003, Wargo et al., 2007a, Reece et al., 2008, Pollitt et al., 2011b), transmission blocking immune factors that affect fertility (Smalley et al., 1981, Paul et al., 1999, Buckling and Read, 2001, Reece et al., 2008), and drugs (Buckling et al., 1997, Buckling et al., 1999a, Wargo et al., 2007b, Peatey et al., 2009, Reece et al., 2010, Sowunmi et al., 2009, Peatey et al., 2013). It is important to understand the reasons underlying this plasticity, because reproductive strategies shape parasite survival within the host (and therefore virulence) and transmission to subsequent hosts (Pollitt et al., 2011a, Mideo and Reece, 2011, Carter et al., 2013) (chapter 2). Being able to predict whether phenotypic plasticity will facilitate or constrain evolutionary change has key implications for the long term efficacy of interventions (Mideo and Reece, 2011, Reece et al., 2009). In chapter 2, I explain the evolutionary concepts behind the effect of environmental "stress" on plasticity in reproductive strategies, summarise the current data and discuss the need for integration between mechanistic approaches and evolutionary ecology (Carter et al., 2013) (chapter 2). Whilst most studies have focused on either the number or sex ratio of gametocytes, evolutionary theory predicts these traits will be simultaneously optimised (Stearns, 1992). Therefore, the data presented in chapter 3 investigates both reproductive strategies. In this chapter, I

explore what cues parasites use to detect and respond to environmental changes and interpret the resulting variation in gametocyte investment and sex ratio in the context of each other (Carter et al., 2014) (chapter 3).

1.5 Sexual reproduction in the vector

Sexual reproduction within the mosquito is essential for the transmission of *Plasmodium* between hosts: when developmentally arrested *Plasmodium* gametocytes enter the mosquito, gametogenesis (the formation of haploid micro- (male) and macro- (female) gametocytes) rapidly ensues. Gametogenesis is common to a number of Apicomplexan parasites such as *Eimeria, Toxoplasma* and *Plasmodium* (Walker et al., 2013). Microgametogenesis involves repeated nuclear division followed by the release of microgametes (flagella) which then fertilise female macrogametes (Walker et al., 2013). Whilst there is a wealth of ultra-structural knowledge for all three organisms, details of the trigger underlying gametogenesis and subsequent mating is lacking for *Eimeria* and *Toxoplasma*, but some lessons can be drawn from *Plasmodium* because a number of molecular mechanisms have been implicated. The expanding knowledge of gene function in malaria gametes (van Dijk et al., 2001, Liu et al., 2008, De Koning-Ward et al., 2008, Ponzi et al., 2009, Straschil et al., 2010, Guttery et al., 2012, Guttery et al., 2014, Eksi et al., 2006, van Dijk et al., 2010) provides an ideal foundation for linking behaviour to mechanism. In addition, early evidence suggests that trypanosomes undergo meiosis and some form of sexual reproduction (mediated by flagella) (Peacock et al., 2014), and so are likely to face many of the same selection pressures

as malaria parasites. However, knowledge of the basic biology and behaviour of microgametes, as well as variation in fertilisation success when exposed to variable environments is significantly lacking.

Surprisingly little is known about how malaria parasites respond to constraints they face during mating. However, with the apparently highly hostile and variable environment (variable RBC densities, immune factors and temperatures) they are exposed to in the mosquito midgut, and their evident success in transmission (Bousema and Drakeley, 2011, Baton and Ranford-Cartwright, 2005), it is likely that parasites have evolved sophisticated strategies to facilitate fertilisation and ensure subsequent ookinete development. Before it is possible to explicitly test for parasite strategies, the fundamental characteristics of parasite biology and behaviour must first be quantified. The ambition of chapters 4 and 5 was to characterise some of the basic features of mating. For example: How do male malaria parasites (microgametes) swim (what is their speed, length, wavelength and swimming direction)? What role do RBCs have for microgamete motility (i.e., do RBCs help or hinder fertilisation success)? And, do microgametes follow non-random paths within the bloodmeal (i.e., are microgametes attracted to female gametes) (chapter 4)? Finally, what factors are important for triggering gametogenesis, how do these affect subsequent ookinete maturation and is there genetic variation in these parameters (chapter 5)?

Vector-parasite interactions are key determinants of parasite fitness, and understanding the details of this process is timely due to the current focus on developing transmission-blocking interventions (Eksi et al., 2006, Ponzi et al., 2009, van Dijk et al., 2010, Guttery et al., 2012). Recent studies suggesting that the accelerated evolution of male-biased genes (compared to universally expressed or female specific- genes), have generated concern that male gametocyte / microgamete- targeted interventions could be particularly vulnerable to parasite counter-evolution (Khan et al., 2013). This emphasises the need for an improved understanding of the basic biology and behaviour of mating within the vector, in order to make interventions as sustainable and as robust to evolution as possible (Williams, 2010).

1.6 Bridging Scales: malaria reproductive strategies

To fully understand and predict the epidemiological consequences of variation in parasite traits and behaviours, linking the within-host and within-vector scales is essential (Alizon and van Baalen, 2008, Mideo and Day, 2008). Clearly, in-host ecology is interesting and important for the evolution of parasite virulence and drug resistance (Paul et al., 2003, Mideo and Reece, 2011). Presumably the ecology of parasite interactions within the vector are equally (if not more-so) important for transmission, and by effecting epidemiology, they can feed back into within-host processes and have important implications for disease control. In addition, vector control programmes select for vector evolution (Gatton et al., 2013), but how this affects parasite evolution remains unclear. Furthermore, with climate change inducing host shifts (Bayoh et al., 2010, Sternberg and Thomas, 2014), unless the

range of phenotypic plasticity of the parasite, and the specific trade-offs the parasites face are quantified, it is impossible to predict how changing vector populations could affect parasite evolution and the spread of disease. In order to fully appreciate the epidemiological impact of variation in traits, the relative importance of host- and vector- derived interactions on parasite behaviour, and how these vary over the course of an infection and between genotypes, must be carefully considered (Poulin, 2007, Matthews, 2011, Mideo and Reece, 2011).

1.7 Thesis outline and aims

Despite the importance of variation in parasite life history traits for understanding their evolution, proximate and ultimate explanations for this variation remain poorly understood. The ambitions of this thesis are to integrate evolutionary ecology, molecular and cellular biology, and biophysics to develop a greater understanding of the transmission of disease. Specifically, this thesis has furthered our understanding of the evolutionary ecology of reproductive strategies of malaria parasites, through the following aims:

i. Examine why parasites adjust investment in gametocytes according to the impact of changing conditions on their in-host survival, and outline the experiments required to test whether plasticity in gametocyte investment enables parasites to maintain fitness in a variable environment (chapter 2).

ii. Identify what factors parasites use to detect environmental changes and make appropriate decisions about investment into gametocytes and their sex ratio (chapter 3).

iii. Quantify the swimming dynamics, mating success in variable environments, and directionality of male malaria gametes (chapter 4).

iv. Investigate variation in gametocyte differentiation and the mating success of parasites when exposed to variable gametocyte activating factors (chapter 5).

2 Stress and sex in malaria parasites. Why does commitment vary?

This chapter is published as:

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2.1 Abstract

For vector-borne parasites such as malaria, how within- and between-host processes interact to shape transmission is poorly understood. In the host, malaria parasites replicate asexually but for transmission to occur, specialized sexual stages (gametocytes) must be produced. Despite the central role that gametocytes play in disease transmission, explanations of why parasites adjust gametocyte production in response to in-host factors remain controversial. We propose that evolutionary theory developed to explain variation in reproductive effort in multicellular organisms, provides a framework to understand gametocyte investment strategies. We examine why parasites adjust investment in gametocytes according to the impact of changing conditions on their in-host survival. We then outline experiments required to determine whether plasticity in gametocyte investment enables parasites to maintain fitness in a variable environment. Gametocytes are a target for anti-malarial transmission-blocking interventions, so understanding plasticity in investment is central to maximizing the success of control measures in the face of parasite evolution.

2.2 Introduction

Plasmodium spp. (malaria parasites) and other Apicomplexans are some of the most serious pathogens of humans, livestock, and wildlife (Garnham, 1966). Cycles of asexual replication inside host red blood cells (RBCs), lasting from 24-72 hours (Mideo et al., 2013), enable parasites to establish and maintain infections. To transmit to new hosts, every cell cycle a proportion of parasites develop into specialized sexual stages called gametocytes, which do not replicate in the host, but are infectious to the mosquito vector (unlike asexual stages). When taken up by the vector, male and female gametocytes differentiate into gametes and mate. The resulting offspring infect the vector and eventually produce stages infective to new hosts (Baton and Ranford-Cartwright, 2005).

It is well known that the production of gametocytes varies during infections and across hosts (Buckling et al., 1997, Buckling et al., 1999b, Buckling et al., 1999a, Pollitt et al., 2011b). However, the factors that induce commitment to produce gametocytes, and why parasites respond to these factors, are long-standing questions (Taylor and Read, 1997, Day et al., 1998, Dixon et al., 2008, Babiker et al., 2008). This information is central to understanding severity and transmission of disease, for predicting how disease control strategies will affect infectiousness (Mideo and Reece, 2011, Bousema and Drakeley, 2011, Churcher et al., 2010), and may also reveal novel ways to target parasites.

Here, we propose that malaria parasites strategically adjust investment into gametocytes (hereafter, the conversion rate) in response to the changeable conditions experienced during infections and that plasticity in the conversion rate enables parasites to optimise their survival and transmission during infections. Our conceptual model stems from the integration of diverse experimental data into an ecological and evolutionary framework, thereby making the predictions of our model and its underlying assumptions explicit and testable. While we focus on malaria parasites, the concepts and approach we outline can be applied more broadly to species for which in-host replication and between-host transmission are achieved by different specialised stages.

2.3 Conversion rate: evolutionary context

Parasites experience rapid and extensive variation in their in-host environment (e.g. in resource availability, competition with other genotypes and species, immune responses, and drug treatment) throughout their infections and while occupying different hosts and vectors. There is mounting evidence that traits underpinning in-host replication and between-host transmission (spanning from immune evasion traits (Lythgoe et al., 2007, Scherf et al., 2008) to investment in transmissible forms (Buckling et al., 1997, Reece et al., 2008, MacGregor et al., 2011)) are adjusted by parasites during infections. This flexibility in traits is called "phenotypic plasticity" defined as the ability of a genotype to produce different phenotypes in response to environmental change (Scheiner, 1993, Pigliucci, 2001). Phenotypic plasticity is an important solution to the challenges of life in a changing environment because it

enables organisms to maintain fitness by altering their phenotype, through mechanisms such as differential gene expression, to match their circumstances (Schlichting and Smith, 2002).

Every cell cycle malaria parasites face a resource allocation trade-off between how much to invest in asexual stages that are required for in-host survival and in sexual stages that are essential for between-host transmission (Koella and Antia, 1995, Reece et al., 2009). This is analogous to the trade-off between survival and reproduction faced by all sexually reproducing organisms (Stearns, 1992, Roff, 1992). Because reproduction is costly, phenotypic plasticity in the conversion rate influences two key fitness components: in host survival and between host transmission (Reece et al., 2009). High conversion early in infections increases the potential for transmission, but this strategy risks insufficient investment in asexual stages to maintain the infection within the host, resulting in a short duration for transmission. Conversely, excessive investment in asexual parasite replication reduces the rate of transmission at any given time, but this may be compensated for by longer infection durations and continued opportunities for transmission (Reece et al., 2009, Klein et al., 2012).

The number of gametocytes produced during infections is generally low (Taylor and Read, 1997) and it has been suggested that high densities of asexual stages are needed to shield gametocytes from transmission blocking immune responses (McKenzie and Bossert, 1998). However, this hypothesis does not explain why

conversion rates vary during infections, between conspecific genotypes, and across species (Reece et al., 2005, Reece et al., 2010, Pollitt et al., 2011b) (figure 2.1). The conversion rate is defined as the proportion of asexual stage parasites that commit to producing gametocytes in subsequent cell cycles (box 2.1), and is called "reproductive effort" in evolutionary biology. Therefore the conversion rate is not synonymous with the density or prevalence of gametocytes; variation in gametocyte densities can be generated by the same level of investment from different numbers of asexual stages (Buckling et al., 1999a).

In multicellular organisms, reproductive effort decisions are based on multiple extrinsic and intrinsic cues, mortality risk, and how these factors vary through an individual's lifetime (Williams, 1966, Stearns, 1992, Roff, 1992, Fischer et al., 2009, McNamara et al., 2009). Evolutionary theory predicts organisms should invest less in reproduction as they age because deterioration in their physiological condition (referred to as "state") means that more resources need to be allocated to maintenance to ensure continued survival (Williams, 1966, Fischer et al., 2009, McNamara et al., 2009). However, when facing an irrecoverable decline in state, or other fatal circumstances, organisms should make a terminal investment to maximise short-term reproduction (Williams, 1966, Creighton et al., 2009, Cotter et al., 2011).

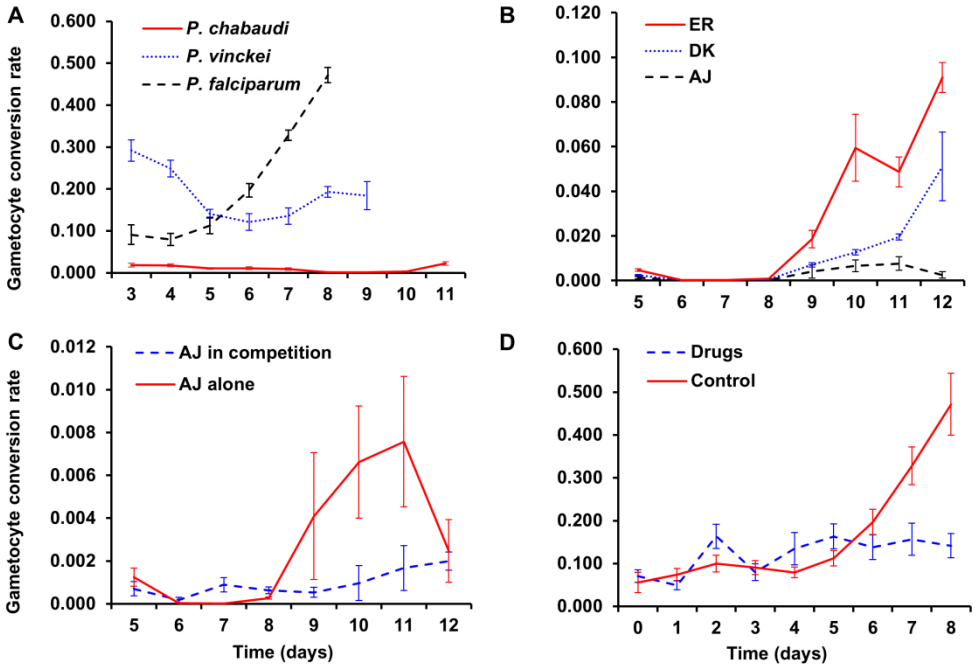

Figure 2.1 *Plasmodium* conversion rates are variable.

The conversion rate (±SEM) represents the proportion of a given cohort of asexual parasites that differentiate into sexual stage gametocytes. Variation in conversion is observed across species and during infections/culture (A). Note: conversion is calculated differently for rodent malaria parasites (*P. chabaudi, P. yoelii, P. vinckei* and *P. berghei, in vivo*) and for *P. falciparum* (*in vitro*) (see box 2.1). Different con-specific genotypes of *P. chabaudi*, in the same experiment, exhibit different patterns for conversion during infections (B). *P. chabaudi* reduces conversion when experimentally exposed to in-host competition (C). The conversion rates of genotype AJ are illustrated; during a single genotype infection (alone), and the mean conversion when in competition with either genotypes ER, AS, or both together (in competition). The reduction in conversion observed when drug sensitive *P. falciparum* isolates are exposed *in vitro* to antimalarial drugs or control conditions (D) (Pollitt et al., 2011b, Reece et al., 2005, Reece et al., 2010).

Box 2.1: Calculating conversion rates

Current protocols for *in vitro* studies of *P. falciparum* calculate the conversion rate on day t as the number of stage II gametocytes observed in 10000 RBCs on day t + 3 (the earliest time point when *P. falciparum* gametocytes are distinguishable from asexual blood stages) divided by the number of ring-stage asexual parasites observed in 10000 RBCs on day t (Carter and Miller, 1979).

For *P. chabaudi*, conversion is calculated from *in vivo* measurements according to (Buckling et al., 1999a). The description of the biological process underlying the model in (Buckling et al., 1999a) overcomes challenges posed by hard-to-quantify parameters (i.e., parasite death rates in the bloodstream and schizont burst sizes) and takes into account the maturation times of gametocytes and asexual blood stages (48 and 24 hours respectively, for rodent parasites). Although the mathematical formulation assumes gametocytes are counted 24 hours into development, current molecular assays count gametocytes of an unknown age (but are likely to be between 24-48 hours old). Ideally we need to know the schedule of development and the precise point at which gametocytes are assayed, since these will determine the exact form of the conversion rate equation. For example, if markers in mature (48h old) gametocytes are used, then conversion rate, ε, should be quantified as:

$$\varepsilon = \frac{\frac{G_{t+3}}{A_t}}{\sqrt[3]{\frac{A_{t+3}}{A_t}} + \frac{G_{t+3}}{A_t}}$$

Where A_i and G_i are asexual and gametocyte densities on day i.

Box 2.1 Continued

Whilst these tools are easy to implement, the assumptions underpinning them are key to making accurate estimates of conversion rates. These assumptions, and their caveats, include:

1. **The probability of asexual parasites producing gametocytes is constant over the period between gametocyte production and detection**. Given the expectation of plasticity in conversion, whereby a different proportion of asexual parasites can commit for every cell cycle, this assumption may often be hard to fulfil.

2. **Both *in vivo* and *in vitro* approaches assume that the death rate of asexual parasites and gametocytes is equal**. Whilst *in vitro* culture conditions do not have the problem of sequestration (disappearance from the circulation) or immune factors that could exacerbate differential mortality rates between lifecycle stages (Taylor and Read, 1997), for *in vivo* assays these factors could confound conversion estimates (Mideo and Day, 2008). Furthermore, conversion rates can be overestimated if the death rate for asexual parasites is higher than for gametocytes (which could well be the case during drug treatment (Reece et al., 2010), or underestimated if early stage gametocytes are mistakenly identified as asexual stages. It is possible to develop mathematical models and formulate predictions for how different survival rates need to be if they are the sole driver of observed patterns in conversion rates. For example for the *in vivo P. chabaudi* data in (Pollitt et al., 2011b), we find that the difference in survival rates between asexual parasites and gametocytes must vary over the course of infections (e.g., immunity sometimes focuses efficiently on killing gametocytes while at other times survival rates across parasite stages are equal) and must vary considerably in different kinds of infection (N Mideo, unpublished work). In particular, to explain the difference in patterns of conversion observed in figure 2.1C, survival rates of gametocytes (relative to asexual parasites) in mixed infections must be several orders of magnitude lower than in single infections. As yet, there is no known mechanism that could underlie such drastically different patterns of survival between parasite stages, during and across infections. Therefore we propose that differential survival is unlikely to be the sole cause of variation in patterns of conversion rates. However, developing a better understanding of immune responses and subsequent parasite death rates remains an important goal.

In the literature, there are considerable discrepancies in how conversion rates for *P. falciparum* have been examined; some studies measure gametocyte density in circulation and others present gametocyte prevalence (reviewed in (Mideo and Reece, 2011)). This is,

in part, due to the difficulties in calculating conversion rates for natural *P. falciparum* infections since repeated samples – at specific time points – are required to assay the number of asexual parasites in a cohort and the number of gametocytes they produce.

Basing inference simply on gametocyte density can be problematic: for example, observations of elevated gametocyte densities post drug treatment could be due to the release of sequestered gametocytes and/or an increase in conversion rate (Taylor and Read, 1997). Data on the timing of gametocytes appearing in the circulation can resolve this issue, but again, requires repeated sampling at specific time points. While there are important ethical and logistical considerations when studying natural infections of humans, monitoring infections, with measurements of conversion and in-host variables (e.g. anaemia and genetic diversity) would be extremely useful.

To address the problems outlined in points 1 and 2, ideally, conversion rates for rodent malaria parasites *in vivo* could be calculated in the same way as is now possible for *in vitro* cultures of *P. falciparum* (using GFP–tagged molecular markers of sexually committed schizonts and flow cytometry to sort fluorescent parasites (Reininger et al., 2012)). However, despite the issues raised, measuring conversion rate remains a more desirable approach than simply analysing gametocyte density or prevalence, because changes in the density of gametocytes can be generated from cohorts that simply differ in asexual parasite number, but invest in the same relative number of gametocytes.

When translating this to malaria parasites, each genotype within a mixed infection is the target of selection and should behave as a multicellular organism (Gardner and Grafen, 2009). The density and/or proliferation rate of parasites is analogous to the "state" of multicellular organisms. During infections, numerous factors, such as competition with unrelated genotypes, other species, drug treatment, immune responses, RBC resource availability, and host nutritional status can all change dramatically and impact upon parasite proliferation in the host. Thus, in-host environmental factors that negatively affect proliferation can be considered as "stressors" which impact on the "state" of parasites.

2.4 Stress induced sex?

Human (*P. falciparum*) and rodent (*P. chabaudi*) malaria parasites elevate gametocyte densities in response to high doses of antimalarial drugs (Buckling et al., 1997, Buckling et al., 1999b, Buckling et al., 1999a Peatey et al., 2009) and an increase in young RBCs (reticulocytes) (Trager et al., 1999, Reece et al., 2010). However, care must be taken when making comparisons as there are discrepancies between the approaches used to estimate conversion rates in different studies (box 2.1). Increasing conversion has been interpreted as a strategy parasites adopt when they experience adverse conditions, enabling them to maximise transmission before the infection is cleared or the host dies (Buckling et al., 1997), a so-called "terminal investment" (Williams, 1966). Whilst this makes intuitive sense in the case of drug treatment, it is not clear whether reticulocytes are, or indicate, adverse conditions.

In contrast, recent experiments (using *P. chabaudi* rodent malaria parasites *in vivo* (Pollitt et al., 2011b, Wargo et al., 2007b), and human *P. falciparum* parasites *in vitro* (Reece et al., 2010)) reveal that when exposed to competition with other genotypes in the host, RBC resource limitation, or low doses of anti-malarial drugs, parasites reduce conversion rates, adopting "reproductive restraint" (figure 2.1). Evolutionary theory predicts that reproductive restraint during periods of mild stress improves the prospects for in-host survival, and therefore the opportunities for future transmission (Mideo and Day, 2008). The experimental data also suggest that parasites respond to the presence of the extrinsic (environmental factors) as well as to

their intrinsic effect (impact on state). Moreover, data from monitoring a cohort of infected patients collected in the same area from which the parasites used in Reece et al. (2010) were isolated provide tentative (*in vivo*) support for the reproductive restraint of *P. falciparum* in response to drug pressure (Ali et al., 2006).

The contrasting observations of increased and decreased conversion rates in response to environmental variation within the host can be reconciled by considering the severity of stress imposed on parasites by in-host factors. This is illustrated in figure 2.2A in which we propose that parasites adjust their conversion rate according to the impact of conditions on their proliferation (state) or via directly detecting the presence of stressors (figure 2.2B). In low stress conditions (e.g., infections of naïve hosts) parasites can afford to invest in gametocytes, and do so at a rate that maximizes transmission. When in-host conditions deteriorate due to the appearance of stressors (e.g., competition with other genotypes and species, immune responses, drug treatment), parasites are constrained to invest in survival, which they achieve by reducing the conversion rate (reproductive restraint) (Koella and Antia, 1995, Pollitt et al., 2011a). By ensuring survival during periods of stress, parasites benefit from the fitness returns of future transmission (i.e., by reducing the rate of transmission in the short term, parasites gain a longer duration for transmission). When faced with attack from immune responses, investing more in replication may also have the added benefit of increasing opportunities for immune evasion via antigenic switching (Mackinnon and Marsh, 2010). However, in very poor conditions, when parasites experience severe stress and their death rate exceeds the capacity for proliferation or

host mortality is imminent, they should make a terminal investment to maximise short-term transmission by diverting resources to gametocyte production.

The pattern of conversion we predict in figure 2.2A is qualitatively similar to that predicted through a mathematical analysis by Koella and Antia (1995). Their analysis relied on strict assumptions: infections are lethal to the host above a threshold density and conversion rates are adjusted to limit asexual parasite densities to just below this threshold. This work raises the point that all else being equal, increasing investment in gametocytes should lead to decreasing virulence of an infection; a large body of theory predicts how virulence should depend on in-host factors (e.g., (Alizon and van Baalen, 2008), reviewed in (Alizon et al., 2009)). However, virulence is only one of many selective forces acting on conversion rates. As only a small proportion of modern human malaria infections are fatal, we predict parasites more often need to respond to in-host factors that are able to clear infections than to imminent host death. The high prevalence of chronic malaria infections and the increasing appreciation of their contribution to the infectious reservoir (Schneider et al., 2006, Okell et al., 2009, Shekalaghe et al., 2009), also suggests that a long duration of transmission matters and producing gametocytes "few but often" results in the greatest lifetime fitness. Transmission success is also heavily dependent on vector availability. In areas where transmission is seasonal, parasites must survive in the host during the dry season. Indeed, parasites have evolved diverse strategies to facilitate long-term in-host survival, from immune evasion mechanisms (e.g. antigenic switching in *P. falciparum* (Scherf et al., 2008)); to resisting competition (e.g. rodent malaria parasites prevent incoming, competing

parasites from establishing an infection via the host iron regulatory hormone hepcidin (Portugal et al., 2011)). In the majority of parasite species, the success of these strategies depends on maintaining asexual replication at a sufficiently high rate, which can be achieved through reproductive restraint.

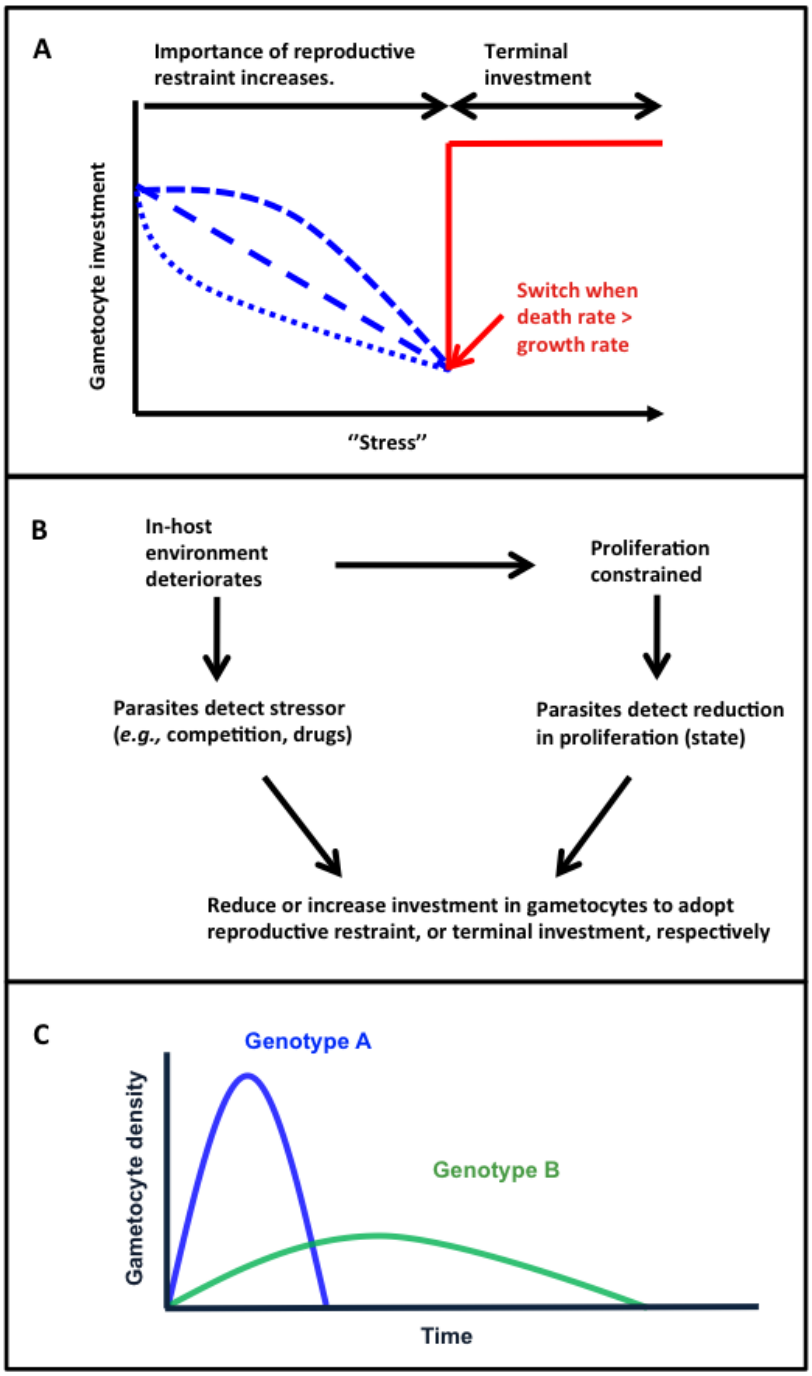

Figure 2.2 Predicted pattern for conversion. A: Under low ‘’stress” (e.g. early in infections of naïve hosts) parasites can afford to invest in gametocytes, but if conditions deteriorate and proliferation is constrained (e.g., when parasites face stressors such as anaemia, competition, or immune responses) parasites reduce conversion, employing reproductive restraint (blue dashed lines), to ensure in-host survival and the potential for future transmission. The form that reproductive restraint takes could follow any of the patterns illustrated with the dashed blue lines, depending on a number of factors (e.g., the cues parasites respond to, how accurately survival probability is determined, and the value of future versus current transmission). When parasites face circumstances likely to be fatal (e.g., when their death rate exceeds the potential for replication during radical drug treatment) or host death is imminent (e.g., due to severe anaemia), parasites should make a terminal investment by investing remaining resources into gametocytes (red solid line). A switch point and step function between reproductive restraint and terminal investment is predicted because investing all remaining resources is the best option in a situation likely to be fatal. Note: the x-axis does not simply translate to “time since infection” because the severity of different stressors fluctuates during infections. **B:** Data suggest that parasites can detect and respond directly to individual stressors and also to the effect they have on proliferation rate. Information from the cues parasites use must be fed into the molecular pathways that underpin commitment to effect a gametocyte investment decision. **C:** The total production of gametocytes (the area under the curve) is equal for both genotypes (Churcher et al., 2010). However, genotype A invests heavily into transmission early in the infection and therefore achieves higher gametocyte densities over a shorter period of time, whereas B has a lower relative investment in gametocytes at each time point, but achieves a longer period for transmission. The optimal balance between these two extremes is predicted to depend on many factors including the frequency of vector blood meals, and the chances of the host clearing the infection or dying.

2.5 Testing the theory: complications and challenges

The model outlined above provides a foundation to explain variable conversion rates when considered in light of several key questions:

(1) Which cues do parasites use to make conversion rate decisions?

(2) What are the mechanisms that enable plasticity in conversion rate?

(3) How finely tuned are conversion rates to the in-host environment and state?

(4) Does adjusting conversion rates in the manner predicted maximise parasite fitness?

We consider answers to these questions in the following sections and outline the challenges required to evaluate these hypotheses in box 2.2.

2.5.1 Cues for conversion decisions

The extent to which parasites respond directly to extrinsic stressors or simply the overall effect those stressors have on state is not known. Experimental data suggest parasites can respond both to state and environmental factors. For example, experiments exposing *P. falciparum* to low doses of different anti-malarial drugs in culture have included both drug sensitive and resistant genotypes but only sensitive genotypes respond. This suggests that parasites do not directly detect each drug, but instead, respond to the negative effect they have on state (Reece et al., 2010). Responding to state seems the more efficient strategy: it avoids the need to integrate information about multiple factors, potentially giving opposing information, to mount an appropriate response. For example, the level of anaemia induced by *P. falciparum* infections varies depending on the type of antimalarial drug administered to patients and whether the parasites are cleared (Ekvall et al., 1998). Because anaemia triggers the formation of reticulocytes, the reproductive strategy employed in response to the presence of drugs may be complicated by the simultaneous change

in RBC age structure. Parasites could be responding directly to the drugs, the resulting changes in RBCs, both, or the overall effect that both factors have on the "state" of the infection (Trager et al., 1999, Reece et al., 2005).

Whether the best measure of state is parasite density *per se* or proliferation (i.e. rate of change in density) is unclear. Data from several *P. chabaudi* genotypes (Pollitt et al., 2011b, Wargo et al., 2007a) and subsequent modeling (Cameron et al., 2013) suggests that parasites alter their conversion rate according to their density in mixed genotype infections. Density could be determined by quorum sensing (Diggle et al., 2007), markers of RBC lysis from burst parasitized cells (Dyer and Day, 2003), immune factors, or metabolic measures such as energy balance or reducing power (e.g., the expression of genes associated with starvation are associated with increased conversion in *P. falciparum* (Daily et al., 2007)). However, detecting the density of a parasite cohort does not necessarily reveal a change in state (i.e., is parasite density increasing or decreasing?).

Measuring proliferation requires that parasites integrate information on density over consecutive cell cycle cohorts. This information may be more accurate for parasite species with synchronous progression through cell cycles than for species with asynchronous cycles. In this case, if proliferation rate information is unreliable, parasites could respond to individual environmental stress factors; either directly or indirectly, by detecting a co-varying factor. For example, parasites may use the onset of anaemia as a signal for the imminent arrival of antibodies and the development of

immune responses (Paul et al., 1999, Reece et al., 2005). Using proxies in this way may also enable parasites to predict future changes in state and respond preemptively (Mitchell et al., 2009). Alternatively, parasites could measure their death rate; although mechanisms for this are more difficult to envision, they could include monitoring the concentration of immune effectors or the release of SOS signals by dying parasites similar to bacteria and *Chlamydomonas* (Swift et al., 2001, Moharikar et al., 2006).

2.5.2 Mechanisms underpinning conversion

The mechanisms regulating the switch to gametocyte production remain elusive. Advances in genomics, transcriptomics, proteomics and functional gene targeting studies have identified several markers of early gametocyte development in human and rodent malaria parasites (reviewed in (Dixon et al., 2008, Babiker et al., 2008, Baker, 2010, Liu et al., 2011, Guttery et al., 2012)). These studies provide further evidence that commitment occurs at or prior to the schizont stage preceding the release of sexually committed merozoites (as has been previously suggested for *P. falciparum* (Bruce et al., 1990, Smith et al., 2000)). Studies using GFP reporters with known gametocyte specific promoters also support this developmental pattern (reviewed in (Babiker et al., 2008, Baker, 2010, Bousema and Drakeley, 2011, Liu et al., 2011)). Recently, the gene *P. falciparum* gametocyte development 1: *pfgdv1*has been identified as a regulator of gametocyte production (and is associated with an increased expression of genes involved in early gametocytogenesis (Pfge genes) (Eksi et al., 2012)), and an ApiAP2 DNA binding protein (Campbell et al., 2010) is required for gametocyte commitment (Kafsack et al., 2014, Sinha et al., 2014).

Whilst identifying molecular markers for commitment is useful for quantifying conversion decisions, the evolution of plasticity in conversion rates is shaped by the nature of the pathways involved in: detecting cues, processing the information, producing a conversion rate phenotype, and the maturation of gametocytes. The critical regulators underlying gametocyte conversion may act within a complex network of interactions between different modules involved in information assimilation and integration to produce a conversion rate phenotype. This level of complexity is very challenging to unravel and made more difficult because gene function and changes in expression must be assessed in the context of variation in both the environment and genetic background of the parasites. Furthermore, it is possible that the environmental sensing mechanisms underlying conversion decisions may also feed information into other plastic life history decisions such as sex ratio, cell cycle arrest and var gene switching (which is responsible for antigenic variation to evade host immune responses), as these traits are sensitive to similar environmental perturbations (reviewed in (Reece et al., 2009)). As these traits are likely to be linked by genetic correlations (e.g. epistasis/pleiotropy: different traits are shaped by the same genes), understanding the nature of these interactions is central to explaining plasticity in these traits.

2.5.3 Parameterising patterns of conversion

The shape and switch point(s) of the reaction norm (how a trait varies across an environmental gradient) reveal how fine-tuned parasite responses are to

environmental variation, including novel stressors. The extent of genetic variation for reaction norms is a determinant of the potential for evolution. Reaction norms are influenced by many interacting factors. This includes the reliability of cues, costs of maintaining detection and response mechanisms, and how much multiple sources of information affect the risk of making the wrong decision (West et al., 2006, Rousset and Roze, 2007, Kümmerli et al., 2009). Differences in reaction norms across species, that have different cell cycle durations, gametocyte development times or RBC age preferences, may reflect how differences in costs and constraints on plasticity shape parasite strategies. As many different factors can independently and simultaneously affect in-host conditions and parasite state, examining the patterns of conversion rates resulting from varying factors individually is useful, but providing cues in different combinations is required to reveal the full picture.

The reaction norm for conversion is predicted to follow a non-linear pattern, with any of the patterns illustrated and at least 1 switch point (reproductive restraint to terminal investment; figure 2.2A) (Koella and Antia, 1995, Pollitt et al., 2011a). This switch should occur when the death rate exceeds the proliferation rate. We expect this point will be influenced by species-specific variation in cell cycle duration and gametocyte development time, and by how quickly the environment and/or state changes. For example, the cell-cycle duration and gametocyte development time of rodent malarias are much shorter than that of *P. falciparum*. Whilst the cell cycle for rodent malaria parasites is 24 hours, and gametocytes reach maturity and are infectious to mosquitoes after 24-48 hours, the cell cycle of the human malaria parasite *P. falciparum* is 48 hours and gametocytes require 10-14 days to reach

maturity (Day et al., 1998, Babiker et al., 2008). Therefore, if *P. falciparum* makes a terminal investment in advance of host death the host is required to survive at least 10-14 days until the investment can pay off (five further asexual cycles), but only 48 hours are required for rodent parasites to produce transmissible gametocytes. As such, *P. falciparum* may ”play it safe” and adopt a more conservative strategy by making a terminal investment in response to lower levels of stress than rodent parasites, whose gametocytes reach maturity within 48 hours (two asexual cycles). If a fast drop in numbers were normally a reliable indicator of a terminal situation, this would explain why increased conversion is observed when parasites are exposed to high, but subcurative, drug doses (Ali et al., 2006, Reece et al., 2010). Also, if the longer cell-cycle duration of *P. falciparum* compared to rodent malarias makes *P. falciparum* more vulnerable to being cleared by the host, reproductive restraint will be induced at lower stress than for rodent parasites.

As shown in figure 2.2C, the characteristics of populations can also influence the shape of reaction norms. For example, a “live fast, die young” strategy in which parasites readily switch to terminal investment may bring greater pay offs in an epidemic setting – where there are plenty of naïve hosts to be transmitted to – than in an endemic setting where parasites will be transmitted to hosts containing competitors and with active immune responses (Boots and Mealor, 2007). This is because genotypes with a high conversion rate risk being unable to establish infections in new hosts, due to being outcompeted by resident genotypes (Alizon and van Baalen, 2008, Mideo and Day, 2008). Furthermore, parasites in hypoendemic areas experience lower levels of in-host competition than those from regions with

high genetic diversity (hyperendemic) and so may be less responsive to novel stressors such as competition and its effect on state (de Roode et al., 2005).

2.5.4 Linking variable conversion rates to fitness

A key prediction to test is whether plasticity in conversion rate is adaptive (Kochin et al., 2010). The extent to which reproductive restraint provides an in-host survival advantage under stress is yet to be determined (e.g., how much does reproductive restraint ameliorate the suppression of a genotype in a mixed infection?). At the between-host level, how different reproductive strategies map to the rate and duration of transmission is hard to assess from data (e.g. gametocyte prevalence) available on natural infections. Therefore, whether (under some conditions) prolonging the duration for transmission enhances fitness, and whether terminal investment benefits parasites in lethal situations through an increase in short-term transmission, remain unknown.

Testing the fitness consequences of variation in traits is notoriously difficult, but identifying the host and parasite factors that elicit a change in conversion rate and the reaction norms generated by different levels of stress will provide the required foundations. For example, by providing a cue that elicits reproductive restraint in different circumstances (e.g. cues for competition provided in single infections) parasites can be induced ("tricked") into making inappropriate responses for their circumstances. The consequences for in-host survival and transmission for parasites responding to fake cues could then be quantified, and compared to the performance

of parasites exposed to cues that accurately reflect their circumstances (Williams, 2010). This framework also opens up the possibility of developing interventions that co-opt plasticity in conversion rates, by manipulating parasites into making suboptimal decisions for their fitness.

The maintenance of mechanisms required to detect and respond to environmental change requires resources that could be otherwise allocated to different functions (deWitt et al., 1998). Evolutionary theory predicts that if these costs are sufficiently high then plasticity is selected against and lost if organisms no longer experience variable environments, but evidence for costs of plasticity is scarce (Auld et al., 2009). Because gametocytes are costly, selection for in-host replication during long-term culture of *P. falciparum* and serial passage of *P. berghei* result in the loss of gametocyte production (Janse et al., 1992). However, whether plasticity is actually lost is unclear because gametocyte production is sometimes recoverable (Ono et al., 1993).

Box 2.2: Challenges & future directions. Whilst our conceptual model is general, testing it requires examining specific circumstances. Here, we outline the main challenges and outstanding questions involved.

Response to drugs: Data for conversion rates – especially from experiments using drugs – are consistent with the basic prediction of parasites adopting reproductive restraint (*P. falciparum in vitro* (Reece et al., 2010)), or terminal investment (*P. chabaudi in vivo* (Buckling et al., 1997, Pollitt et al., 2011b) and *P. falciparum in vitro* (Buckling et al., 1999b)), in response to different levels of stress. However, further work is required to explicitly test the effects of varying dose within the same experiment - both for rodent models and *in vitro* for *P. falciparum*. Furthermore, not all drugs appear to induce changes in conversion rate (Buckling et al., 1999a, Peatey et al., 2009). This may be because drugs with different modes of action differentially affect the capacity of survivors to detect/respond to changes in state, or the capacity of dying parasites to provide signals.

Response to competition: In-host competition is a stressor with a negative effect on state because the densities of all genotypes (individually and when combined) are reduced in mixed infections compared to single infections. This is due to a mixture of competition for RBC and the action of immune responses that are not genotype-specific. Competition within the host could occur via a single bite from a mosquito infected with multiple genotypes (to a naive host). Alternatively, competition can be established when a mosquito infected with one genotype bites an individual already infected with a different genotype. The latter example of sequential infection would be less stressful for the resident genotype than the newcomer, even if it the resident genotype is competitively inferior to the incoming genotype (Hellriegel, 1992). This is because the incoming genotype will enter a RBC resource depleted environment with cross-reactive immune responses already in place (de Roode et al., 2005). *In vivo* studies of simultaneous in-host competition using *P. chabaudi* reveal reproductive restraint across several genotypes (Wargo et al., 2007a, Pollitt et al., 2011b), but there are no reports of increased conversion in response to competition. Adopting reproductive restraint in response to competition might be the only strategy required because in-host competition is never stressful enough to merit terminal investment. Alternatively this may be an artefact of experimental design in which mixed infections do not result in competitive exclusion, even for the weakest genotypes (Bell et al., 2006, Barclay et al., 2008, Pollitt et al., 2011b). Experiments using genotypes that vary in competitive ability, inoculated at different starting doses and times during infections are needed to test whether in-host competition can induce terminal investment. At the host population level, the consequences of different investment strategies would be much harder to test experimentally, but theory demonstrates that there will be feedback from the within- to

between-host levels, and vice versa (e.g., (van Baalen and Sabelis, 1995)). For instance, if mixed infections really do promote reproductive restraint, then this should result in less transmission and, consequently, fewer mixed infections. Some of the variation observed in conversion rates may be a consequence of this sort of dynamic feedback.

Response to reticulocytes: Conversion has been observed to both increase and decrease in response to reticulocytes. For some species (e.g. *P. berghei* and *P. vivax*) that preferentially invade reticulocytes, an increase in conversion upon exposure to reticulocytes is consistent with parasites making use of available resources. However, species able to infect a wide range of RBC ages, such as *P. falciparum* and *P. chabaudi,* also increase conversion in response to reticulocytes (Trager et al., 1999, Reece et al., 2005). This may be because reticulocytes are also exploitable resources. However, the lifespan of gametocytes in *P. falciparum* is at least 5 times that of asexual stages, so the longer expected lifespan of reticulocytes may provide a better resource to support the development of gametocytes than mature RBCs. Alternatively, for all species, increased reticulocytaemia could indicate severe anaemia leading to imminent host death, and thus, terminal investment is the best strategy. For example, the poultry malaria parasite *P. gallinaceum* appears to be able to determine whether the host will survive or die from severe anaemia because it produces different sex ratios in these different circumstances (Paul et al., 1999). However, an influx of reticulocytes could also indicate the opposite – that the host is generating an appropriate erythropoietic response and will recover from severe anaemia. In this case, reproductive restraint maximises the potential for the parasites to survive.

When in-host survival does not rely on asexual parasite replication: Parasite species producing dormant stages that persist in the liver (hypnozoites) and dendritic cells, such as the human malaria parasites *P. vivax* and *P. ovale* (Cogswell, 1992, Wykes et al., 2011), may not adopt reproductive restraint in response to stress because survival in the host does not depend on blood stage replication. Terminal investment due to imminent clearance will also be unnecessary but may be required to cope with host death. To our knowledge there are no data on the conversion rates of *P. vivax* experiencing different in-host conditions. However, during natural *P. vivax* infections, higher gametocyte densities are correlated with a mixture of seemingly favourable and unfavourable conditions, including younger (immunologically naive) patients, those with higher parasite densities, lower haemoglobin levels, lower platelet counts and an absence of fever (reviewed in (Mideo and Reece, 2011)). *P. vivax* gametocyte densities are also generally much higher compared to those recorded for *P. falciparum*, but each gametocyte circulates for a shorter time; a maximum of 3 days (reviewed in (Mideo and Reece, 2011)). These observations suggest that *P. vivax* may have a non-plastic strategy of a relatively high conversion during the short-lived erythrocytic stage of their infections.

2.6 Conclusions

That in-host ecology shapes the dynamics of infections (Read and Taylor, 2001, Harrison, 2007) and patterns of transmission is well known (Mideo and Reece, 2011, Lloyd-Smith et al., 2005, Matthews, 2011). However, why the density of circulating gametocytes in malaria is generally low (Taylor and Read, 1997, Mideo and Day, 2008) has eluded explanation. We provide an evolutionary theory-based model, which predicts that parasites can rarely afford to invest in more because their life history spreads reproduction across multiple attempts over a relatively long time.

Given renewed interest in transmission blocking interventions, understanding parasite strategies for gametocyte investment is central to making such measures as resilient to parasite counter evolution as possible (Mideo and Day, 2008, Alizon and van Baalen, 2008). For example, inducing all parasites to commit to gametocytes (ideally of the same sex) would reduce the virulence of the infection and could also produce an effective transmission-blocking immune response that acts against future infections. For example, this could be useful for travellers returning to non-malarious countries. Inducing commitment *in vitro* could also generate material to inform the development of other transmission blocking interventions such as vaccines and drugs with gametocytocidal action.

Finally, it is often not appreciated that plasticity in parasite life history traits can also shape evolutionary responses to environmental change. For example, if plasticity in conversion rate acts as a buffering mechanism to minimise the impact of drug

treatment, this may weaken selection for other forms of resistance. This may be favourable from the perspective of maximising the timespan of efficacy of antimalarial drugs. However, such infections will likely be harder to treat than if malaria parasites exhibited a higher, fixed, conversion rate.

3 Information use and plasticity in the reproductive decisions of malaria parasites.

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3.1 Abstract

Investment in the production of transmissible stages (gametocytes) and their sex ratio are malaria parasite traits that underpin mosquito infectivity and are therefore central to epidemiology. Malaria parasites adjust their levels of investment into gametocytes and sex ratio in response to changes in the in-host environment (including red blood cell resource availability, host immune responses, competition from con-specific genotypes in mixed infections, and drug treatment). This plasticity appears to be adaptive (strategic) because parasites prioritize investment (in sexual *versus* asexual stages and male *versus* female stages) in manners predicted to maximize fitness. However, the information, or 'cues' that parasites use to detect environmental changes and make appropriate decisions about investment into gametocytes and their sex ratio are unknown. Here, single genotype *Plasmodium chabaudi* infections were exposed to 'cue' treatments consisting of intact or lysed uninfected red blood cells, lysed parasitized RBCs of the same clone or an unrelated clone, and an unmanipulated control. Infection dynamics (proportion of reticulocytes, red blood cell and asexual stage parasite densities) were monitored, and changes in gametocyte investment and sex ratio in response to cue treatments, applied either pre- or post-peak of infection were examined. A significant reduction in gametocyte density was observed in response to the presence of lysed parasite material and a borderline

significant increase in sex ratio (proportion of male gametocytes) upon exposure to lysed red blood cells (both uninfected and infected) was observed. Furthermore, the changes in gametocyte density and sex ratio in response to these cues depend on the age of infection. Demonstrating that variation in gametocyte investment and sex ratio observed during infections are a result of parasite strategies (rather than the footprint of host physiology), provides a foundation to investigate the fitness consequences of plasticity and explore whether drugs could be developed to trick parasites into making suboptimal decisions.

3.2 Introduction

Malaria parasites proliferate in the blood through cycles of asexual replication, but every cell cycle a small proportion of progeny commit to developing into male and female gametocytes (which do not replicate in the host) (Smith et al., 2002, Talman et al., 2004, Dixon et al., 2008, Liu et al., 2011). This means that, like all sexually reproducing organisms, malaria parasites face resource allocation trade-offs between survival and reproduction and between producing males and females (Hamilton, 1967, Charnov, 1982, Stearns, 1992, Roff, 1992). Specifically, every cell cycle parasites make decisions about how much to invest in gametocytes (which are essential for reproduction and transmission) *versus* asexuals (which are essential for in-host survival) and in males *versus* females. These decisions are sensitive to variation in the in-host environment (Paul et al., 2003, Reece et al., 2009).

Extensive variation in gametocyte investment (also known as the ‘conversion rate’ or

'reproductive effort') and sex allocation (proportion of male gametocytes) of *Plasmodium spp.* has been observed across different species, strains, and during infections (Buckling et al., 1997, Paul et al., 2000, Reece et al., 2005, Eisen and Schall, 2000, Reece et al., 2009, Reece et al., 2010, Neal and Schall, 2010, Pollitt et al., 2011b). Understanding variation in gametocyte investment and sex ratio (collectively referred to as 'reproductive strategies') is important because they are key fitness-determining traits, shaping survival within hosts and the success of transmission to new hosts (Pollitt et al., 2011a, Mideo and Reece, 2011, Carter et al., 2013, chapter 2). Experiments using rodent malaria parasites *in vivo* and *Plasmodium falciparum in vitro* suggest that parasites alter investment in gametocytes and their sex ratio in response to: changes in red blood cell (RBC) resource availability (Drakeley et al., 1999, Trager et al., 1999, Paul et al., 2000, Robert et al., 2003, Reece et al., 2005), host derived transmission blocking immune (TBI) responses (Reece et al., 2008, Paul et al., 1999, Smalley et al., 1981, Buckling and Read, 2001), competition from con-specific genotypes in mixed infections (Dyer and Day, 2003, Pollitt et al., 2011b, Reece et al., 2008, Wargo et al., 2007a) and drug treatment (Buckling et al., 1999b, Buckling et al., 1999a, Buckling et al., 1997, Wargo et al., 2007b, Sowunmi et al., 2009, Peatey et al., 2009, Reece et al., 2010, Peatey et al., 2013). Observational data from natural infections also suggests that *P. falciparum* sex ratios and gametocyte investment differ between single and mixed infections and are altered in response to variation in RBC density (Bousema and Drakeley, 2011).

Evolutionary theory offers explanations for why parasites adjust their reproductive strategies in response to the changing environmental conditions encountered in the

host (Antia et al., 2008, Mideo and Day, 2008, Reece et al., 2009, Kochin et al., 2010, Mideo and Reece, 2011). For example, parasites increase gametocyte investment in response to anaemia, reticulocytes and exposure to sub-lethal anti-malarial therapy (Trager and Gill, 1992, Buckling et al., 1997, Buckling et al., 1999a, Buckling et al., 1999b, Trager et al., 1999, Paul and Brey, 2003, Reece et al., 2005, Peatey et al., 2009). This has been interpreted as a strategy of 'terminal investment' during extreme stress (Williams, 1966): investing heavily in gametocytes maximizes transmission potential in a situation likely to be lethal (e g., before the infection is cleared or the host dies) (Buckling et al., 1997, Buckling et al., 1999b, Peatey et al., 2009,). However, recent evolutionary theory predicts that this may be an oversimplification and that less severe stress induces parasites to reduce investment, as a strategy of 'reproductive restraint' (Mideo and Day, 2008). Reproductive restraint is predicted to facilitate in-host survival and therefore future transmission opportunities (Mideo and Day, 2008). Empirical work supports these predictions, revealing that when parasites experience competitive suppression, RBC limitation, and low doses of anti-malarial drugs, they reduce gametocyte investment (Wargo et al., 2007a, Reece et al., 2010, Pollitt et al., 2011b). The sex allocation decisions of parasites are sensitive to many of the same factors as gametocyte investment. For example, different sex ratios bring the highest fitness returns in single- *versus* mixed-genotype infections (Hamilton, 1967, Read et al., 1992, Godfray and Werren, 1996, West et al., 2001, Nee et al., 2002, Reece et al., 2008) and when hosts are mounting immune responses that differentially affect male and female gametocytes (Ramiro et al., 2011). Experiments with *Plasmodium chabaudi* reveal that sex ratios are precisely allocated according to the number of co-infecting genotypes and their

relative representation within a mixed-genotype infection (Reece et al., 2008). Therefore, sex ratio data suggest that parasites can determine the genetic diversity of their infections and measure the number (or replication rate) of asexual stages belonging to their genotype (Reece et al., 2008).

Whilst evolutionary theory can explain why parasites adjust investment into gametocytes and their sex ratio, it does not explain how they do so. Whether parasites identify and respond to individual factors (e.g., RBC density and age structure, the presence of competing parasites and the dose of drugs), or the overall impact the environment has on their proliferation rate (i.e., 'state') is not known (Carter et al., 2013). A further complication is that the in-host environment is complex and many factors change simultaneously. For example, both anaemia and immunity develop as parasite number increases (Haldar and Mohandas, 2009, Paul et al., 1999), competition in mixed infections brings RBC limitation and suppresses asexual proliferation (Paul et al., 2003, de Roode et al., 2004, Bell et al., 2006, Raberg et al., 2006), and different drugs kill parasites in dose-dependent ways and can alter anaemia (Ekvall et al., 1998). For the parasite, more accurate information may be obtained from directly measuring individual environmental factors, but measuring changes in overall state may be the most efficient strategy, as it does not require the assimilation of information from multiple environmental variables that could elicit contradictory parasite responses (Carter et al., 2013).

The experiments presented here investigate the cues that parasites use to make their reproductive decisions by examining whether the gametocyte investment and sex ratio of a single clone infection change in response to material ('cues') derived from uninfected RBCs, RBCs infected with con-generic parasites, and RBCs infected with a con-specific genotype. The experiments were designed to build on previous work (Reece et al., 2008, Pollitt et al., 2011b) to more specifically test 'what' parasites sense in their in-host environment. For example, in previous experiments conversion rates (Pollitt et al., 2011b) and sex allocation (Reece et al., 2008) were compared in single and mixed genotype infections to ask whether parasites respond to in-host competition. However, numerous factors vary between single and mixed infections (e.g., anaemia, the age structure of RBCs, the concentration and balance of cytokines and the density of parasites), in complex ways. This makes it difficult to pinpoint exactly which factor(s) parasites are responding to. Furthermore, these changes in the in-host environment offer different opportunities and constraints to parasites that could be incorrectly interpreted as a parasite response. For example, parasites may not respond directly to anaemia, but may appear to do so, because a lack of preferred RBCs available for parasites to invade could directly interfere with their replication rate. The experiments presented here were designed to minimise the problem of simultaneously changing multiple aspects of the in-host environment, with the aim of getting closer to identifying the factor(s) which parasites are sensitive to.

3.3 Methods

3.3.1 Hosts and parasites

The rodent malaria parasite *P. chabaudi,* genotypes AJ and ER were used (from The University of Edinburgh's malaria reagent repository http://malariaresearch.eu/). These wild-type clonal genotypes were originally isolated from areas where mixed infections were frequent (Carter, 1978). Male MF1 mice, between ten and 12 weeks of age (in-house supplier, The University of Edinburgh), were kept in groups of two to five under a 12-hour light/dark cycle, at 21^{o}C and provided *ad libitum* with food and water containing 0.05% para-aminobenzoic acid (PABA); a growth factor for parasites. Dynamics of the *P. chabaudi* AJ infections were monitored when exposed to treatments consisting of material derived from self, non-self (genotype ER), and RBCs (detailed below and in table 3.1). AJ was chosen as the focal genotype, because it has been shown to respond to competition from unrelated strains with large changes in gametocyte investment and sex ratio (Reece et al., 2008, Pollitt et al., 2011b). All procedures were carried out in accordance with the UK Home Office regulations (Animals Scientific Procedures Act 1986) and approved by the ethical review panel at The University of Edinburgh.

Table 3.1 Summary of cue treatment groups, sample sizes, rationales, and classifications. The analysis involved comparing individual cue treatments and comparing treatments grouped in different ways to test whether parasites respond to lysed parasite material (P *vs.* NP) and/or to lysed RBC material (L vs NL). N = number of mice that received a particular treatment.

Cue treatment	N	Rationale	Classification		
			Treatment	Lysed Parasites	Lysed
Control	5	No-treatment control for the stress of handling and injections.	C	NP	NL
Uninfected RBC	5	Control for the stress of handling and injecting the host with blood.	U	NP	NL
Uninfected lysed RBC	10	To test for a response to RBC debris	UL	NP	L
AJ-infected lysed RBC	10	Compare AJ to UL to test for a response to high density of self	AJ	P	L
ER-infected lysed RBC	10	Compare ER to AJ to test for a response to non-self	ER	P	L

3.3.2 Cue treatments

The experiment consisted of five treatment groups that received different cues injected into hosts (table 3.1). The cue treatments, and the acronyms they are hereafter referred to as, are: (i) unmanipulated control, 'C'; (ii) uninfected whole RBCs control, 'U'; (iii) uninfected lysed RBCs, 'UL'; (iv) AJ-infected lysed RBCs, 'AJ'; and, (v) ER-infected lysed RBCs, 'ER'. Note that these cues do not include the administration of additional live self (AJ) or competing (ER) parasites, nor do they

directly affect the amount of RBC resources available to the focal AJ parasites. This avoids the potential problem of incorrectly interpreting a change in gametocyte investment or sex ratio as a parasite strategy when, for example, competition limits the availability of RBCs for gametocyte development, or induces immunity that increases gametocyte mortality.

The use of lysed *P. chabaudi* infected RBCs was inspired by recent demonstrations that asexual stages contain products that are packaged into 'exosomes' or 'microvesicles' to stimulate sexual differentiation in recipient parasites (Regev-Rudzki et al., 2013, Mantel et al., 2013). AJ infected RBCs (AJ) and ER infected RBCs (ER) were chosen to examine whether parasite products can be used to discriminate kin from non-kin (i.e., determine the presence of a con-specific genotype) in mixed infections, as suggested by previous experiments (Wargo et al., 2007a, Reece et al., 2008, Pollitt et al., 2011b, Cameron et al., 2013). It is also possible that the high concentration of parasitized material in the AJ and ER cues mimicked a high density infection or high parasite mortality. Lysed, uninfected RBCs (UL) were intended to act as a control for the lysed, parasitised material, to distinguish whether any responses to the AJ and ER cues were due to parasite products or the lysed RBCs themselves. It is also possible that the administration of lysed uninfected RBCs mimics anaemia because many uninfected RBCs are lysed during an infection and gametocyte investment and sex ratio correlate with RBC resource availability (Trager et al., 1999, Paul et al., 2003, Reece et al., 2005). Cells (RBCs and parasites) and the serum of the blood they were collected in were present in the cues. This was to maximize the chance that the cue material contained all

potentially relevant factors, for example molecules released from inside cells, membrane components, or immune factors in the plasma.

To prepare the cue material, eight mice were infected via intraperitoneal (IP) injection with 1 x 10^6 AJ parasitized RBCs, and eight separate mice with 1 x 10^6 ER parasitized RBCs; both passaged from donor mice. When these infections reached their peak densities (on day 7 or 8 post infection (PI)), blood (infected with parasites at ring and trophozoite stages) was extracted from anaesthetized mice via cardiac puncture. Total blood volume, RBC density and parasite density were recorded for each mouse. The AJ and ER infected blood was pooled separately. The density of parasites in the pooled blood for each strain was similar; for AJ this was 1.61 x 10^9 parasitized RBCs/ml of cue and for ER-infected blood this was 1.31 x 10^9 parasitized RBCs/ml of cue. RBC densities were also similar, with an average RBC density for the AJ cue of 5.14 x 10^9 RBCs/ml blood and 4.77 x 10^9 RBCs/ml blood for the ER cue. Blood from naïve mice was collected for the UL cue. The RBC density for blood from naïve mice was much higher (9.06 x 10^9 RBCs/ml blood) than for the AJ- and ER-infected mice. Therefore, to ensure RBC density was consistent across all cues, the blood for the UL cue was diluted with serum from uninfected mice, to give a final RBC density of 4.53 x 10^9 RBCs/ml blood. For each of the cue treatment groups requiring lysed material (AJ, ER, UL) the cues went through four cycles of freeze–thaw, to ensure lysis of RBC and parasite membranes. Lysed cues were confirmed not to contain any live parasites capable of initiating an infection prior to the experiment, as follows. Three naïve mice each received 2 x 100 µl IP injections of the AJ cue with a four-hour gap between injections. PCR analysis of blood DNA

samples (Drew and Reece, 2007) taken from the three mice confirmed that no parasite material was present in the blood 48 hours after injection of the cue and no infections appeared over the subsequent two weeks. Finally, for the U cue treatment group, blood was obtained via cardiac puncture from a naïve mouse immediately before it was injected as a cue.

On treatment days, 2 x 100 µl of cue material was administered to hosts via IP injection, with a four-hour gap between the injections. For the AJ cue, each host received a total of 1.03×10^9 lysed RBCs, of which 3.21×10^8 had been parasitized. For the ER cue, each host received a total of 9.53×10^8 RBCs, of which 2.62×10^8 were parasitized. The lysed parasite material that was administered in both the AJ and ER cues was at least at the density that is typically observed at the peak of live AJ infections (assuming some cue material is cleared by innate immune factors before reaching the bloodstream). For example, the mean parasite density at the peak of infection for the control group, in cohort 2, of this experiment was 5.95×10^7 parasites/ml blood. The cue administration regime (2 x 100 µl IP injections), with a four-hour gap between injections was chosen from pilot studies because it results in parasite material being detectable (by PCR) in the blood from 20 minutes and up to 24 hours post administration of the first cue; ensuring that cues are present in the bloodstream during the ring and trophozoite stages of the asexual cycle. Exposing a large proportion of the asexual cycle to cue treatments was necessary, because it is not known which stage is responsible for detecting the environmental signals that influence gametocyte investment and sex ratio decisions.

3.3.3 Experimental design

Two cohorts, each containing 40 mice, were used to compare the effect of the cues administered during the pre-peak phase (day 4 PI; cohort 1) and post-peak phase (day 10 PI; cohort 2) of AJ focal infections (table 3.1). Whilst transmission can occur throughout *P. chabaudi* infections, these time-points were chosen specifically because previous studies have revealed that this is when the largest effects of mixed-genotype infections on gametocyte investment and sex ratio have been observed (Reece et al., 2008, Pollitt et al., 2011b). On day 0, all mice were infected with 1 x 10^6 AJ parasitized RBCs via IP injection, and mice were randomly allocated to the cohorts and cue treatment groups. Gametocyte density and sex ratio were examined on the days of cue administration to verify that there was no significant variation across treatment groups that could confound the detection of parasite responses. For *P. chabaudi*, it is thought that committed parasites differentiate into gametocytes in the cycle following the detection of a cue, that gametocytes require approximately 48 hours to reach maturity, and gametocytes remain infectious for a further 24 hours (Buckling et al., 1999a). Therefore, to cover the period over which the focal AJ parasites could detect cues, adjust their reproductive strategies in response, and for the resulting gametocyte investment and sex ratios phenotypes to be detected, infections were monitored over the three days (i.e., three asexual cycles) following cue administration. To check whether aspects of the in-host environment (known to influence reproductive strategies, which could confound parasite responses to the cues given) varied across the treatment groups, the densities of RBCs, asexual stages and the proportion of RBCs that were reticulocytes were also monitored for three

days post cue administration. The experiment was designed so that the responses to all cues could be compared to each other, and so that some cues could be combined to test for general responses to lysed parasites and/or lysed RBCs by grouping cue treatments into those containing parasite material ('P') or not ('NP'), and those containing lysed RBC material ('L') or not ('NL'), (table 3.1).

3.3.4 Data collection and analysis

Blood samples (taken from tail snips) were collected for thin smears (to count reticulocyte proportion), to measure RBC densities (using flow cytometry, Beckmann Coulter Counter), and for DNA and RNA to quantify parasites, gametocytes and sex ratios. Samples were collected daily, from day 2 to day 15 PI for both cohorts, but analyses were restricted to day 4 to day 7 PI for cohort 1, and day 10 to day 14 PI for cohort 2. Mouse weight was monitored every other day for both cohorts. All samples were obtained in the morning when parasites were at ring stage, before DNA replication for the production of daughter progeny had occurred. The density of reticulocytes was calculated from examination of blood smears and coulter count readings. DNA and RNA were extracted from blood samples using the ABI Prism 6100 Nucleic Acid PrepStation and the Bloodprep chemistry (for DNA, Life Technologies) or total RNA chemistry system (RNA, LifeTechnologies) as described in (Drew and Reece, 2007). cDNA was generated from RNA and quantitative PCR was used to quantify DNA or cDNA, according to the protocols outlined in (Drew and Reece, 2007). Real-time PCR was performed a) on DNA using CG2 primer pairs (Wargo et al., 2007a) to quantify asexual parasites, b) on cDNA using CG2 primer pairs to quantify total gametocytes, and, c) on cDNA using

MG8 primer pairs to quantify male gametocytes, according to the protocols outlined in (Drew and Reece, 2007). Sex ratios were calculated by dividing the number of male gametocytes by the total number of gametocytes in any given sample.

Data were analysed using R version 3.0.2. Response variables were $\log_{10}$ transformed (gametocyte density) or arcsine square root transformed (sex ratio) to meet the assumptions of normality. ANOVAs were performed to compare RBC densities, reticulocyte densities and asexual densities across cue treatment groups. Comparisons were made on the day of cue administration before cues were given, and for the following three days. The cumulative gametocyte densities for three days post cue administration were used to compare gametocyte investment decisions across treatments. In this case, it was appropriate to use gametocyte density as a measure of gametocyte investment because asexual densities did not vary significantly across the treatment groups before cue administration (see table 3.2). This means that any observed differences in gametocyte density must result from different levels of gametocyte investment (i.e., given that all else is equal, variation in gametocyte densities can only result from variation in investment in response to cues). This approach also avoids the difficulties of accurately calculating gametocyte investment (Carter et al., 2013), especially when the time period between parasites detecting cues and their response being measurable is uncertain. Similarly, for sex ratio, the time between parasites detecting cues and their response being measurable is uncertain, so the mean sex ratio for the three days post cue administration was compared across groups. Finally, Welch's unpaired T test (for unequal variances) was used to compare the effects of parasitized *versus* non-parasitized cues and lysed

versus non-lysed cues on cumulative gametocyte densities and mean sex ratios for both cohorts 1 and 2. The number of samples analysed varied between tests because (a) some mice died during the experiment, and (b) total and male gametocyte densities below the lower limits of detection for the PCR were excluded, because quantification was unreliable.

Table 3.2 Summary of ANOVA analyses.

Asexual density and the in-host environmental parameters of RBC density and proportion of reticulocytes did not vary significantly across the treatment groups - either prior to, or post cue administration, in either cohort. Furthermore, gametocyte density and sex ratio did not vary significantly prior to cue administration. This means that the effects of the cue treatments were not confounded by unintended variation in the in-host environment or pre-existing variation in gametocyte density and sex ratio (see also appendix figure 1).

	Cohort 1		**Cohort 2**	
	Prior: day 4	**Post: days 5-7**	**Prior: day 10**	**Post: days 11-13**
Asexual density	$F_{4, 34} = 1.13$, $p = 0.36$	$F_{4, 34} = 0.79$, $p = 0.54$	$F_{4, 25} = 0.59$, $p = 0.68$	$F_{4, 24} = 0.14$, $p = 0.97$
RBC density	$F_{4, 34} = 1.00$, $p = 0.42$	$F_{4, 34} = 1.70$, $p = 0.17$	$F_{4, 28} = 1.62$, $p = 0.20$	$F_{4, 24} = 0.45$, $p = 0.77$
Reticulocyte proportion	$F_{4, 34} = 1.05$, $p = 0.40$	$F_{4, 34} = 0.32$, $p = 0.86$	$F_{4, 28} = 0.77$, $p = 0.56$	$F_{4, 24} = 1.53$, $p = 0.23$
Gametocyte density	$F_{4, 34} = 0.17$, $p = 0.95$	$F_{4,34} = 0.39$, $p = 0.81$	$F_{4, 28} = 1.60$, $p = 0.20$	$F_{4,20} = 1.73$, $p = 0.18$
Sex ratio	$F_{4,31} = 1.27$, $p = 0.30$	$F_{4,34} = 0.60$, $p = 0.67$	$F_{4,28} = 0.63$, $p = 0.64$	$F_{4,26} = 0.22$, $p = 0.93$

3.4 Results

3.4.1 Asexual densities and in-host environmental variables

Asexual density, RBC density, and the proportion of RBCs that are reticulocytes all correlate with reproductive decisions and so variation in these parameters across treatment groups could potentially confound any responses to the cue treatments. However, there was no significant variation in these parameters across treatment groups, either before cue administration, or over the subsequent three-day period, for either cohort (table 3.2, appendix figure 1)

3.4.2 Gametocyte investment

Gametocyte densities were not significantly different between treatment groups either pre peak of infection (cohort 1) or post peak (cohort 2) on the days of cue administration (figure 3.1A and table 3.2). This result, together with the validation that asexual densities and in-host environmental variables were not significantly different prior to cue administration means that, in this study: gametocyte density is synonymous with gametocyte investment. For the three days following cue administration, there were no significant differences in cumulative gametocyte densities between the five cue treatment groups in either cohort 1 or cohort 2 (figure 3.1A and table 3.2). When treatments were grouped to compare the effect of cues containing parasitized (P) *versus* non-parasitized (NP) material, there were no significant differences in gametocyte densities in cohort 1 ($t(35.8) = 0.83$, $p = 0.41$) (figure 3.1B). However, in cohort 2, gametocyte density was significantly 50% lower in infections that received parasitized cues (378 ± 75 gametocytes/µl blood),

compared to those that received non-parasitized cues (753 ± 125 gametocytes/µl blood), ($t(22.9) = -2.19$, $p = 0.04$) (figure 3.1B). Finally, when treatments were grouped to compare cues containing lysed (L) or non-lysed (NL) material, there were no significant differences for cohort 1 ($t(12.8) = 0.12$, $p = 0.91$) or cohort 2 ($t(6.6) = -1.47$, $p = 0.19$) (figure 3.1C).

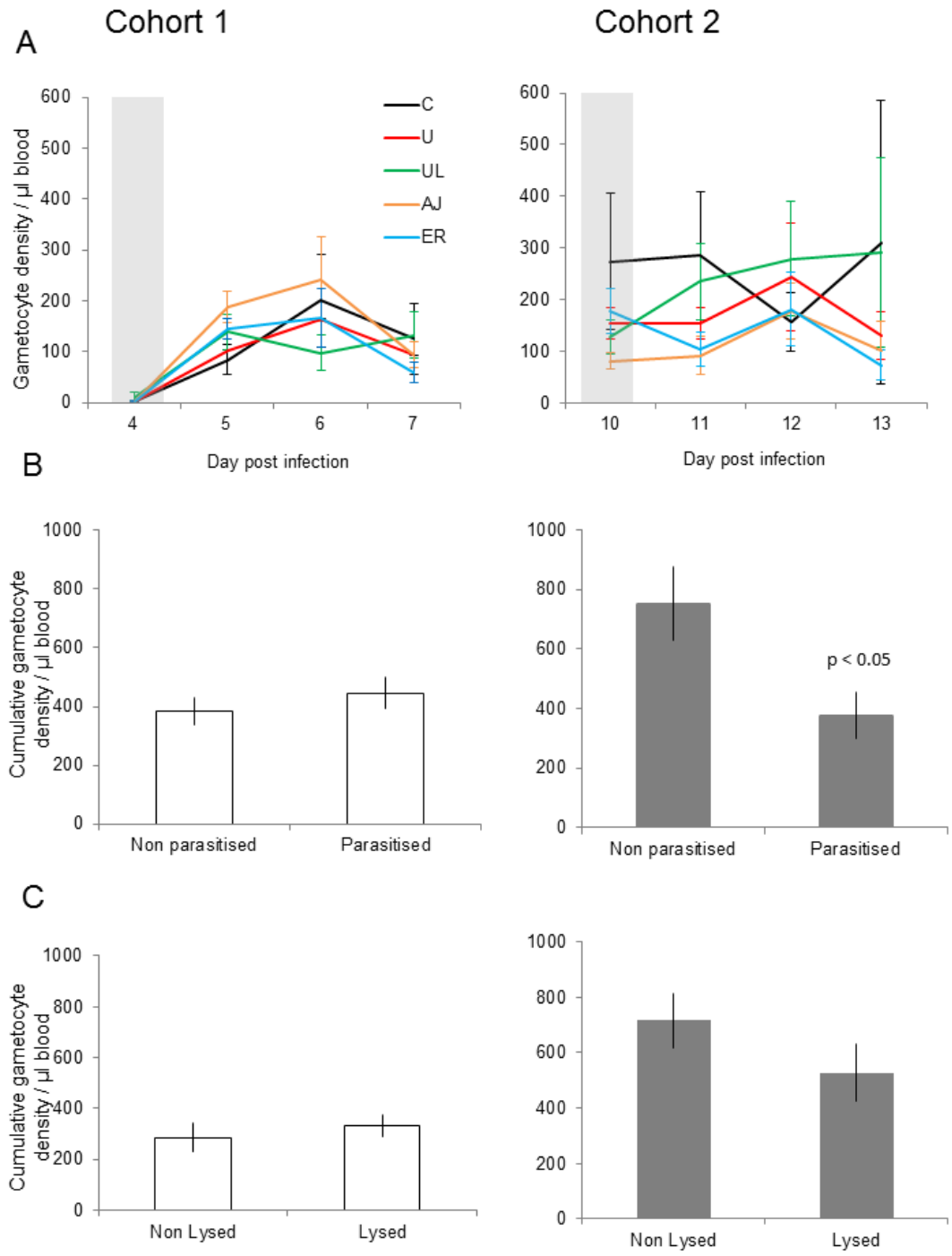

Figure 3.1 *Plasmodium chabaudi* AJ gametocyte density dynamics. (± SEM) from the day of administration of five cue treatments: C: control, U: uninfected RBCs, UL: uninfected lysed RBCs, AJ: AJ infected lysed RBCs and ER: ER infected lysed RBCs. Grey bars indicate the days when cues were administered - on day 4 PI for cohort 1 (left) and day 10 PI for cohort 2

(right) **(A)**; cumulative gametocyte densities (± SEM) for three days post treatment with cues containing parasitized material (P: AJ, ER) or non-parasitized material (NP: C, U, UL) for cohort 1 (left) and for cohort 2 (right: where gametocyte density was significantly lower in the P group than NP group) **(B)**; cumulative gametocyte densities (± SEM) for three days post treatment with either lysed RBC material (L: UL, AJ, ER) or non-lysed material (NL: C, U) for cohort 1 (left) and cohort 2 (right) **(C)**.

3.4.3 Sex ratio

Sex ratios (proportion of male gametocytes; figure 3.2A) were not significantly different between cue treatment groups for cohort 1 or cohort 2 on the days of cue administration (table 3.2). Therefore, as for gametocyte density, there was no pre-existing significant variation in sex ratios that could have confounded any changes in sex ratio following the cue treatments. For the three days following cue administration there were no significant differences in mean sex ratios between the five treatment groups in cohort 1 or cohort 2 (figure 3.2A and table 3.2). When cue treatments were grouped to compare the effect of parasitized (P) *versus* non-parasitized (NP) material, there were no significant differences in mean sex ratio in cohort 1 ($t(36.7) = 0.66$, $p = 0.51$), or in cohort 2 ($t(27.8) = -0.35$, $p = 0.73$) (figure 3.2B). However, when treatments were grouped to compare the effects of cues containing lysed (L) or non-lysed (NL) material, there was a borderline significant increase in sex ratio (of 45%) in infections that received lysed material (0.11 ± 0.02), compared to those that received non-lysed cues (0.06 ± 0.01) in cohort 1 ($t(27.0) = 2.04$, $p = 0.05$), but not in cohort 2 ($t(9.87) = -0.13$, $p = 0.90$) (figure 3.2C).

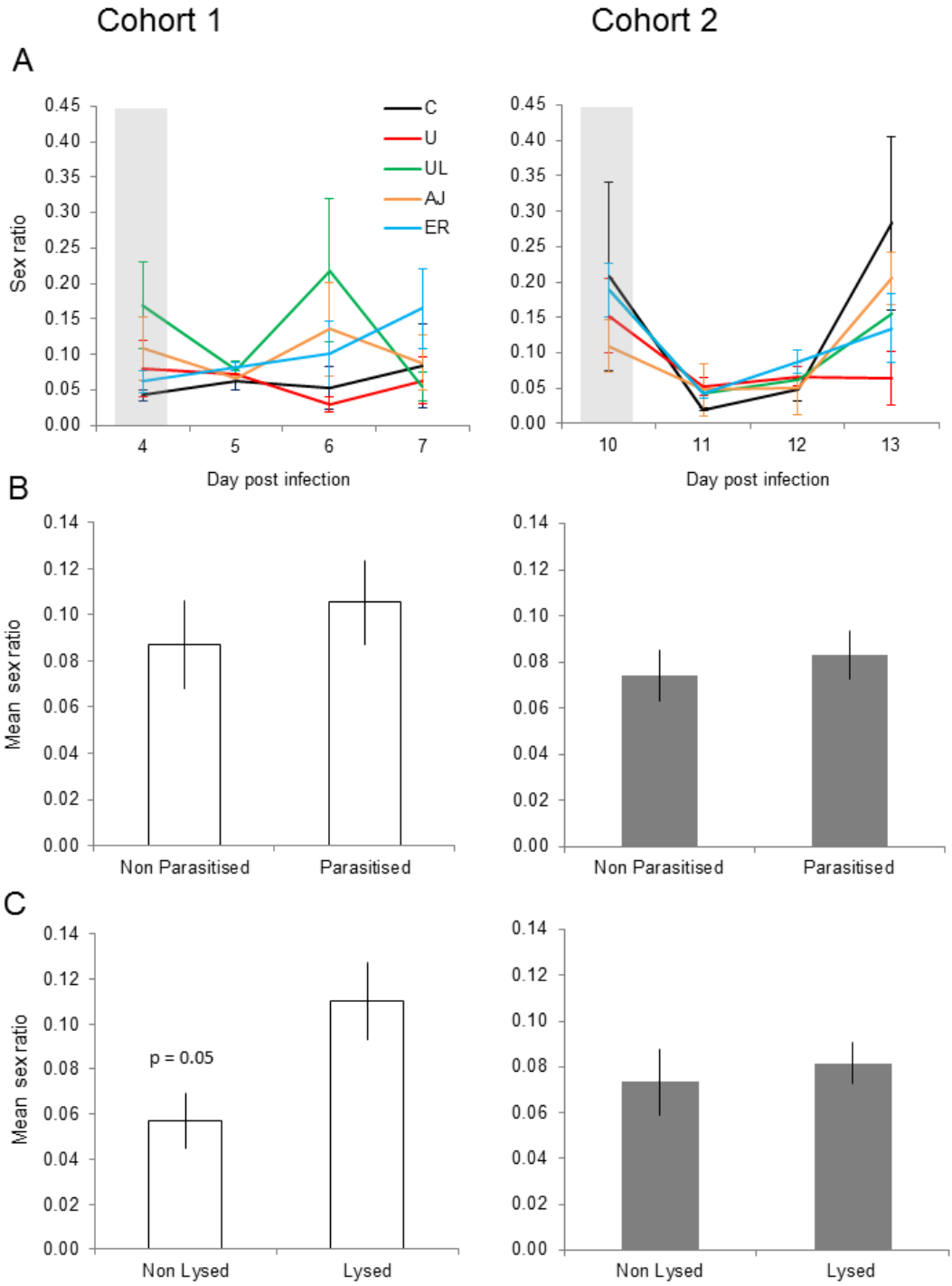

Figure 3.2 *Plasmodium chabaudi* AJ sex ratio (proportion of male gametocytes) dynamics. (± SEM) from the day of administration of five cue treatments: C: control, U: uninfected RBCs, UL: uninfected lysed RBCs, AJ: AJ-infected lysed RBCs and ER: ER-infected lysed RBCs). Grey bars indicate the days when cues were administered - on day 4 PI for cohort 1

(left) and day 10 PI for cohort 2 (right) **(A)**; mean sex ratio (± SEM) for three days post treatment with cues containing parasitized material (P: AJ, ER) or non-parasitized material (NP: C, U, UL) for cohort 1 (left) and cohort 2 (right) **(B)**; mean sex ratio (± SEM) for three days post treatment with either lysed RBC material (L: UL, AJ, ER) or non-lysed material (NL: C, U) for cohort 1 (left: where sex ratio was significantly (borderline) higher in the lysed group), and for cohort 2 (right) **(C)**.

3.5 Discussion

The experiments presented here reveal that: (i) gametocyte investment is reduced by 50% in response to lysed material containing parasites (P) compared to material without parasites (NP); (ii) the change in gametocyte investment in response to parasitized material occurs post peak of infections but not during the growth phase; (iii) there was a borderline significant increase (45%) in the proportion of male gametocytes in infections given lysed (L) compared to non-lysed (NL) material; and, (iv) the potential sex ratio adjustment in response to lysed material only occurred in the growth phase of infections. The following paragraphs discuss how these results compare to studies of human and rodent infections that report changes in sex ratio and gametocyte investment in response to variation in RBC resource availability, drugs, competition, and parasite density (Buckling et al., 1997, Trager et al., 1999, Buckling et al., 1999b, Buckling et al., 1999a, Paul et al., 2000, Robert et al., 2003, Dyer and Day, 2003, Reece et al., 2005, Wargo et al., 2007b, Reece et al., 2008, Sowunmi et al., 2009, Peatey et al., 2009, Neal and Schall, 2010, Reece et al., 2010 Pollitt et al., 2011b).

In the post-peak phase of infections, why do parasites make different gametocyte

investment decisions when exposed to material derived from non-parasitized (NP) blood compared to parasitized blood (P, figure 3.1B)? Gametocyte investment is lower in the P group compared to NP group which suggests that either the parasites in the P group are adopting reproductive restraint (i.e., actively reducing investment) or the parasites in the NP group are making a terminal investment (i.e., actively increasing investment). The former scenario is the most likely for the following reasons. When parasites are faced with adverse, but not lethal, circumstances either due to resource limitation or death rates that do not exceed the capacity for replication, they are predicted to adopt a strategy of reproductive restraint (Mideo and Day, 2008, Pollitt et al., 2011a, Carter et al., 2013) (chapter 2). Lysed parasite material in the P group could signal that many parasites are being killed (e.g., due to immune attack or drugs) and reproductive restraint enables the replication rate to exceed the death rate. The ability to predict future scenarios may seem highly sophisticated for parasites, but this is one of the main evolutionary drivers of adaptive phenotypic plasticity (Scheiner, 1993, McNamara et al., 2009). Preparing for environmental change in advance avoids fitness costs incurred by delays involved in waiting for the environment to change and then reacting, or not reacting to environmental change at all (deWitt et al., 1998). Second, the gametocyte investment of parasites in the NP group appears too low to be explained by terminal investment. This is because the NP group includes the unmanipulated control group and most studies use such infections as a baseline to demonstrate that increased investment (i.e., terminal investment) occurs in response to drugs. In summary, gametocyte investment appears to be reduced in response to material from parasitized blood, which is consistent with parasites adopting reproductive restraint to maximize

survival during stressful, but not lethal, challenges during infections (Wargo et al., 2007a, Reece et al., 2010, Pollitt et al., 2011b).

Instead of parasites actively adjusting gametocyte investment, could differential immune responses in the P and NP groups explain the observed differences in gametocyte investment? It is possible that the administration of lysed parasitized material induced the host to produce the pro-inflammatory cytokines interferon gamma (IFN-gamma) and tumour necrosis factor-alpha (TNF-alpha), which are known to be involved in killing gametocytes (Naotunne et al., 1991, Naotunne et al., 1993, Long et al., 2008). However, data from *in vitro* studies suggest this would be unlikely, as the induction of TNF and IFN-gamma is much reduced when exposed to lysed parasitised RBCs, compared with exposure to live intact parasitised RBCs (Scragg et al., 1999, Hensmann and Kwiatkowski, 2001, O'Dea and Pasvol, 2003, Waterfall et al., 1998). Furthermore, the induction of TNF by lysed parasites in culture is negligible when the parasitised erythrocytes were harvested and lysed at ring and / or trophozoite stages (compared to lysis at schizont stage) (Scragg et al., 1999). As such, the P group (a lysed mixture of ring and trophozoite infected erythrocytes) is unlikely to have induced TNF to a level that was sufficient to clear gametocytes. Furthermore, the gametocytocidal activity of TNF is rapid (Naotunne et al., 1991) and would therefore have produced a sharp drop in the P group on day 11 only, which was not observed. Finally, the cue treatments were the same in cohort 1 and 2 and so should elicit the same immune responses. If these responses killed gametocytes then fewer gametocytes would have been observed in the P group of cohort 1 as well, but this was not the case.

The question of why parasites only adopted reproductive restraint in response to parasite material in the post-peak phase (i.e., in cohort 2) of infections requires further work. This timing is consistent with previous studies showing that the difference in gametocyte investment between parasites in control and sub-lethal conditions increases over time (Reece et al., 2010, Pollitt et al., 2011b). Furthermore, the timing suggests a biologically significant difference in phenotype with real epidemiological relevance, as it is at this later stage of *Plasmodium chabaudi* infections where transmission is typically most successful in laboratory studies (Ferguson et al., 2003). Furthermore, a twofold reduction in gametocyte density in *Plasmodium falciparum* infections can have a significant impact on the proportion of mosquitoes infected (Churcher et al., 2013). The lack of any effect in the pre peak phase of the infection may be due to the difficulty in detecting small effects at low parasite densities (as is the case early in infections), or because parasites become increasingly able to detect, or respond to, environmental changes as infections progress. The latter is perhaps the most parsimonious explanation because cumulative gametocyte densities are very similar between all of cohort 1 and the P of cohort 2 (figure 3.1B; ($t(41.5) = -1.02$, $p = 0.31$). This may reflect a necessity to maintain a baseline level of gametocyte production to ensure no transmission opportunity is wasted, even during reproductive restraint.

Why might parasites make different sex ratio decisions when exposed to material derived from lysed cells (L; parasites and RBCs), and why is this only observed in

the growth phase of infections? Further work is required to confirm whether parasites do produce a less female-biased sex ratio when exposed to lysed cues (because significance was borderline), but this pattern is predicted by evolutionary theory and consistent with other data (West et al., 2001, Gardner et al., 2003, Reece et al., 2008). Lysed material could either represent host anaemia, or the material could have stimulated innate host immune responses that reduce the fertility of males more than females. In these situations, males become a limiting resource for fertilization and so parasites are predicted to partially compensate by increasing their investment in male relative to female gametocytes (West et al., 2001, Paul et al., 2002, West et al., 2002, Gardner et al., 2003, Reece et al., 2008, Ramiro et al., 2011). That extra males are required to ensure females are fertilized when transmission blocking immune factors have more severe effects on males is intuitive, but why are more males required when hosts are anaemic? Each male gametocyte can produce up to eight gametes, but each female only produces one gamete, which means that the number of parasite progeny is maximized at a ratio of eight female gametocytes to one male gametocyte (Hamilton, 1967, Godfray and Werren, 1996). However, when there are eight-fold fewer male gametocytes circulating in the host and gametocyte density is very low, or hosts are anaemic, there is a stochastic risk that blood meals do not contain enough males to ensure the females are fertilized (West et al., 2001, Gardner et al., 2003). Therefore, if lysed material represents anaemia and/or immune factors, parasites will be most sensitive to these scenarios when gametocyte density is low (i.e., in cohort 1; figure 3.1C). In summary, similarly to the gametocyte investment results, the sex ratio data suggest lysed cell material (parasitized and non-parasitized) is interpreted as a cue for adverse conditions.

Based on previous observations of mixed genotype infections and evolutionary theory (Hamilton, 1967, Godfray and Werren, 1996, Schall, 2000, Eisen and Schall, 2000, Reece et al., 2008, Mideo and Day, 2008, Neal and Schall, 2010, Pollitt et al., 2011b), parasites were predicted to adopt different reproductive strategies when exposed to cue material derived from self (AJ) *versus* a non-self, con-specific genotype (ER). However, there were no significant differences either in gametocyte investment (figure 3.1A) or sex ratio (figure 3.2A) when parasites were exposed to AJ *versus* ER cue material, in either cohort. This could be due to a number of (non-mutually exclusive) reasons. First, there may not have been a high enough concentration of lysed ER parasite material in the bloodstream in the ER group for live AJ parasites to discriminate kin from non-kin. Alternatively, the cue to discriminate kin may be something that is only actively secreted by live parasites in direct response to competitors (which were not present in the cue-generating infections), or degraded in the freeze-thaw process. For example, malaria parasites could employ a similar quorum-sensing strategy to that observed in bacteria (Miller and Bassler, 2001, Diggle et al., 2007) and use microvesicles (Mantel et al., 2013) or exosome-like vesicles (Regev-Rudzki et al., 2013) derived from infected RBCs as a carrier for the cue. However, microvesicle or exosome structures may have been destroyed during cue preparation lysis. The cue treatments were designed simply to test whether parasite responses could be elicited, rather than to identify precisely what they are detecting, so it is possible that the live AJ parasites could discriminate kin, but the AJ and ER cues also represented other scenarios (e.g., a high death rate), that provided a stronger stimulus and resulted in the responses detected.

3.6 Conclusions

Despite decades of investigating gametocytes, how the genes and molecular pathways underpinning commitment to gametocytes and sexual differentiation interact with environmental sensing has proved difficult (Dixon et al., 2008, Ranford-Cartwright and Mwangi, Liu et al., 2011, 2012, Guttery et al., 2012), although recent characterisation of the ApiAP2 gene family in *Plasmodium falciparum* and *Plasmodium berghei* is promising (Kafsack et al., 2014, Sinha et al., 2014). The difficulty may be partly due to different genes and pathways being involved in: (a) sensing environmental cues relevant to decisions about reproductive strategies; (b) processing information and making decisions; and, (c) producing the gametocyte investment and sex ratio phenotypes resulting from the decisions made (Carter et al., 2013). Breaking down treatments to isolate the molecule(s) used as a cue(s) within the morass of lysed cells and serum used in this study could facilitate further characterization of molecular mechanisms underpinning commitment and differentiation into gametocytes. Repeating the experiments presented here *in vitro,* to expose synchronous parasites at different time points within the cell cycle could reveal which developmental stages are responsible for sensing and responding to changes in the in-host environment. More broadly, it may be possible to harness cues to 'trick' parasites in an infection into producing gametocytes instead of asexuals, or only producing gametocytes of a single sex (Williams, 2010, Carter et al., 2013). The former strategy could be useful for treating returned travellers in hospital (without malaria vectors) because the virulence of infections will be reduced, and the latter strategy would prevent fertilization and subsequent transmission. Finally, precisely identifying the cues that parasites use to make reproductive decisions is required to

quantify the costs and benefits (fitness consequences) of their strategies, which is central to understanding their evolution.

4 Biology and behaviour of *Plasmodium berghei* microgametes

4.1 Summary

Despite sexual reproduction in the mosquito midgut being essential for their transmission, little is known about the mating behaviour of malaria parasites. Once inside the mosquito vector, gametocytes immediately differentiate into male and female gametes and motile male gametes must swim through the hostile environment of the bloodmeal to find and fertilise female gametes. Developing drugs and/or vaccines that prevent transmission by disrupting mating are major goals of biomedicine and the male gamete (microgamete) is thought to be an attractive target for such interventions. Therefore, knowledge of fundamental aspects of microgamete morphology and behaviour is key to developing successful transmission blocking interventions. Here, I describe three projects to elucidate key characteristics of the microgamete. First, I demonstrate how digital holographic microscopy can be used to characterise the morphology and motility of microgametes. The observations I make about microgamete motility raise questions about the consequences of their interactions with red blood cells in the blood meal, and how microgametes find females. I therefore carried out two further projects which experimentally manipulated the mating environment *in vitro*; to test whether mating is hindered by obstacles (such as red blood cells) and to test whether microgametes preferentially swim towards female gametes.

4.2 Introduction

To transmit to new vertebrate hosts, malaria parasites must produce specialised sexual stages (gametocytes) which are taken up in the blood meal of the mosquito vector. Gametocytes are produced continuously (but in varying numbers) throughout infections (Carter et al., 2013) and circulate in the blood stream for several days whilst waiting to be taken up when an insect vector bites the host and takes a blood meal (Baton and Ranford-Cartwright, 2005, Bousema and Drakeley, 2011). As soon as male and female gametocytes are ingested by a mosquito, gametogenesis is triggered by the drop in temperature, change in pH, carbon dioxide tension and mosquito midgut factors such as xanthurenic acid (Billker et al., 1997, Arai et al., 2001), but see chapter 5 for a more in-depth analysis of this. Within a few minutes of ingestion, male and female gametocytes round up and shed their surrounding RBC membranes. Female gametogenesis does not involve any DNA replication; a single female gamete simply emerges from the residual gametocyte infected RBC (facilitated by pre-synthesized secretory organelles called osmiophilic bodies) (Ponzi et al., 2009), but male gametocytes undergo a process termed exflagellation; producing up to eight flagellated and motile male gametes (microgametes) from each gametocyte (MacCallum, 1897, Laveran , 1881).

4.2.1 Microgametes

Within ten minutes of activation, male gametocytes have undergone three rapid rounds of endomitosis and axoneme assembly within the cytoplasm (Sinden et al., 1976). A single microtubule organising centre (MTOC) located on the cytoplasmic

side of the nuclear pore consists of eight basal bodies which are the nucleation sites for the growth of microtubules to make up the tubulin-based axonemes of microgametes (Sinden et al., 2010). Unlike the basal bodies of most eukaryotic cilia which have nine microtubule triplets, the malaria microgamete axoneme has a set of nine singlets surrounding two central microtubules (Sterling and Aikawa, 1973) (figure 4.1C). Chromatin condensation, axoneme motility and cytokinesis are the final stages before the nucleated, flagellated microgametes are extruded from the gametocyte body (Sinden et al., 2010). The flagellum is forced through the gametocyte membrane, tearing the adjacent haploid genome along with some of the nuclear envelope from the parental nucleus into the emerging gamete (Sinden et al., 1976). As a result, nuclear material is distributed along the axoneme over a length of 1-2μm (Sinden et al., 2010, Straschil et al., 2010) and is surrounded by a plasma membrane (Sinden et al., 1976, Sinden et al., 2010) (figure 4.1). Despite the complex processes involved in microgamete development, both genetic and micrographic evidence indicate that the microgamete is a very simple cell, containing no other organelles than the axoneme, nucleus and plasma membrane (Sinden et al., 1976, Okamoto et al., 2009) (figure 4.1A,B). During the process of gametogenesis, microgametes are frequently observed interacting with RBCs, other parasite infected cells, and emerged female gametes *in vitro*. However, the reason for these temporary but strong interactions, termed 'exflagellation centres' (Templeton et al., 1998) is not known, and has not yet been examined *in vivo*. Because of this, it remains unclear whether these interactions have a requisite function, are a hindrance to mating success or simply an artefact of *in vitro* conditions (in which, cells are typically less dense than the mosquito midgut).

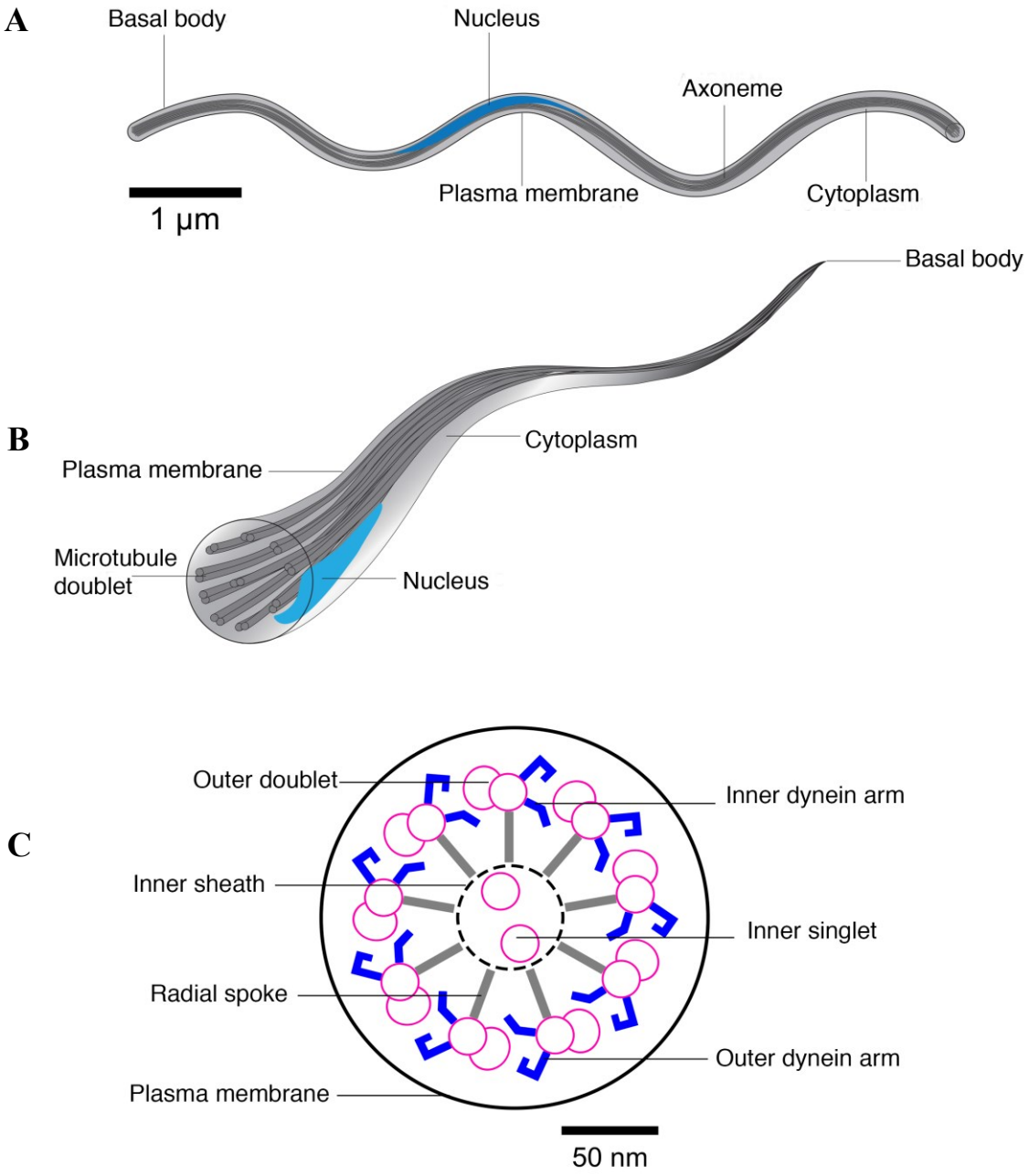

Figure 4.1 Microgamete morphology Longitudinal (A) and angled (B) cross-sectional illustrations show the simplicity of a typical *P. berghei* microgamete (credit: Francesca Bourne). The sketches are based on electron micrographs in studies by Straschil et al. (Straschil et al., 2010) and Sinden et al. (Sinden et al., 2010), and are labelled to show the key features of the flagellum. (C) Cross-sectional schematic diagram of a microgamete detailing the elements common to a typical 9+2 axoneme (credit: Laurence Wilson). The flagellar waveform is driven by microtubule doublets, which, in

turn, are driven by the shearing force generated by the dynein arms. Figure is adapted from (Wilson et al., 2013).

4.2.2 Microgamete morphology and behaviour

Once released from the residual gametocyte body, flagellated microgametes must locate and fertilise the non-motile female gametes within a brief (approximately 30-60 minute) time window before the gametes stop swimming and die (Carter and Nijhout, 1977, Sinden et al., 2010). It is not exactly clear what determines microgamete lifespan, but the mosquito midgut it is assumed to be extremely hostile for microgametes. A limited supply of resources such as glucose may constrain swimming duration (Nijhout and Carter, 1978, Wass et al., 2012), and a number of host-derived immune factors are taken up in the bloodmeal which can inhibit and kill microgametes (reviewed in (Aikawa et al., 1981, Mendis et al., 1990, Naotunne et al., 1991, Naotunne et al., 1993, Motard et al., 1993, Ramiro et al., 2011, Long et al., 2008). Furthermore, mosquitoes perform diuresis to concentrate the blood (Vaughan et al., 1991), which is likely to dramatically change the biophysics of the bloodmeal to a more colloidal composition and therefore reduce the ease of microgamete motility. Due to the difficulties parasites face during mating, fertilization appears to be a significant bottleneck in the parasite life cycle, and so interventions that target the fertility of microgametes are thought to offer a good opportunity to stop disease transmission (Eksi et al., 2006, Ponzi et al., 2009, van Dijk et al., 2010).

However, there is a lack of knowledge about fundamental and diverse aspects of microgamete morphology and behaviour. For example, definitive values for the length, diameter, speed, wavelength, fundamental chirality (clockwise / counter

clockwise motion) and beating pattern of microgametes are unclear. Why microgametes interact with RBC and whether microgametes follow non-random paths when searching for females are also unknown. I address these issues in this chapter: section 4.3 demonstrates how digital holography can be used to improve resolution of known microgamete features and to reveal new characteristics; section 4.4 explores the effect of increasing blood and microparticle density on fertilisation success *in vitro*; and section 4.5 investigates whether microgametes follow non-random paths when searching for females. A better understanding of microgamete morphology and behaviour might reveal novel targets for interventions as well as provide insight into making interventions robust to parasite evolution.

4.3 Microgamete structure and motility

This section is adapted from the supplementary information that I wrote for (Wilson et al., 2013). The supplementary information provided a broader context of the measurements of microgametes obtained by high speed digital holographic microscopy, as well as their implications for the mating biology. It also emphasises the interdisciplinary nature of this work, and proposes how results obtained from biochemical and genetic studies of malaria parasites can help to answer longstanding questions in physics, by allowing an unprecedented level of control over the structure of the axoneme.

4.3.1 Background

The ambition of this interdisciplinary project was to highlight the utility of the 'simplicity' of malaria microgametes to understand the fundamental mechanics of axonemes (which underlie flagellar waveforms). Understanding the biophysics of flagella will inform research across a wide range of biological disciplines and medical disorders. All eukaryotic cilia and flagella have the same basic structure, but they facilitate cells to move and pump fluid to perform a number of different roles; ranging from sensory detection, fluid transport in the brain and sperm propulsion (Satir, 1965, Sanchez et al., 2011). Therefore, understanding how flagella beat is critical in understanding medical ciliopathies, including male and female infertility, hydrocephalus and kidney diseases.

Flagella waveforms are also important for parasitology because microgamete swimming characteristics have implications for the evolution of parasite mating strategies and success of transmission blocking interventions. Previous work on flagellar waveforms has been limited to two-dimensional data, typically from model

systems that are constrained by accessory structures which obscure the fundamental waveform patterns. Therefore, as isolated swimming flagella, without any structures such as a cell body (as in *Chlamydomonas* algae) which potentially restrict or alter their natural motility; *P. berghei* microgametes offer a unique model system for studying basic flagellar motility.

Using a new approach; with high speed digital microscopy and 3D holographic reconstruction (developed by (Wilson and Zhang, 2012) and used in (Wilson et al., 2013)), several key physical characteristics of the microgamete of *P. berghei* were quantified (summarised in table 4.1); some for the first time (e.g., swimming direction, beating pattern and chirality of the microgamete) and others (e.g., length, diameter, wavelength, speed) in a more robust way than with previous attempts (Sinden, 1975, Straschil et al., 2010). The following sections outline the approach used, the measurements made, and their implications for the mating biology of malaria parasites.

4.3.2 Methods

The rodent malaria parasite *P. berghei* line 820 (from The University of Edinburgh's malaria reagent repository http://malariaresearch.eu/) was used to initiate infections in male MF1 mice (8-10 weeks old), which had been pre-treated with phenylhydrazine (PHZ) at 125 mg/kg (2 days prior to infection) to enhance the production of gametocytes (Reece et al., 2008). Five independent infections were initiated with 10^7 parasitized red blood cells. Infected blood was collected by tail snip when gametocytes reached maturity (day 4 or 5 post infection). For each sample, to

stimulate the differentiation of gametocytes into microgametes, 2µl infected blood was added to 1 ml of complete ookinete culture media (RPMI + 10% foetal calf serum, pH 8) and incubated at room temperature (20^{o}C; the optimal temperature for transmission of *P. berghei*) *in vitro*. Microgametes were filmed using a Nikon Ti inverted microscope and a complementary oxide semiconductor (CMOS) camera. Unlike conventional microscopy techniques, digital holographic microscopy (DHM) does not simply record the projected image of the microgamete. Instead, the wave front information originating from the microgamete is digitally recorded as a hologram from which the microgamete is later reconstructed using an algorithm (Sheng et al., 2007, Wilson and Zhang, 2012,Wilson et al., 2013). DHM allows three-dimensional imaging at frame rates limited by the imaging device (rather than mechanical translation); in this case a CMOS camera (Sheng et al., 2007). Furthermore, unlike previous holographic studies that have tracked the *average* positions of flagella in 3D, this approach allowed measurement of individuals; resulting in detailed information on the variation in microgamete swimming strokes (Wilson et al., 2013).

4.3.3 Results & Discussion

Using DHM , several key physical characteristics of the microgamete of *P. berghei* were quantified (summarised in table 4.1). Whilst some observations are completely novel (e.g., swimming direction, beating pattern and chirality of the microgamete), the length, diameter, wavelength and speed of microgametes were quantified in a more robust way than with previous attempts (Sinden, 1975, Straschil et al., 2010).

Table 4.1. Summary of the key characteristics of the *Plasmodium berghei* microgamete identified by previous estimates (*(Straschil et al., 2010), ^(Sinden, 1975)) and by the novel method of digital microscopy and holographic reconstruction (Wilson et al., 2013).

Characteristic	*Previous estimates*	New estimate
Length	*14 ± 3μm^*	8.4 ± 1.4μm, n= 24
Wavelength	*N/A*	5μm, n=19
Frequency	*~6 Hz**	9.6 ± 0.7Hz, n = 19
Diameter	*0.21±0.02μm^*	0.2μm, n= 19
Speed	*~ 9μm /s**	Fast and forward: 5 ± 0.4μm/s, n=19 Slow and reverse: 0.937μm/s, n=5
Swimming direction	*N/A*	Towards the active end
Beat pattern	*Irregular*^*	Irregular
Chirality	*N/A*	Alternating waves of left and right, n = 24

4.3.3.1 Structure

The mean length of the *Plasmodium berghei* microgamete in this study was 8.4 ± 1.4μm (SEM, n = 24). This variation in length could be attributed to a number of factors, not least the potential errors accumulated due to the speed at which the microgametes are assembled and released (Sinden et al., 2010). Variable lengths may also be due to a limited availability of resources for producing 8 full-length microtubules per microgamete at the time of synthesis. The microgamete length of 8.4μm measured in this study is slightly shorter than the previous estimate of 14 ± 3μm (Sinden, 1975). However, the microgamete diameter of 0.2μm is the same as

the previous estimate (Sinden, 1975). That the microgametes bend into an asymmetrical sinusoidal shape with a wavelength of 5.6μm are novel observations. Furthermore, although microgametes have no defined 'head', the analysis revealed clear 'active' and 'passive' ends (figure 4.2). The passive tail end is associated with the basal body, where the microgamete detaches from the residual gametocyte (Sinden et al., 2010). This is consistent with other sperm, where the basal region is the least active part of the flagellum; owing to the increased stiffness caused by accessory structures located there. It is also possible that the passive end exhibits lower flexibility because this is where the microgamete's DNA is located. However, on balance, this seems unlikely; DNA is drawn into the cell through the tail end during the final stages of gametogenesis and the nucleus is usually distributed along the centre of the cell (Sinden et al., 2010).

Because previous studies have suggested that 60% of *P. berghei* microgametes are either malformed, anucleate, or contain multiple axonemes (Sinden et al., 2010), there was risk of analysing aberrant gametes. Therefore, the probability that all the microgametes in the sample were aberrant was calculated as follows. In any given sample that was imaged, it was assumed that 60% of gametes were malformed, that one third of these 60% were anucleate (according to the observations of (Sinden et al., 2010) described above), and that it is impossible to visually distinguish between nucleate and anucleate microgametes (L. Wilson, personal communication). Given these assumptions, the chance that a single anucleate microgamete from a mixed population was imaged is predicted to be at approximately 33%. While this suggests it is likely that some (around one-third) of the microgametes in the sample were

anucleate, there were not two distinct populations in the estimates and it can be concluded either that the presence or absence of nuclear material has little impact on the swimming behaviour, or that anucleate microgametes are not as common as previously estimated. Finally, it should be noted that it is highly unlikely (~ one in a billion) that all 19 of the forward-swimming microgametes analysed were anucleate.

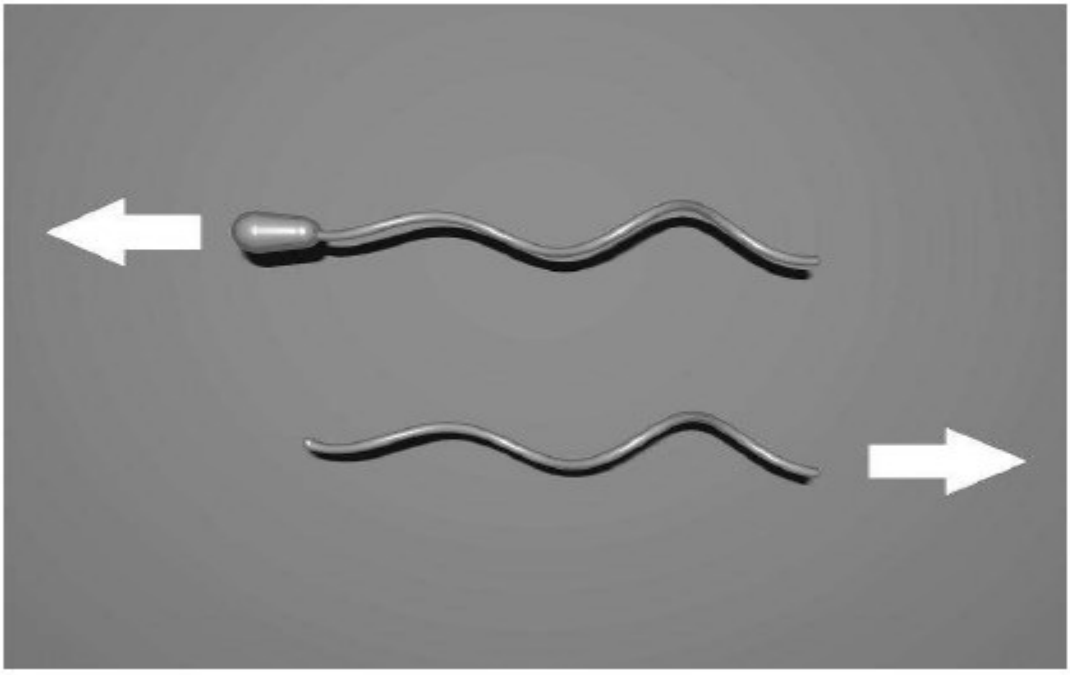

Figure 4.2: Microgamete swimming direction of mammalian sperm (*top*) and the *Plasmodium* microgamete (*bottom*), where waves of curvature propagate from the active to the passive end (basal body).

4.3.3.2 Swimming direction

In contrast to the conventional direction of sperm motility, the microgamete swims in the direction of the "active end," that is, the end with the higher average curvature. Waves of curvature propagate from the active end to the passive end as the microgamete swims (figure 4.2). This mode of swimming is analogous to the flagellum "pulling" the cell through the medium, rather than being "pushed" by a

greater activity at the tail end. The variation in average curvature is likely to be due to the presence of a mechanically distinct basal body, or a non-uniform distribution of molecular motors along the length of the flagellum such as that found in *Chlamydomonas* (Bui et al., 2012). However, further work is necessary to resolve this.

Swimming in the direction of the active end of the flagellum is rare among sperm, although it has been observed in other microorganisms, such as trypanosomes (Rodríguez et al., 2009). . However, in a blood meal environment with tightly packed RBCs, having the active end at the front may enable microgametes to probe the environment more effectively to find spaces to pass between cells and therefore move more efficiently around the blood meal. Furthermore, it is not yet known how microgametes locate females, but if chemotaxis (or other) cues are involved (see section 4.5), travelling in the direction of the active end may maximize the likelihood of detecting a chemotaxis gradient. This is because the active end describes a larger arc and so could 'sample' more of the environment than the passive end (which covers a smaller area). Similarly, the ability to swim in reverse may also be useful for finding pathways between RBCs and/or tracking chemotaxis gradients. Currently, there is no evidence for chemotaxis, but the calculations below and investigation of these issues (reported in sections 4.4 and 4.5) suggest it is unlikely that microgametes randomly encounter female gametes.

4.3.3.3 Speed

Microgametes displayed both fast and slow swimming patterns as previously described (Sinden and Croll, 1975). The more waves of curvature that travel along the microgamete in a given period, the faster it swims. For the majority of the time, active microgametes moved with a fast beat, which is defined here as "forward swimming", at an average speed of 5.0 ± 0.4μm/s (n = 19) and a mean frequency of 9.6 ± 0.7 Hz. A previous study (from a smaller number of independent infections and microgametes) estimated the same parameters by hand from videos at 16 frames/second, finding a speed of ~9μm/s and beating at ~6 Hz (Straschil et al., 2010). Although these results are broadly consistent with findings from this experiment (and variation may be introduced by differences in sample preparation), the methods presented here have three important advantages for comparative studies: (i) they are completely automated, making estimations substantially less painstaking and less subjective than estimation of parameters by hand; (ii) because the data is 3D, and was obtained at higher frame rates, out-of-plane motion and motion blurring artefacts are removed; and (iii) because the data are in the form of coordinates that specify microgamete position as a function of time, it is far easier to investigate new motility metrics systematically without recourse to the raw data.

The wavelength of microgametes (5μm) matches the average range of the diameter of murine RBCs (4–7μm) (Fox et al., 2006), which may represent an adaptation to enhance microgamete motility in the blood through interactions with RBCs. A similar interaction has been suggested in trypanosomes where flagella have a wavelength and amplitude, that matches the distance between RBCs in the blood,

enabling them to use the friction generated from physical interactions with RBCs to swim eightfold faster in blood (up to 40μm/s) than in a Newtonian fluid (cell culture medium) (Heddergott et al., 2012). The experiments presented in section 4.4 provide initial data on the impact of RBCs on mating success, to explicitly test their mechanical effect on microgamete motility. However, future imaging of *P. berghei* gametes could involve measuring differences in motility parameters when exposed to polydimethylsiloxane (PDMS) pillar arrays of different densities and dynamics, in a similar manner to the experiments performed for trypanosomes (Heddergott et al., 2012).

Occasionally, microgametes were observed to swim in the opposite direction, which is defined here as "reverse swimming," at a slower speed and lower frequency (up to threefold lower) than the forward, fast-swimming microgametes. However, these measurements are preliminary because they come from a small sample of microgametes (n = 5, whereas n = 19 for forward swimmers). Again, similarities can be drawn between microgamete and trypanosome motility here. Heddergott et al. (2012) demonstrated that when trypanosomes get trapped in a dense matrix (stimulating a high blood density) their beating direction is reversed.

4.3.3.4 Beat plane and waveform chirality

Unlike other sperm flagella, microgametes do not have a discernible beating pattern (beat plane); it is complex and irregular (Wilson et al., 2013). All microgametes that were imaged (including those in fast and slow beating modes) had alternating

chirality; with waves propagating in a left- or right-handed character on alternate beats, and successive waves had dissimilar shapes. Whilst the mechanisms and factors that determine chirality and beat pattern are still a subject of debate, the results from this study contradict claims that chirality is hard-wired into the axoneme structure (Hirokawa et al., 2009), and instead suggest that accessory structures (that are absent from microgametes) play a critical role in shaping the more 'consistent' flagellar beats observed in other species, compared to the irregular beat plane of the *P. berghei* microgamete.

Two non-mutually exclusive aspects of mating biology that may explain the irregular beat plane of microgametes are as follows. First, the relatively dilute *in vitro* environment, with a low RBC density could have a similar effect on the microgametes as for trypanosomes, where in the absence of any obstacles, the flagella beat is slow and irregular (Heddergott et al., 2012). Microgametes could also be affected by immune factors that are taken up by the mosquito in a blood meal (Mendis et al., 1990, Naotunne et al., 1991, Motard et al., 1993, Naotunne et al., 1993, Ramiro et al., 2011, Long et al., 2008). Even though immune factors are likely to be diluted in *in vitro* culture conditions, any binding to one part of a microgamete could potentially alter the beat plane and affect swimming direction (Bohring et al., 2001).

4.3.3.5 Probability of microgametes randomly encountering females

The parameters described above, and the calculations below enable us to estimate whether mating can rely on microgametes moving randomly around the blood meal in search of female gametes to fertilise. The approximate lifetime of a microgamete is 30 minutes (Carter and Nijhout, 1977, Sinden et al., 2010); the flagellar beats are randomly oriented with an amplitude (peak to trough) of ~ 5µm. If the microgamete swims at the speed that was measured in this study (5.0µm/s) for 30 min (1,800s), it sweeps out a cylindrical volume according to equation (1).

$$V_{gam} \sim \pi \cdot \left(2.5 \times 10^{-6}\right)^2 \cdot 1,800 \cdot \left(5 \times 10^{-6}\right) \sim 2 \times 10^{-13} \mathrm{m}^3. \quad (1)$$

The swimming speed may increase in a blood meal (e.g. via interactions with RBC; as observed in trypanosomes (Heddergott et al., 2012)), but still, the microgametes swim in viscous-dominated environments (i.e., at a low 'Reynolds' number) (Wilson et al., 2013). This means that microgametes cannot swim faster than the speed at which waves propagate along the flagellum (around 50µm/s), regardless of whether interactions with RBCs enhance speed. The volume explored will be 10-fold larger if the maximum (50µm/s) swimming speed is used. Assuming a blood meal size of 2µl ($2\times 10^{-9}\mathrm{m}^3$), this equates to a microgamete being able to explore between 1/1,000 and 1/10,000 of a blood meal in 30 minutes. It would take more than 1 month for the microgamete to explore an entire blood meal at 5.0µm/s, assuming it never retraces its steps. However, blood meals contain multiple gametocytes and assuming a

gametocyte density of 10^5 gametocytes/µl blood and a sex ratio of 30% males, approximately 400000 microgametes will be present the blood meal. In this case, it is likely that at least 1 microgamete visits everywhere in the blood meal in 30 min.

These calculations are a "best case scenario" estimate based on the gametocyte density of *P. berghei* infections. Gametocyte densities of human malaria (e.g., *P. falciparum*) in natural infections are variable but generally much lower (e.g., 500 gametocytes /µl blood (including males and females)) (Schneider et al., 2007), which translates to only approximately 1,800 microgametes in the blood meal. An additional limiting factor here is the ratio of male to female gametocytes present in the blood meal. The resulting trade-off is between the area of the blood meal that the microgametes can cover (increased when the proportion of males is high) *vs*. the density of female gametocytes available for the microgametes to locate (which is decreased when the proportion of males is high). Also, the estimates above do not account for the negative effects of transmission-blocking immune factors (Aikawa et al., 1981) and the high failure rate in the production of viable microgametes (Sinden et al., 2010).

Given the increasing appreciation that transmission to mosquitoes occurs readily from sub-microscopic gametocyte densities (<5 gametocytes /µl blood (Schneider et al., 2007)), these results suggest that the evolution of mechanisms to facilitate encounters between microgametes and female gametes would be favoured by natural selection. These could include (i) the use of interactions with RBCs to increase

swimming speed (as for trypanosomes (Heddergott et al., 2012), see section 4.4), (ii) locating females non-randomly by a mechanism such as chemotaxis (Eisenbach, 2007) (see section 4.5) or nanotubes (tubular filaments extending from cell membranes, thought to establish contact for communication between gametocytes and gametes) (Rupp et al., 2011, Kuehn and Pradel, 2010) (iii) gametocyte aggregation in the circulation of the vertebrate host maximizing the densities of gametocytes in blood meals of vectors that become infected (Pichon et al., 2000, Gaillard et al., 2003) (although this mechanism trades off against the proportion of infected mosquitoes). Even if microgametes swim at the maximum speed of 50μm/s, successful mating in the absence of such mechanisms would seem unlikely for sub-microscopic gametocyte densities. For example, if 5 gametocytes enter the mosquito midgut, even if 4 of these are males, this would result in a maximum of only 32 microgametes, with each exploring 1/1,000 of the blood meal on average, in the 30 minute window.

4.3.4 Conclusions and future directions

Given the drive to develop transmission-blocking interventions by interfering with the fertility of microgametes, a better understanding of the behaviour of microgametes is central to making interventions as "evolution-proof" as possible. Using high speed digital microscopy and 3D holographic reconstruction (Wilson and Zhang, 2012), several key physical characteristics of the microgamete of *P. berghei* were quantified (summarised in table 4.1). Some of these measurements such as the length, diameter, wavelength and speed were quantified in a more robust way than with previous attempts (Sinden, 1975, Straschil et al., 2010), and some are

completely novel (e.g. swimming direction, beating plane and chirality of the microgamete). Two key parasitological questions that emerge from this work concern how microgametes interact with the blood meal environment in the search for females:

(i) What role do RBCs have for microgamete motility? (which is addressed in section 4.4)

(ii) Do microgametes follow random paths in the blood meal? (which is addressed in section 4.5)

Finally, the contribution of biology to physics in this context should not be underestimated. The microgamete is an ideal model system for understanding the axoneme on a mechanical level. The small number of components in a microgamete, coupled with genetic control over its structure and assembly (Straschil et al., 2010) allows for a rigorous test of current physical theories. In particular, the ability to disrupt genes responsible for the central pair of microtubules (in other words, producing "9+1" or "9+0" axonemes) offers the chance to resolve a longstanding debate about the role they play in determining flagellar waveforms, and their influence on the flagellar beat (Omoto et al., 1999). For example, an ortholog of the flagellar protein PF16 (first characterized in *Chlamydomonas*) in *P. berghei* (PbPF16) is crucial for flagellar motility in malaria parasites (Straschil et al., 2010). The majority of the PbPF16 mutant microgametes lacked at least one central microtubule and were either immotile or had slower swimming speeds (Straschil et al., 2010). Such mutant lines are ideal for identifying the role of the central pair in flagellar motility, compared with wild type *P. berghei* microgamete motility.

4.4 Role of parasite interactions with red blood cells during mating

4.4.1 Introduction

RBCs are one of the main components of blood meals, and so microgamete - RBC interactions (physical and chemically-mediated) are likely to be very common; both during exflagellation and when microgametes are freely swimming. These interactions and their potential consequences for microgametes and mating success are described below and explored in this section.

To maximise their intake of RBCs without overly increasing their total mass (which would inhibit their capacity to fly), some mosquito species perform diuresis (anal excretion of fluid) during and after feeding (Vaughan et al., 1991). This has been demonstrated in *A. gambiae* and *A. stephensi*, which concentrate ingested RBCs by factors of 1.8 and 1.7, respectively (Vaughan et al., 1991), but large variations in these values have been found both within and between species (Chadee and Beier, 1996). This concentration of RBCs is predicted to dramatically change the biophysics of the bloodmeal to a more colloidal composition and significantly increase the pressure due to peristaltic contraction of the midgut. Because this is occurring simultaneously to exflagellation and fertilisation, it may have significant consequences for the ability of the microgamete to navigate through the bloodmeal in search for a female to fertilise. RBCs are potentially a significant physical barrier to the navigation of microgametes through the bloodmeal. As diuresis increases the density of RBCs, the longer it may take males to search a given volume of blood meal to find female gametes to fertilise. Furthermore, some microgametes

themselves are excreted through diuresis (Sarah Reece, personal communication); thereby reducing the probability of fertilisation success even further.

Another phenomenon commonly observed upon the initiation of gametogenesis is the interaction of microgametes with RBCs, other parasite infected cells, and emerged female gametes; both during exflagellation and once microgametes are free from the residual gametocyte. During exflagellation, microgametes form temporary but strong interactions with RBCs, in what are termed 'exflagellation centres' (Templeton et al., 1998). Whether exflagellation centres occur in the blood meal is yet to be examined so it is not known whether this adhesion has a requisite function, is a hindrance to mating success, or is simply an artefact of *in vitro* conditions. Evidence suggests against the interaction simply being an *in vitro* artefact, because it has been shown to be species specific and dependant on an interaction between the gamete and the negatively charged sialic acid on the surface of the RBC membrane (Templeton et al., 1998). Adhesion to RBC may benefit parasites by aiding microgamete detachment from the residual gametocyte microgametes and/or microgametes could use the RBC surface as a substrate to migrate through the dense blood meal; thereby covering more distance than by simply "swimming" through a bare medium (Carter and Graves, 1988). Indeed, as described in section 4.3, the wavelength of microgametes (5μm) matches the average diameter of murine RBCs (4–7μm) (Fox et al., 2006), which is a pre-requisite for an adaptation of microgametes to enhance motility in the blood meal using interactions with RBCs (Wilson et al., 2013). Similar suggestions have been proposed for the motility of trypanosomes (Heddergott et al., 2012). In addition, being surrounded by RBCs may act as a barrier to protect microgametes from transmission blocking immune factors

or phagocytic cells (Sinden and Smalley, 1976, Naotunne et al., Mendis et al., 1990, 1991, Naotunne et al., 1993, Motard et al., 1993, Ramiro et al., 2011, Long et al., 2008).

Previous *in vivo* studies suggest that increasing the concentration of RBCs in the blood meal (using a range of *Anopheles* species with different prediuresis abilities) has a negative effect on *P. falciparum* ookinete density (Vaughan et al., 1991, Sinden et al., 1996). Whilst these results suggest that a high density of RBCs hinder fertilisation success (i.e., 'ookinete conversion'), there are many confounding factors in such studies. The current data do not disentangle the effects of (a) other blood components and characteristics affected by diuresis (pH, glucose availability and immune factors), (b) simple physical- (i.e., RBCs acting as an obstacle to microgamete motility) or chemical- microgamete – RBC interactions (i.e. RBCs acting as a substrate mediated by sialic acid), or (c) adaptation/coevolution of parasites to only some of the vector species examined. Furthermore, the potential pros and cons of RBCs in the blood meal are not mutually exclusive and any combination could be operating. In fact, as is the case with trypanosomes (Heddergott et al., 2012), it may be that an intermediate density of RBCs could be optimal for successful exflagellation and subsequent fertilization success. For example, RBCs could simultaneously provide a substrate for- (via sialic acid interactions) and act as an obstacle to- (as a physical barrier) microgamete motility. The experiments presented in this section use biocompatible particles ('microparticles') that are similar to the size, shape and hydrophillic surface of

murine RBCs but without the sialic acid surface coat to disentangle the consequences of physical and chemical interactions with RBCs for mating success.

4.4.2 Methods

4.4.2.1 Parasite preparation

Infections of the rodent malaria parasites *P. berghei* WT ANKA and *P. berghei* 820cl1m1cl127 (*P. berghei* 820) (from The University of Edinburgh's malaria reagent repository http://malariaresearch.eu/) were initiated in male MF1 mice (8-10 weeks old, from an in house supplier, The University of Edinburgh). These two lines (derived from the same parental line) were chosen because they are both known to have a high gametocyte conversion rate. The mice had been pre-treated with phenylhydrazine at 125 mg/kg (2 days before infection) to enhance the production of gametocytes (Reece et al., 2008). Eighteen independent infections (10 *P. berghei* WT ANKA and 8 *P. berghei* 820) were initiated with 10^7 parasitized RBCs from donor mice infected with cryopreserved parasites. Exflagellation assays were performed on days 3, 4 and 5 post infection to verify that there was a sufficient density of mature, exflagellating gametocytes in the infected blood for successful fertilisation and culturing. This involved culturing 2µl of tail blood in 100µl fresh ookinete media (RPMI + 10% foetal calf serum, pH 8) at 20^{o}C and vortexing to stimulate gametogenesis (Janse et al., 1985). Ten minutes post initiation of this culture, 8µl was placed under the cover slip of a haemocytometer and the number of exflagellation events observed in 1/9 of the haemocytometer grid (100nl culture) was recorded. An exflagellation event was defined as a haphazard, rapidly moving parasite extruding flagella; often forming clumps (exflagellation centres) with nearby

RBCs. When more than 20 exflagellation events were counted in 1/9 of a haemocytometer, these infections were considered suitable for culturing. Culturing always occurred on day 4 or 5 post infection. Thin blood smears were also taken and stained with giemsa on days 3, 4 and 5 post infection, and the asexual parasitaemia (number of parasites/RBC) and gametocytaemia recorded. In addition, RBC density counts were taken of the infected blood, to monitor anaemia of the mice and to allow calculations of parasite densities.

4.4.2.2 Microgamete purification and culture

For each mouse, blood was used to initiate cultures across seven different treatment groups, as detailed in table 4.2**.** RBC densities and blood smears were taken from all mice contributing to cultures. The number of female gametocytes/ml culture was calculated from reading blood smears and accounting for RBC densities and culture conditions. Initially, two control treatment groups were set up with blood straight from the mouse tail. 2µl tail blood was placed into either 100µl or 20µl ookinete culture media (RPMI + 10% foetal calf serum, pH 8 at 21^{o}C) to create cultures at a concentration of 2% (routine culture conditions) and 10% (high blood concentration culture conditions) blood (table 4.2). Both cultures were incubated at pH 8 and at 20^{o}C for 18-20 hours before a haemocytometer was used to calculate the number of ookinetes/ml culture. For the remaining four treatment groups, *P. berghei* infected blood was collected via cardiac puncture from anaesthetised mice (total blood volume collected ranged from 0.5ml to 1.6ml) and was added to 10ml of gametocyte stasis media (RPMI + 5% foetal calf serum, pH 7.25, at 37^{o}C). The culture was then immediately passed through a magnetic column (MACS LS separation columns,

Miltenyl Biotech) to which the heam–rich magnetic gametocytes adhered, whilst the serum, asexuals and uninfected RBCs cells passed though. 3ml gametocyte stasis media was then added to wash any remaining non – gametocyte stages (non-magnetic) from the column. The column was then removed from the magnet; 1ml ookinete media was added and immediately forced through the column to flush out the gametocytes into a sterile centrifuge tube. This was repeated three times, resulting in 3mls of purified gametocytes. The gametocytes were then spun down at 2500rpm for 3 minutes at 37^{o}C, the supernatant was removed and from the remaining pellet of pure gametocytes, 3µl was aliquoted into pre- prepared ookinete media cultures (total volume: 23µl, RPMI + 10% foetal calf serum, pH 8, 20^{o}C) containing different ratios of microparticles : media (detailed below and in table 4.2). Tail blood cultures were set up from the same infections that were used for the purified gametocyte cultures to a) confirm that the gametocytes successfully fertilised and developed into ookinetes in 'routine' culture conditions and b) to qualitatively compare the effect of increasing the concentration of whole blood *vs.* increasing the density of microparticles on ookinete conversion.

Four different culture conditions were prepared with increasing densities of biocompatible polymethyl methacrylate (PMMA) microparticles, diameter 6.33 µm (microParticles GmbH): 0% (no microparticle control), 1% (similar to routine culture conditions), 35% (similar to the host haematocrit), 60% (similar to an increased RBC density due to prediuresis in the mosquito midgut) (table 4.2). Pilot data showed that microgametes do not form exflagellation centres with these microparticles, which indicates that the RBC-microgamete interaction is mediated by a molecular

interaction with sialic acid as a specific ligand (rather than simple electrostatic attraction to the hydrophilic RBC coat (Templeton et al., 1998). In high microparticle-density cultures, after fertilisation, ookinete development could be limited by a lower concentration of resources (e.g. glucose) due to a lower proportion of media in the culture, so this was tested for by replenishment of media after mating. Two hours after culture initiation (i.e., once fertilisation was complete) an additional 80µl ookinete media was added to half (54/108) of all cultures (across all treatment groups).

All cultures were incubated at pH 8 and at 20^{o}C for 18-20 hours before a haemocytometer was used to calculate the total number of ookinetes/culture. Some cultures were diluted before counting to avoid missing ookinetes masked by high densities of microparticles. After adjusting for dilution factors, the conversion rate of female gametocytes to ookinetes (total ookinetes / total female gametocytes) for each culture was calculated, as a measure of fertilisation success, but for clarity, this is referred to as 'ookinete conversion' from here on. Any ookinete conversion values over 1 were removed from analysis (9/108 cultures) because this is biologically impossible. This was most likely to be due to the risk of underestimating female gametocytes in very low density populations, which is a well-known problem in microscopy when reading blood smears. I chose the most conservative option of removing these values (and therefore reducing the sample size), instead of artificially inflating the values of all female densities.

Whilst flow cytometry techniques could enable quantification of zygote densities post fertilisation, it is not as amenable as haemocytometry for the high-throughput nature of this experiment. Zygote conversion rate may be the most direct measure of fertilisation success, but ultimately the conversion rate of female gametocytes to mature ookinetes is a more accurate indicator of transmission potential. Furthermore, assaying ookinete conversion rates facilitated an analysis of the unexpected but significant and large effect of replenishing media post fertilisation for zygote maturation / ookinete formation.

4.4.2.3 Analysis

Data were analysed using R version 3.0.2 using Generalized linear mixed model (GLMM) (for the microparticle data) and linear mixed effect models, (for the blood data) treating each infection (mouse) as a random effect to account for pseuodoreplication of blood from each mouse contributing to each treatment. For the microparticle data, GLMMs were constructed with the binomial response variable as "Ookinetes per ml blood / females per ml blood" after both values had been rescaled (divided by 1000) in order to conform to the representable range of integers R is restricted to using ($+/-2x10^9$). As the response variable data was strictly bounded (between zero and one), had non-constant variance and non-normal errors, a binomial error structure with a logit link function was used. For the blood culture data, the response variable (ookinete conversion) was $\log_{10}$ transformed to conform to the assumptions of normality. For both GLMM and LME, models were minimised following stepwise deletion of the least significant term and using log likelihood

ratio (χ^2) tests to evaluate the change in model deviance until only significant terms remained in the model.

Table 4.2 Summary of treatment groups, sample sizes (N) and rationales. Half of the cultures received media replenishment (80µl fresh ookinete media) 2 hours after culture initiation (across all treatment groups). N= number of *P. berghei* infections.

Treatment	Density	Volume Of culture	Gametocyte sample used to initiate culture	N (*Strain*) (WT)	N (*Strain*) (820)	Rationale
Blood	2%	100µl	Tail blood	8	8	Control - routine blood culture.
	10%	20µl	Tail blood	8	8	Medium density blood culture.
Microparticles	0%		Purified gametocytes	10	8	Absence of microparticles (~Newtonian fluid).
	1%	20µl	Purified gametocytes	10	8	Microparticle density similar to routine blood culture RBC density.
	35%	20µl	Purified gametocytes	10	8	Microparticle density similar to healthy host haematocrit.
	60%	20µl	Purified gametocytes	10	8	Microparticle density similar to the increased density of RBCs within a blood meal in the mosquito midgut due to diuresis.

4.4.3 Results

4.4.3.1 Purified gametocyte and microparticle initiated cultures

Ookinete conversion (proportion of female gametocytes that developed into mature ookinetes) was measured when purified *P. berghei* gametocytes were cultured in the

presence of four different densities of microparticles (treatments): 0%, 1%, 35% and 60%. In addition, the effect of replenishing media post fertilisation on ookinete development to maturity was tested. Data from the two lines *P. berghei* 820 and *P. berghei* WT ANKA were grouped because their ookinete conversion did not differ significantly (line: $\chi^2_{1,9} = 2.786$, p= 0.10). The effect of microparticle density on ookinete conversion was dependant on whether media was replenished post fertilisation (treatment*media: $\chi^2_{3,6} = 1.3x10^5$, p<0.001) (figure 4.3). Overall, ookinete production was reduced by more than 80% when microparticle density was increased from 0% to 60%. Specifically, for media replenished cultures, mean ookinete conversion (95% confidence interval range) when microparticle density was: 0%: 0.81 (0.61-0.92), 1%: 0.65 (0.5-0.85), 35%: 0.28 (0.13-0.51), 60%: 0.12, (0.05-0.27) (figure 4.3).

Ookinete conversion was much lower for all cultures that were not replenished with media and followed a similar pattern for the negative effect of increasing microparticle density. Mean ookinete conversion (CI range) for non-media replenished cultures, when microparticle density was 0%: 0.04 (0.02-0.11), 1%: 0.03 (0.01-0.09), 35%: 0.03 (0.01-0.09), 60%: 0.01 (0.003-0.02) (figure 4.3). However, replenishment resulted in a larger increase in ookinete conversion at low microparticle densities. Specifically, when microparticle density was 60%, media replenishment increased the probability of ookinete conversion by 0.11, and by 0.77 at 0% microparticle density (figure 4.3). An additional, unquantified observation was that in the 60% microparticle density cultures, the proportion of retort- shaped (immature) ookinetes was higher than in the lower density microparticle cultures.

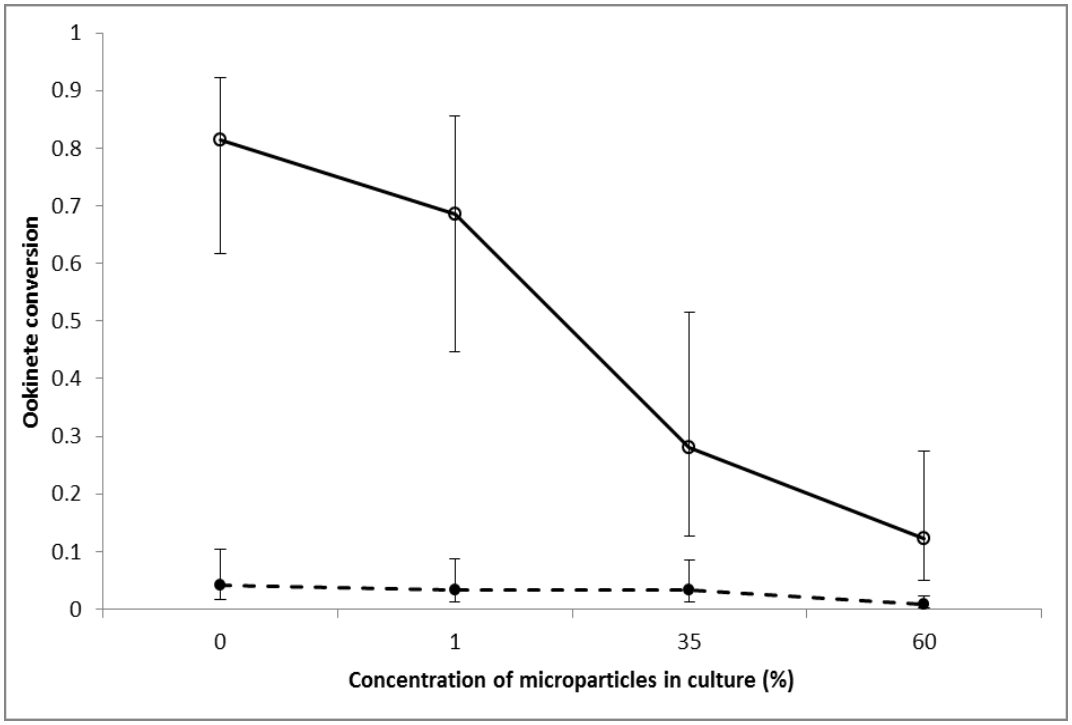

Figure 4.3 Ookinete conversion when MACS purified gametocytes were exposed to increasing densities of microparticles in cultures in which media was replenished post fertilisation (white circle, solid line, n ranges from 5 to 9) or not replenished (black circle, dotted line, n = 9). Estimated mean (± asymmetric binomial CI). Ookinete conversion: probability of a female gametocyte developing into an ookinete.

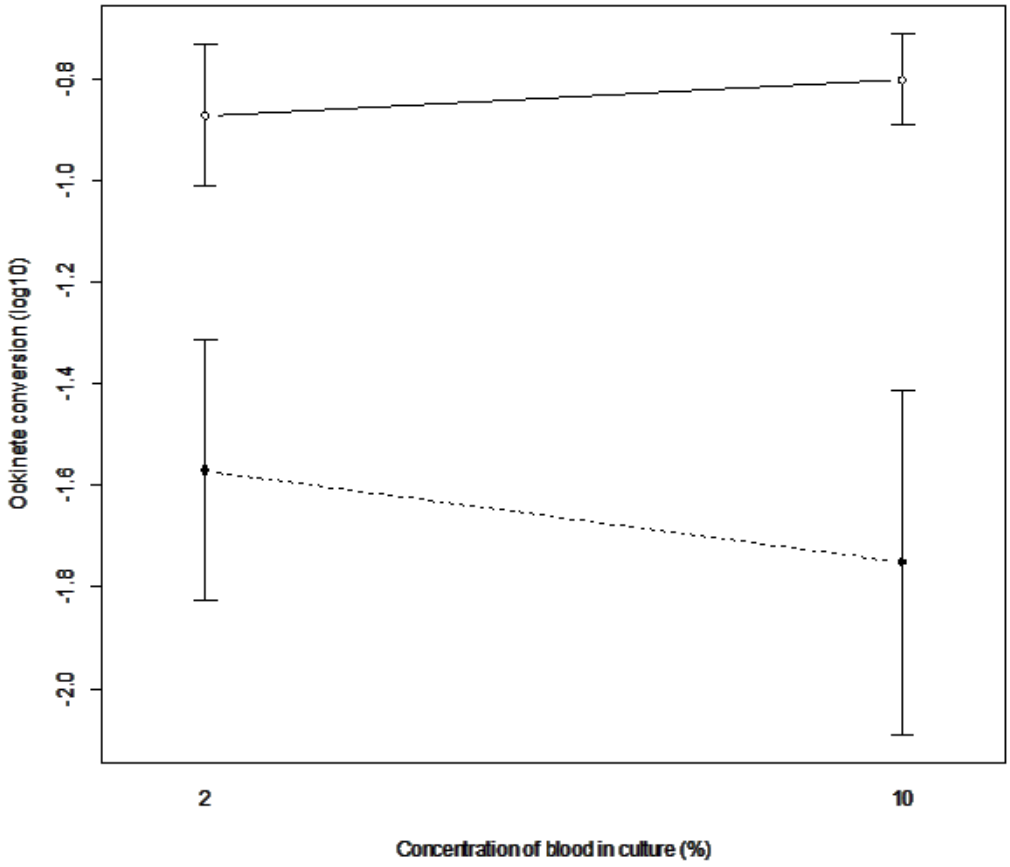

Figure 4.4 Ookinete conversion (log_{10}) when gametocyte infected whole blood was added to cultures in which media was replenished post fertilisation (white circle, solid line, n = 9) or not replenished (black circle, dotted line, n = 6), at either 2% or 10% blood density. Mean (± SEM). Ookinete conversion: probability of a female gametocyte developing into an ookinete.

4.4.3.2 Whole blood initiated cultures

Ookinete conversion was measured when *P. berghei* infected whole blood (from the same infections used to initiate the purified gametocyte cultures (4.4.3.1)) was cultured at two different concentrations of blood in media (treatments): 2% and 10%. This was to check that gametocytes successfully fertilised and developed into ookinetes in 'routine' tail-blood culture conditions (2%), and to qualitatively

compare the effect of increasing the concentration of whole blood *vs* increasing the density of microparticles on ookinete conversion. Again, the effect of replenishing media post fertilisation on ookinete development was investigated. There was no significant difference in ookinete conversion between the two parasite lines (*P. berghei* 820 and *P. berghei* WT ANKA) (line: $\chi^2_{1,7} = 0.27$, p= 0.60), so the data from the two lines were combined.

Ookinete conversion was higher in media replenished cultures and the effect of blood concentration depended on whether media was replenished or not (treatment*media: $\chi^2_{1,5} = 6.11$, p= 0.01) (figure 4.4). When media was replenished ookinete conversion was similar at 2% (mean: 0.18 ± 0.13 ookinetes/female) and 10% blood cultures (mean: 0.19 ± 0.13 ookinetes/female). When media was not replenished post fertilisation, ookinete conversion was also similar in 10% (mean: 0.02 ± 0.05 ookinetes/female) and 2% blood cultures (mean: 0.03 ± 0.07 ookinetes/female) (figure 4.4).

4.4.4 Discussion

The experiments presented here reveal that increasing microparticle density from 0% to 60% reduced ookinete conversion of purified *P. berghei* by >80%. Furthermore, when media was replenished post fertilisation, ookinete conversion for all microparticle densities was increased, but the magnitude of the effect decreased as microparticle density increased. Increasing the concentration of blood in cultures (from 2 to 10%) produced a similar reduction in ookinete conversion, but only when media was not replenished.

4.4.4.1 Effect of microparticle and blood density on fertilisation success

Whilst the proportion of female gametocytes that develop into mature ookinetes is reduced when microparticle density is increased, whether microgametes or female gametes were affected is unknown. This would be very hard to test, because it is not possible to target only one sex during fertilisation. However, the most parsimonious explanation is that the increase in microparticle density resulted in physical barriers to microgamete movement. If this is true, and if microparticles do accurately mimic the effect of RBCs in the blood meal, this suggests that a high density of RBCs hinders mating, which is simply a constraint that natural selection cannot overcome. An alternative explanation could be that the microparticle environment did not accurately represent the mosquito bloodmeal environment. The exact composition of blood within the mosquito midgut remains unknown, because it is not yet possible to observe internal processes within the mosquito. Bloodmeal structure could vary from a clotted mass (as observed when mosquitoes are dissected and exposed to air), to a single cell suspension (similar to the microparticle environment that was tested here). Future work could involve utilising sophisticated imaging techniques optimised for within –host processes (Frischknecht et al., 2004) to elucidate bloodmeal composition. Indeed, if it does more closely resemble a clotted mass, further work could involve testing fertilisation success at different densities of matrigel (as used in Trager and Williams (1992).

The observation that ookinetes were less well developed ('retort'- shaped (Gass, 1979)) in cultures where the microparticle density was highest (60%) suggests that RBCs hinder mating. Retorts may have been a result of resource limitation and/or a delay to mating leading to a delay in zygote formation and ookinete formation. In turn, this may have been a result of microgametes spending longer navigating around a higher density of microparticles to find females. Resource limitation can be ruled out because retorts were observed even when media was replenished post fertilisation. Nevertheless, an alternative explanation for the presence of retort shaped ookinetes could be that the presence of microparticles at 60% caused ookinetes to arrest during their development. Time course analyses (investigating whether the retort shaped ookinetes eventually developed into mature ookinetes after ~ 2 more hours in culture) is necessary to explicitly test this.

Furthermore, that the highest ookinete conversion rates occurred in 0% microparticle cultures (0.81 (CI range: 0.61-0.92), ookinetes/female) suggests that RBC-microgamete interactions (mediated by sialic acid adhesion) do not have a large, if any, effect on mating success by facilitating microgamete detachment from the residual gametocyte (Templeton et al., 1998). Testing the effect of coating the microparticles in sialic acid on ookinete conversion could resolve whether sialic acid plays any role in mating. In addition, investigating whether there is any role of heparin and heparan sulphate (which are involved in merozoite invasion of RBCs and sporozoite invasion of salivary glands (Atkinson et al., 2012)) during exflagellation and fertilisation may provide alternative explanations for the RBC-microgamete interactions.

An alternative explanation for the reduction in fertilisation success as microparticle density increases is that microgametes were nutrient limited at high microparticle densities. To keep the culture volume constant (to avoid confounding the effect of microparticles on fertilisation success with different culture volumes) the media component of cultures decreased as microparticle density increased. Glucose transport is thought to be essential for micro gametogenesis: proteomic analysis indicates that glycolysis provides the power for microgamete motility (Khan et al., 2005), and the hexose transporter has been shown to play an essential role in utilizing the environmental glucose supply (Nijhout and Carter, 1978, Slavic et al., 2010, Talman, 2010, Slavic et al., 2011). However, even in cultures at the highest microparticle concentration (60%), the concentration of glucose was 4mM. Previous exflagellation studies found no difference in exflagellation intensity between cultures at glucose concentrations ranging from 1.5mM to 10mM (Slavic et al., 2011). Therefore glucose limitation can be ruled out as a cause for the reduction in fertilisation success. This is not surprising, because glucose concentrations within the bloodmeal are likely to be much lower than those used in *in vitro* experiments, so microgametes should have evolved to cope with low glucose concentrations.

As observed in the microparticle data, ookinete conversion was reduced when blood concentration was increased. However, in contrast to the microparticle data, this was only observed in the non-media replenished cultures. Delayed fertilisation as an explanation for higher ookinete conversion in media replenished cultures *vs* non

media replenished cultures can be ruled out because within each microparticle density treatment, there were no more retort- shaped ookinetes in non-replenished than replenished cultures. Thus, media replenishment does not affect mating success (because replenishment occurred post fertilisation) but does affect the ability of zygotes to transform into ookinetes. Therefore, the replenished groups give a more accurate picture of fertilisation success whereas the non-replenished groups demonstrate the influence of the environment on subsequent ookinete development. In this case, there is no influence of increasing blood concentration from 2 to 10% on mating success. This is not surprising because the difference between 2 and 10% blood is relatively small, and still at a much lower density than in the mosquito midgut during a blood meal (Vaughan et al., 1991, Templeton et al., 1999). Investigating the effect of RBC concentration is important, but is complicated because blood contains RBC, immune factors, and resources that can affect mating and ookinete development in many different ways (Baton and Ranford-Cartwright, 2005, Ramiro et al., 2011). Nevertheless, for this study, the data for the media replenished microparticle data are the best conditions in which to test how the physical conditions in a viscous bloodmeal influence mating success.

4.4.4.2 Media replenishment

For all microparticle and blood densities, the proportion of female gametocytes that developed into ookinetes was higher when media was replenished post fertilisation. Furthermore, the increase in ookinete conversion was greatest at low microparticle and blood densities, suggesting that when ookinetes are at high densities, their development is limited by environmental factors. Identifying the reasons for a

density dependant effect of media replenishment requires further work. Whist the need for more resources when ookinete density is high is the most obvious explanation, it is unlikely that glucose is limiting. Increasing glucose concentration (from 1.5 to 22mM has no effect on post fertilisation ookinete development) (Slavic et al., 2011), and the glucose concentrations in this experiment (which ranged from 4mM in 60% microparticle cultures to 10mM in 0% microparticle cultures) fall well within this range. Another explanation could be that media-replenishment dilutes host derived immune factors (Ramiro et al., 2011) (although the majority of immune factors would have been removed during the gametocyte purification process), and/ or waste metabolites (although very little is known about this).

4.4.4.3 Conclusions and future directions

Whilst the negative effect of microparticles on ookinete conversion rates is clear, translating this to the impact of RBC density is more difficult. Understanding the impact of RBC density on transmission success matters, not only in relation to the effect of host anaemia on mating success, but it is also increasingly important given the number of vector species (with different diuresis capacities) that are able to transmit malaria. For example, vector control measures may cause parasites to encounter novel vector species (as is occurring due to insecticide use) (Bayoh et al., 2010) with different diuresis behaviours (up to 55% of the fluid ingested can be excreted (Clements, 1992)); thus, different packing densities of RBCs could affect transmission.

The effect of media replenishment has implications for future experiments that require high densities of ookinetes *in vitro*. Currently, routine culture conditions are represented by the 2% blood, non-media-replenished treatment in this study. The mean ookinete conversion rate for this method (0.03 ± 0.07 ookinetes / female) is much lower than what can be achieved using purified gametocytes (up to 0.82 (CI range: 0.61-0.92) ookinetes/female).

4.5 Directional microgamete movement

4.5.1 Introduction

The data presented in sections 4.3 and 4.4 provide support for the hypothesis that microgametes and female gametes do not meet simply by chance. Section 4.3 suggests that even if microgametes travel at their maximum possible speed (50μm/s), a single microgamete could only explore 1/1,000 of a 2μl blood meal in the 30 minute window that fertilisation can occur. Section 4.4 suggests that ecologically realistic RBC densities are likely to slow microgamete motility down and reduce the volume searched by each microgamete. Furthermore, host derived immune factors in the blood meal also interfere with microgamete motility. Given that mating clearly does happen, even when small numbers of gametocytes are taken up in a blood meal, the existence of a mechanism to facilitate microgametes in the search for female gametes is likely.

Three mechanisms that may facilitate encounters between microgametes and female gametes include (but are not limited to) i) microgamete interactions with RBCs increasing swimming speed (as suggested for trypanosomes (Heddergott et al., 2012)); ii) gametocyte aggregation in the peripheral circulation of the vertebrate host maximizes the density and proximity of male and female gametocytes in the blood meals of vectors that become infected (Pichon et al., 2000, Gaillard et al., 2003), iii) microgametes navigate the blood meal non-randomly by using chemotaxis (Eisenbach, 2007) and / or nanotube-like filaments of gametes (FiGs) (Rupp et al., 2011, Kuehn and Pradel, 2010) to locate female gametes. Data from section 4.4 suggest that it is unlikely that microgametes benefit from physical interactions with

RBCs, and hypothesis ii) is unlikely to evolve because it would trade off against the reduced probability of mosquitoes picking up parasites to get infected. However, the latter option iii) is perhaps the most likely, as mechanisms such as chemotaxis are ubiquitous across eukaryotic flagella (Eisenbach, 1996). Whilst there is no direct evidence for malaria chemotaxis to-date, filamentous protrusions (FiG) on the surface of *P. falciparum* activated gametocytes and gametes of both sexes have been observed. These protrusions form immediately upon activation and may establish long-distance (> 100µm) contacts between gametes (Rupp et al., 2011). FiGs exhibit adhesion proteins on their surface which may be a mechanism to locate and connect activated gametocytes and gametes within the bloodmeal (Rupp et al., 2011). However, due to the very low gametocyte densities in natural malaria-infected blood meals (see section 4.3 for calculations), an additional mechanism such as chemotaxis is likely to be required to attract microgametes towards the 100µm circumference around female gametes, before FiGs can act to adhere the gametes and facilitate fertilisation. Further support for the existence of a female gametocyte-derived chemotactic gradient, and / or nanotube – mediated mechanisms comes from the observation that host derived monocytes and neutrophils appear to migrate distances in excess of 40µm towards female gametocytes upon ingestion by the mosquito (Sinden and Smalley, 1976). If phagocytic cells can direct their movement towards female gametes, it seems plausible that microgametes could too. Furthermore, detecting chemotaxis gradients could be facilitated by the motility parameters outlined in section 4.3 where microgametes travel in the direction of their "active end", and their ability to swim in reverse may maximize the likelihood of detecting and following a chemotaxis gradient (Wilson et al., 2013). Whilst the ability to detect

and follow chemotactic gradients may seem unlikely for the simple-structured microgamete, malaria parasites are known to employ complex behaviours and flexible (plastic) strategies for survival and reproduction in changing environmental conditions (chapters 2 and 3). Due to its simple structure (section 4.3), microgametes are unlikely to have chemotaxis receptors (and none have been identified to date), however, chemotaxis-like behaviour is thought to be possible in the absence of chemotaxis receptors, for example, if cells are forced to swim more slowly in some regions than in others (Cates, 2012).

4.5.2 Methods

Microgametes were observed when exposed to female gamete material *vs* non-female gamete material. Specifically, the experiment tested whether microgametes preferentially aggregate in zones of live female gametes, and/or lysed female gametes, when compared to live asexuals, lysed asexuals, or uninfected RBCs.

4.5.2.1 Microgamete preparation and experimental design

Infections of *P. berghei* WT ANKA (from The University of Edinburgh's malaria reagent repository http://malariaresearch.eu/) were used to prepare parasite material at a sufficient density for the assays, following the protocols outlined in section 4.4.2.1. On day 4 or 5 post infection (when the density of exflagellation was judged sufficient) tail blood was taken from 13 independent infections and microgametes were purified from this blood (as described in section 4.5.2.3). The purified microgametes were aliquoted and exposed to 5 different treatments ("cues"; table 4.3). The aim of the experiment was to test whether microgametes aggregate in the

vicinity of the cue treatment material. Five different cues were prepared (table 4.3); uninfected RBCs (to act as a control cue and to test whether microgametes are attracted to a group of cells, regardless of its composition); live asexuals (a control cue of live parasites); lysed asexuals (a control cue of material liberated from lysed parasites); live female gametes (to test if microgametes are attracted to live female gametes); and lysed female gametes (to test if microgametes are attracted to material liberated from lysed female gametes three hours after activation). The experiment was designed so that the responses to all cues could be compared to each other, and so that some cues could be combined to test for general responses to female material vs control material by grouping treatments according to the classifications outlined in table 4.3. RBC densities and blood smears were taken from all mice contributing microgametes. The change in microgamete position when exposed to each treatment was examined over 20 minutes (as described in section 4.5.2.3 and illustrated in figure 4.5).

Table 4.3 Summary of treatment groups, sample sizes, rationales, and classification. The analysis involved comparing the effect of individual interface treatments as well as comparing treatments grouped into 'control' vs 'female' on microgamete density. N = number of independent infections that provided microgametes to test a particular treatment. All 13 mice did not contribute to all treatments due to a lack of treatment material on some assay days.

Treatment	N	Rationale	Classification
Uninfected RBC	10	Control interface. To test whether microgametes are attracted to a zone of cells, regardless of its composition	Control
Live asexuals	13	Control for live females. Purified asexual- stages in ookinete media.	Control
Lysed asexuals	12	Control for attraction to lysed material liberated from parasites. Asexual–stage parasites were cultured in ookinete media for 3 hours before lysis (to match conditions used to prepare the lysed female treatment group).	Control
Live females	9	To test if microgametes are attracted to live female gametes. Purified female gametes in ookinete media.	Female
Lysed females	10	To test if microgametes are attracted to material liberated from lysed females. Female gametes were cultured in ookinete media for 3 hours to allow for the production and secretion of any potential chemotactic cues as well as all other cell contents, before being lysed.	Female

4.5.2.2 Preparation of treatments

Each 'live' treatment was freshly prepared on the same day of the assay and lysed cues were prepared in advance. All cues were prepared as follows:

4.5.2.2.1 Uninfected RBCs (RBC):

20µl uninfected blood was collected from the tail of a naive mouse and placed in 20µl ookinete media. This was then centrifuged at 10,000rpm for 2 minutes and the supernatant (serum) was removed; leaving uninfected RBCs only. These uninfected RBCs were added to slides for assaying, as described below.

4.5.2.2.2 Live female gametes and live asexuals:

On day 4 or 5 post infection, 0.5 to 1.6ml blood was collected from *P. berghei* infected mice by cardiac puncture from anaesthetised mice and added to 10ml of ookinete media at 20^{o}C for 20 minutes to exflagellate (and to ensure that the microgametes could be separated from females). This culture was then passed through a magnetic column (MACS, Miltenyl Biotech) to which the female gametes and any remaining non – activated male gametocytes adhered. Three x 3ml washes of ookinete media were then passed through the column to wash the non-magnetic uninfected RBCs and asexual stage parasites out, along with microgametes. This flow through was collected and spun down at 10000rpm for 2 minutes. The supernatant (serum and media) was removed; leaving the pellet of asexual and uninfected RBCs, which formed the 'live asexual' treatment group, as described in table 4.3. Aliquots of this pellet were also taken to prepare the 'lysed asexuals'

treatment group as described below. After the last wash through the column, it was removed from the magnet and 1ml ookinete media was added and immediately forced (via a plunger) through the column to flush out the activated female gametes and gametocytes into a clean centrifuge tube. This was repeated three times; resulting in 3mls of female culture. This was spun down in a centrifuge at 2500rpm for 3 minutes at 37^{o}C. The supernatant was removed and the remaining pellet of pure female gametes was resuspended in 1ml of ookinete media containing aphidicolin (Sigma-Aldrich, UK) at a concentration of 5 x 10^{-4}M, and incubated at 21^{o}C for 10 minutes. Aphidicolin was used to irreversibly inactivate any remaining male gametocytes (and prevent microgamete formation) that may have been present (Ramiro et al., 2011). The culture was spun again and the pellet washed with ookinete media to remove the aphidicolin. Thus, the pellet contained a high density of purified and activated female gametes which was confirmed by smears. The females were then aliquoted into two tubes; one for immediate use for the 'live female' treatment assay, and the second to form the 'lysed female' treatment. This second tube was incubated at 20^{o}C for 3 hours to allow the female gametes to produce and release any potential chemotactic cues into the pellet before further aliquots were made to prepare the 'lysed females' treatment group, as described below.

4.5.2.2.3 Lysed females and asexuals

Aliquots of pure live female gametes or asexuals (prepared as described above) were lysed via three rounds of freeze thaw at -80^{o}C and 37^{o}C. This method has been shown to completely lyse parasite infected RBCs (see section 3.3.2 (Carter et al.,

2014)). These preparations were then frozen at -80^{o}C before being used in the experiment.

4.5.2.3 Chamber preparation and assay set up

Glass chambers were constructed based on advice from Laurence Wilson (The Rowland Institute, Harvard University) to create an assay environment without 'flow', which means that any microgamete movement that is observed would be due to Brownian motion and their own motility. Glass slides and cover slips were arranged as detailed in figure 4.5 and optical adhesive (Norland) fixed by UV light was used to secure the coverslips in position.

For each assay, microgametes were isolated from infected blood according to the following method. 20µl blood was taken from the tail of a mouse and placed into 20µl ookinete media (RPMI + 10% foetal calf serum, pH 8, 20^{o}C) for 20 minutes (to allow sufficient time for the activation of male gametocytes, exflagellation and release of microgametes), and then spun down at 2000 rpm for 1 minute, to produce a supernatant containing purified microgametes. 7µl of the supernatant was placed in the chamber, immediately followed by 2µl of the treatment material (detailed in table 4.3). The set-up is detailed in figure 4.5.

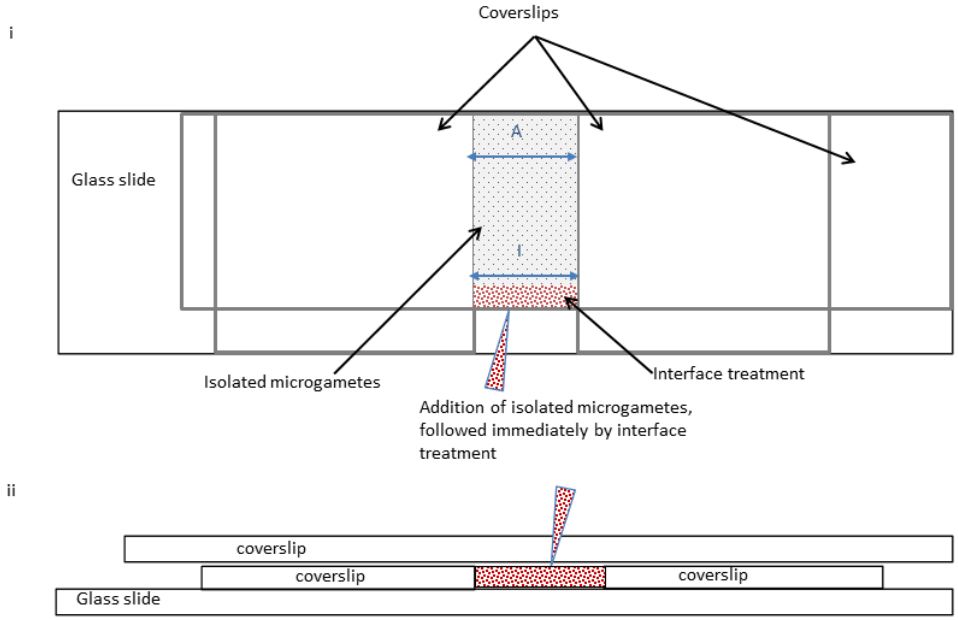

Figure 4.5 Chamber design and assay set up. Three coverslips (1 large, rectangular and 2 small and square) were fixed on to each slide to create a chamber at the centre. For each assay, 7µl isolated microgametes was placed into the chamber, followed immediately by 2µl of the treatment to create an interface between male gametes and the treatment. "A" represents the area counted for the away location, and "I" for the interface location. (i) View from above, (ii) view from the side (not to scale).

The number of microgametes/field (microgamete density) for 'time = 0' was recorded immediately upon placing microgametes on the slide at two locations: the interface ("I") with the cue treatment and as far away ("A") from the interface as possible (at least 12.5mm). The location counted first was randomly selected and counting in each location was limited to duration of 3 minutes. For the t=0 time point, counts were taken over a range of 0-6 minutes post assay initiation. Twenty to 26 minutes after adding microgametes to the slide, microgamete density counts were taken again at both locations, starting with the same location that the t=0 counts were taken first.

For example, for assay X, microgamete density was counted at the interface with the treatment (I) from 0-3 minutes, and away from the interface (A) from 3-6 minutes, then from 20-23 minutes post assay initiation, microgamete density was counted at (I), and then (A) from 23-26 minutes. The 20 minute time period (covering the 20 – 40 minute period after gametogenesis was initiated) was chosen because microgametes have approximately 30 minutes between being taken up in a bloodmeal and for fertilisation to occur (Carter and Nijhout, 1977, Sinden et al., 2010). The probability of any particles (cue material/cells) placed at the interface diffusing into the 'away location' during the 20 minutes between t=0 and t=20 counts was calculated to be less than 6×10^{-13} (appendix equation 1). Calculations assumed typical molecular diffusivity, ignored any edge effects, and assumed that distance along the chamber is the only relevant quantity (i.e., concentration is constant as a function of channel width and height). These assumptions were based on advice from Laurence Wilson (The Rowland Institute, Harvard University), and standard diffusion theory (Fick, 1855) (see appendix equation 1 for details of the diffusion probability calculation).

4.5.2.4 Analysis

Microgametes were evenly distributed across the chamber for all treatments at the start of all assays (appendix figure 2). Given this, it would have been possible simply to analyse differences in the microgamete densities at the interface (I) over time. However, instead, a generalised linear mixed model (GLMM) was fitted. Data were analysed using R version 3.0.2 and the package 'lme4'. The GLMM tested whether

there were any differences between treatments in the proportion of microgametes at the interface over 20 minutes in order to a) account for any other potential changes in the chamber environment over time, b) to make full use of all the data collected and c) allow the use of a binomial error structure to weight the analysis based on the density of microgametes observed at the start of the assay. GLMMs were constructed with the response variable as the proportion of the total microgametes at the interface (I_{md} / (I_{md}+A_{md})), where 'I_{md}' is the microgamete density at the interface and 'A_{md}' is the microgamete density away from the interface. As the response variable data was strictly bounded (between zero and one), had non-constant variance and non-normal errors, a binomial error structure with a logit link function was used. Explanatory variables included the assay time (either t=0, or t=20) and the cue interface treatment. All models included 'infection ID' as a random effect to account for repeated measures from infections from the same mouse, thereby avoiding pseudoreplication. Models were minimised following stepwise deletion of the least significant term and using log likelihood ratio tests (χ^2) to evaluate the change in model deviance, until only significant terms remained in the model. All 13 mice did not contribute to all treatments due to a lack of treatment material on some assay days due to problems with purification.

4.5.3 Results

The aim of the experiment was to test whether microgametes preferentially move towards (aggregate) at an interface (I; treatments) of live female gametes, and/or lysed female gametes, when compared to live asexuals, lysed asexuals, or uninfected RBCs. There was no overall effect of treatment, so treatment groups were collapsed

as follows to simplify the model: There was no significant difference in the proportion of microgametes at the interface when it consisted of live female gametes *vs* lysed females ($\chi^2_{2,9}$=0.194, p= 0.907). Likewise, the proportion of microgametes at interfaces of live asexuals *and* lysed asexuals were not significantly different ($\chi^2_{2,7}$=2.326, p=0.313). Therefore, treatments were grouped into three categories: "RBCs", "asexuals" and "females." Further model simplification found no significant difference in the proportion of microgametes at the control interfaces of "RBC's" vs "asexuals" ($\chi^2_{2,5}$=1.814, p=0.404) and so treatments were grouped into two categories: "control" (RBCs, live asexuals and lysed asexuals) and "female" (live and lysed). When the proportions of microgametes at "female" and "control" interfaces were compared there was a borderline significant interaction between treatment and time ($\chi^2_{1,5}$=3.83, p= 0.051) (figure 4.6). Specifically, after 20 minutes, the proportion of microgametes at the interface with female material increased by 10%, from 0.505 ± 0.11 to 0.563 ± 0.11, and reduced by 7% at the interface with the control material (start: 0.490 ± 0.08, end: 0.460 ± 0.08).

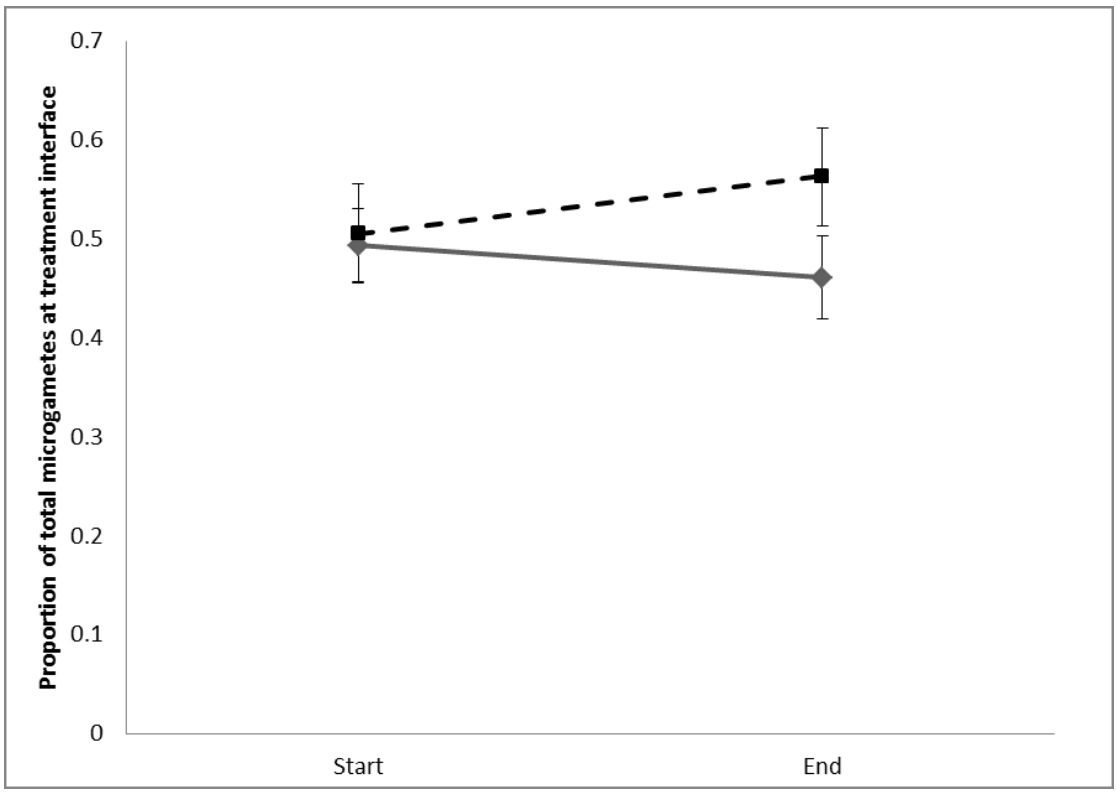

Figure 4.6 Change in microgamete density over time. The mean proportion of microgametes (± asymmetric 95% Confidence interval) at the interface with either a control treatment (RBC and asexual stage material: solid line) or female material (a mixture of live female gametes and lysed female gametes: dashed line) at the start of the assay (Start: time = 0-6 minutes) or after 20 – 26 minutes in the chamber (End).

4.5.4 Discussion

The aim of this experiment was to test whether exposing microgametes to material from various isolated components of the blood meal (live female gametes, lysed female gametes, live asexual parasites, lysed asexual parasites, or uninfected RBCs) had any effect on the direction of their movement. The density of microgametes at an interface with female material increased by 10% over a 20 minute period, compared to a 7% decrease when exposed to control (RBC and asexual stages) material. Whilst the effect is relatively small and of borderline significance, it is consistent with predictions that microgametes are attracted to female gametes and suggests that microgametes non-randomly search the blood meal to find female gametes.

Microgametes could be attracted to a gradient of chemoattractant released by the activated female gametes ("chemotaxis"), in a similar mechanism to bacterial food searching (Eisenbach, 1996). Chemotaxis has been observed in many other 'externally fertilizing' systems, such as marine invertebrates where sperm are guided toward eggs by their secretions (Zimmer and Riffell, 2011). Whilst these results are not a conclusive demonstration of the existence of a mechanism that facilitates fertilisation, it does suggest that the process by which microgametes find females is not completely random. In order to definitively identify whether chemotaxis is involved, a choice experiment is required; simultaneously exposing microgametes to two interfaces: female material and control material and assaying the change in microgamete density at each interface over several time points. Extending the assay time beyond 20 minutes post initiation may provide more insight (and perhaps

increase the effect size observed), as my observations suggest microgametes may continue swimming for one hour post initiation.

Another important consideration is that it is difficult to know how the concentration of treatments within the chambers compares to a natural blood meal, especially as the dilution and removal of any potential chemo-attractants during female material preparation is likely. Intuitively, due to the time- sensitive nature of exflagellation and fertilisation, female-derived chemo-attractants are most likely to be released immediately upon egress of the female gamete from the residual gametocyte. Gamete egress is known to be facilitated by a local increase in Ca^{2+}, activation of calcium dependant protein kinases and the localisation of osmiophilic bodies at the plasma membrane (Billker et al., 2008, Olivieri et al., 2014). Osmiophilic bodies release their contents upon emergence and are virtually non-existent once the female gametocyte has differentiated into the gamete (De Koning-Ward et al., 2008). In this study, the contents of osmiophilic bodies may have been degraded during purification and / or lysis of cue material. The cue treatments were designed simply to test whether microgamete responses could be elicited, rather than to identify precisely what they are detecting. It is possible that the female material did contain a chemoattractant, but there may have been other components within the material that overrode the chemoattractant stimulus (e.g., lysed RBCs), which may explain the responses detected. Identifying whether a chemo-attractant is released from osmiophilic bodies (or simply whether osmiophilic bodies are involved) could be facilitated by comparing microgamete motility at interfaces with activated wild type female gametocytes and Pfg377-KO female gametocytes which lack the full

complement of osmiophilic bodies (De Koning-Ward et al., 2008). Similarly, testing the effect of different Ca^{2+} concentrations (at an interface) on microgamete motility could provide clues as to whether microgametes use Ca^{2+} as a cue for the presence of activated female gametes.

In addition to chemotaxis, another potential mechanism that facilitates mating success is the presence of a compound that changes the motility (i.e. speed, swimming direction, amplitude, wavelength, or beating pattern) of microgametes *in a non-vectorial manner;* i.e., unlike chemotaxis ("chemokinesis") (Petrie et al., 2009). For example, variable glucose concentrations have been shown to affect microgamete motility (Talman et al., 2014). Clarification on whether chemokinesis is involved is not possible from the assays described above (because it would not be expected to result in an aggregation of gametes at an interface), but instead, should be available following the holographic reconstruction and comparison of microgamete motility (specifically swimming direction, speed, wavelength and beating pattern) in the presence of different factors that may affect microgamete motility (e.g., different concentrations of glucose, Ca^{2+}, or live, activated female gametocytes (Billker et al., 2008, Olivieri et al., 2014, Talman et al., 2014)), as an extension of the work presented in section 4.3.

If chemotaxis, chemokinesis and / or nanotube-like filaments of gametes (FiGs) (see section 4.5.1) (Rupp et al., 2011, Kuehn and Pradel, 2010) can be definitively demonstrated as mechanisms that facilitate mating, one future question would be to

ask how species –specific the strategies are? For example, are *P. berghei* microgametes attracted to female gametes of another species (e.g. *P. yoelii*), and vice versa? Data from sperm studies in other organisms suggest this would be unlikely, because chemotaxis (for example) has been shown to be highly dose dependant and species specific (Zimmer and Riffell, 2011). Nevertheless, examining whether these mechanisms play a role in facilitating fertilisation would open up several new lines of investigation for identifying transmission blocking interventions targeting chemo-attractants. For example, the egg-derived tryptophan signal to attract male sea urchin sperm is relatively easily extinguished by the addition of the enzyme tryptophanase (Zimmer and Riffell, 2011). If a putative malaria female gamete / gametocyte chemo-attractant can be identified, then one definitive test would be to observe the effect of inhibiting the chemo-attractant on microgamete motility. Finally, 'optical tweezers' are one possible tool that could be used to measure the force of attraction between isolated microgametes and female gametes (Altindal et al., 2011). Advances in microscopy and microfabrication, often originating from physics laboratories, are ideally suited to addressing these questions.

5 Linking gametogenesis activating factors and reproductive success

5.1 Summary

Sexual differentiation is crucial in the transmission of malaria parasites through the mosquito vector, but how this process is initiated is unclear. When ingested by a mosquito, gametocytes rapidly differentiate into male and female gametes in a process called gametogenesis. Developing drugs and/or vaccines that prevent transmission by disrupting this process are major goals of biomedicine, but understanding the environmental factors that are involved in initiating gametogenesis is essential for any intervention to be sustainable. A number of gametocyte activating factors (GAFs) have been identified *in vitro* (e.g., lower temperatures and a rise in pH), but factors experienced by parasites *in vivo* are less clear. Several tryptophan metabolites (in particular, Xanthurenic acid, XA and Kynurenic acid, KA) have been implicated as natural GAFs, but the source of GAFs, the identification of their receptors and the mechanism by which they reach the bloodmeal remain unresolved. There are discrepancies across the literature in the extent to which putative GAFs trigger exflagellation in different species and subspecies. Furthermore, the knock-on implications of GAFs (e.g., on ookinete development) have not been explicitly tested in controlled conditions. Here, *P. berghei* gametocytes were cultured over a wider concentration range of XA and KA than previously tested, and tryptophan itself was tested for the first time. By following each independent culture from exflagellation (male gametogenesis) to ookinetes, the experiments directly connected gametocyte activation and reproductive success (ookinete yield: ookinete density / exflagellation density) for the first time. The data reveal significant variation in exflagellation and ookinete yields when exposed to XA, KA and tryptophan, as well as highlighting the

importance of pH. Finally, variation in responses to GAFs across species (*P. berghei vs. P. yoelii*) as well as genetic variation between three subspecies of *P. yoelii* was observed.

5.2 Introduction

When a mosquito takes a bloodmeal from a malaria-infected host, gametocytes are immediately activated and the process of gametogenesis is initiated. After emergence from the RBC, male gametocytes undergo three rounds of DNA replication and construct flagella; forming up to eight microgametes which emerge after 10-20mins in a process called exflagellation (see chapter 4, section 4.1 for details) (Sinden et al., 2010). In contrast, female gametocytes simply emerge from the RBC, in a process mediated by osmiophilic bodies (De Koning-Ward et al., 2008). Although the production of microgametes involves activation of a calcium signaling pathway via calcium dependant protein kinase 4 (cDPK4), cGMP-dependent protein kinase (PKG), phospholipase C and inositol (1, 4, 5) triphosphate (IP_3) (Kawamoto et al., 1990, Martin et al., 1994, Billker et al., 2004, McRobert et al., 2008, Raabe et al., 2011), the primary environmental trigger(s) for gametogenesis remain unclear.

5.2.1 Gametocyte activating factors

To avoid initiation of gametogenesis within the host (which would waste crucial resources invested into transmission and stimulate the host to produce transmission blocking immune factors (Mendis et al., 1990, Naotunne et al., 1991, Naotunne et al., 1993,)), gametocytes must possess an environmental sensing mechanism to recognise when they have moved from the host environment to the mosquito. The nature of these triggers are poorly understood, though, given the short time period in

which they have available to mate, it is likely that gametocytes use obvious cues ("gametocyte activating factors: GAF"), and are highly sensitive to them. The most obvious GAF is the drop in temperature by 5 - 17^{o}C (from the host to the mosquito, or *in vitro* culture), which is sufficient to induce a low level of exflagellation (Baton and Ranford-Cartwright, 2005). However, exflagellation is significantly increased by the addition of one, or a combination of the following factors: a fall in carbon dioxide tension as the blood equilibrates with the atmosphere, thereby increasing the pH (Carter and Nijhout, 1977); setting the pH of culture media to 8 (Billker et al., 1997); and the addition of sodium bicarbonate to the culture media (Butcher et al., 1996). Nevertheless, the relative importance of each of these factors in triggering exflagellation and their relevance to natural infections remains unclear (Arai et al., 2001).

Whilst creating a pH8 environment is convenient for inducing exflagellation in laboratory experiments, a pH change is not thought to play any significant role as a natural GAF for the following reasons. The pH of uninfected mouse blood does not change dramatically between the host (pH 7.3 - 7.4) to the mosquito blood meal (7.4 – 7.7) (Billker et al., 2000), but when infected with *P. berghei*, blood is significantly lower than pH 7.3 (Chang et al., 2001), and so the cause behind the pH of infected blood in the vector is unclear. Furthermore, pH changes have unknown consequences for subsequent ookinete development. During the search for GAFs that are relevant *in vivo,* Xanthurenic acid (XA) (and similarly structured compounds within the tryptophan metabolism pathway) have been implicated (Billker et al., 1998, Garcia et

al., 1998). In addition to XA there is likely to be at least one more GAF, and it is most likely to be derived from the host (Arai et al., 2001).

5.2.2 The tryptophan metabolism pathway

In mammals, tryptophan is an essential amino acid required for protein synthesis as well as a biochemical precursor for a range of compounds including neurotransmitters such as serotonin, kynurenic acid (KA) and XA (figure 5.1 summarises the tryptophan metabolism pathway). KA and XA result from the oxidation of kynurenine or 3-hydroxykynurenine (3-HK) by kynurenine aminotransferase (KAT) (Schwarcz et al., 2012). Whilst a definitive role for XA as a neurotransmitter is yet to be defined, KA acts as an antagonist to prevent overstimulation of the central nervous system (Schwarcz et al., 2012, Han et al., 2007). In the mosquito, 3-HK is readily oxidised to produce reactive radical species which can accumulate to toxic levels. Mosquitoes lack kyneurinase (which hydrolyses the 3-HK in mammals), so they convert it to the chemically stable XA via transaminase instead (Han et al., 2007) (figure 5.1).

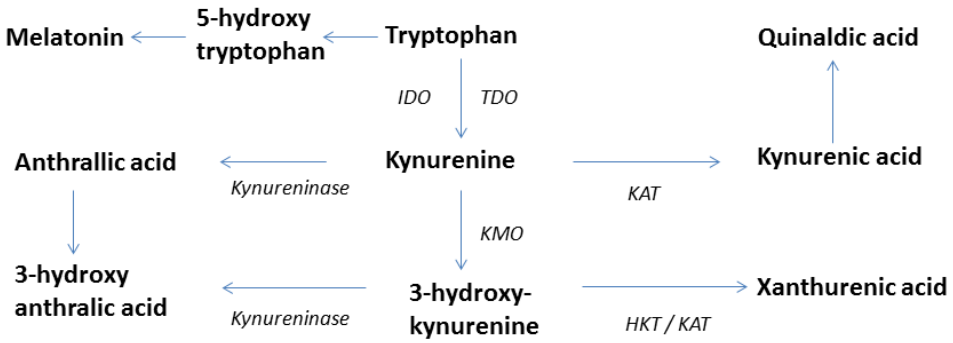

Figure 5.1 Summary of the tryptophan metabolism pathway, catalyzed by a number of enzymes: IDO, Indoleamine 2,3-dioxygenase; TDO, tryptophan dioxygenase; KFM, kynurenine formamidase; KMO, kynurenine monooxygenase; KAT, kynurenine aminotransferase; HKT, 3-hydroxykynurenine transaminase. The pathway presented here occurs in both the host and the vector, except that mosquitoes do not have the enzyme kynureninase, so 3-hydroxy-kyneurenine is oxidised to XA by HKT (Han et al., 2007).

5.2.2.1 XA and related compounds as GAFs

Table 5.1 summarises the current data on exflagellation induced by tryptophan metabolites at various concentrations, *in vitro*. For all *Plasmodium* species that have been tested (rodent*: P. berghei* and *P. yoelii*, chicken: *P. gallinaceum* and human: *P. falciparum* -infecting parasites), tryptophan metabolites induce exflagellation in a dose dependent manner, but there is variation between species and some discrepancy between studies. XA induced the highest level of exflagellation compared to all other compounds and 10^{-4}M XA induced *P. berghei* exflagellation to the same level as pH 8. However, the concentration of XA required to induce maximum exflagellation varied between species (Arai et al., 2001) and studies (Billker et al., 1998). *P. gallinacuem* appears to be the most sensitive of all species to XA (Arai et al., 2001), but there are some discrepancies in the patterns observed across studies, with a value

of 42% exflagellation at the same concentration reported elsewhere as inducing maximum exflagellation (Garcia et al., 1998, Arai et al., 2001). For *P. falciparum*, exflagellation at 10^{-4}M XA is 100% across studies, but reports are more variable at lower concentrations (ranging between 26 and 49%) (Garcia et al., 1998, Bhattacharyya and Kumar, 2001). The pattern for rodent parasites (*P. berghei* and *P. yoelii*) is more repeatable across studies, with 90-100% exflagellation consistently induced from 5 x 10^{-5} to 1 x 10^{-3}M XA (intermediate to high concentrations) (Billker et al., 1998, Arai et al., 2001).

For *P. berghei*, quinaldic acid (QA) induced the second highest exflagellation at an intermediate concentration (~80% of the 10^{-4}M XA level) (Billker et al., 1998), and KA followed (Billker et al., 1998). *P. berghei* was the only species tested that reached 100% exflagellation when exposed to high concentrations of KA. Other compounds such as 3-hydoxyanthralic acid (3-HAA) and 3-hydroxy kynurenine (3-HK) also induced some exflagellation in *P. berghei*, but to a much lesser extent, and only at the highest concentration (Billker et al., 1998). For *P. falciparum,* KA induced approximately 50% maximum exflagellation regardless of compound concentration, and induced approximately 30% maximum exflagellation in *P. gallinaceum* regardless of concentration. However, there are no data with a sufficient range of concentrations to make detailed comparisons between other compounds, for any species.

5.2.2.2 Relevance of XA *in vivo*

The majority of research on the relationship between tryptophan metabolites and exflagellation has been carried out *in vitro*, but XA has been implicated as a mosquito – derived GAF which is required for transmission *in vivo* (Billker et al., 1998, Garcia et al., 1998). Transmission rates are reduced in eye-colour-mutant *Anopheles stephensi* mosquitoes compared to wild type *A. stephensi.* This has been attributed to the lower concentrations of XA within their eye-pigment pathway, which results in lower levels of exflagellation and reduced transmission (Billker et al., 1998). Furthermore, XA levels vary depending on mosquito age and species, which could explain variation in exflagellation and transmission success across different parasite and vector combinations (Siden-Kiamos and Louis, 2004, Matsuoka et al., 2007).

However, the mechanism by which XA enters the midgut remains unclear. In *Drosophila* (and therefore presumably also in mosquitoes), XA is deposited in Malpighian tubules and excreted in the faeces (Billker et al., 1998); thereby bypassing the midgut. Some XA may be secreted into the saliva (0.28ng XA in salivary gland) which could be ingested back by the mosquito as it takes a bloodmeal (Okech et al., 2006, Matsuoka et al., 2007), but whether this could result in a sufficient blood meal XA concentration to induce exflagellation is unclear. Uninfected mosquito midgut XA concentration is estimated to be 2.5-3µM XA (Arai et al., 2001, Okech et al., 2006) which would only induce approximatley 1- 10 % of the maximum level of exflagellation observed for *P. berghei*, but approximately 50% for *P. gallianceum* and *P. falciparum* (table 5.1). These levels are unlikley to be

sufficient for fertilisation success and transmission (especially for *P. berghei*) (Billker et al., 1998).

5.2.3 Questions outstanding

5.2.3.1 Is XA the (only) natural GAF?

Despite wide acceptance that XA is the natural cue responsible for triggering exflagellation (Billker et al., 1998, Garcia et al., 1998, Bhattacharyya and Kumar, 2001, Arai et al., 2001, Okech et al., 2006, Matsuoka et al., 2007), there is no definitive proof that it is the *actual* GAF and also whether it is the *only* GAF acting within the mosquito midgut, for the following reasons:

- The receptor for XA is yet to be identified (Smith and Jacobs-Lorena, 2010, Raabe et al 2011), and pH 8 and XA cultures trigger exactly the same downstream calcium signalling pathways involved in exflagellation (Billker, 2004). It is unclear how these pathways would be triggered by such diverse cues.

- It is not known how parasites avoid premature exflagellation in the blood. The combination of a drop in temperature of the peripheral circulation at night-time, and XA at a concentration of 0.6 -2μM (Truscott and Elderfield, 1995, Williams et al., 1984) poses a risk of inadvertently triggering exflagellation.

- The concentration of XA in an infected bloodmeal has not been quantified, neither has the mechanism by which XA reaches the blood meal.

Table 5.1 Comparison of tryptophan metabolite - induced exflagellation across a range of *in vitro* studies. Values represent exflagellation as a % of that induced when parasites were exposed to 10^{-4}M XA (**bold**). XA: xanthurenic acid, KA: kynurenic acid, QA: quinaldic acid, KYN: kynurenine, 3-HK: 3-hydroxy-kynurenine. 3-HAA: 3-hydroxy anthralic acid. The baseline pH for all cultures ranged from 7.4 -7.5.

Concentration (M)	Rodent parasites								Bird parasites			Human parasites			
	P. berghei							*P. yoelii*	*P. gallinaceum*			*P. falciparum*			
Source	(Billker et al., 1998)						(Arai et al., 2001)	(Arai et al., 2001)	(Garcia et al., 1998)		(Arai et al., 2001)	(Arai et al., 2001)	(Bhattacharyya and Kumar, 2001)		
Compound	XA	KA	QA	KYN	3-HK	3-HAA	XA	XA	XA	KA	XA	XA	XA	XA	KA
6.2×10^{-8}									0	0	2				
1.0×10^{-7}	0	0	0	0	0	0	0	0			90	1			
1.3×10^{-7}									5	0					
2.5×10^{-7}									9	0					
5.0×10^{-7}									42	0	100	2			
1.0×10^{-6}	1	0	0	0	0	0	1	1	105	0	100	26	65	49	43
5.0×10^{-6}	10	0	0	0	0	0	10	10			100	60			
1.0×10^{-5}	30	0	0	0	0	0	65	65	91	0	100	80		67	
5.0×10^{-5}	90	0	10	0	0	0	100	100			100	100			
1.0×10^{-4}	**100**	**10**	**80**	**0**	**0**	**0**	**100**	**100**	**100**	**26**	**100**	**100**	**100**	**100**	**52**
5.0×10^{-4}	100	90	100	0	0	0	100	100			100	100			
1.0×10^{-3}	100	100	100	0	10	5	100	100	89	34		90		44	

5.2.3.2 Why is there so much variation in sensitivity to GAFs?

Sections 5.2.2.1 and 5.2.2.2 summarise variation and discrepancies in the literature. Exposing all species to a wider (and higher) concentration range of KA and QA (and ideally other members of the tryptophan metabolism pathway) is required for a detailed comparison of these metabolites with the activity of XA, and to assess whether there is any toxic effect of compounds at high concentrations. The trigger of exflagellation by a range of compounds suggests that gametocytes may have evolved 'generalist' receptors, receptors for multiple compounds, or that exflagellation is triggered by different GAF in different parasite species. Determining whether there is genetic variation in exflagellation responses is important, because for behaviours and traits to evolve, genetic variation is required (chapter 1). Determining the extent of genetic variation in GAF sensitivity may also facilitate a better understanding of the selective forces shaping mating success.

5.2.3.3 What are the post fertilisation effects of XA?

Medium to high XA concentrations induce a high level of exflagellation in all species tested to date, but this could trade off against a possible toxicity of high XA concentrations that impair ookinete development (as suggested by the inhibition of exflagellation when *P. gallinaceum* was exposed to undiluted mosquito head and gut extracts containing GAFs (Garcia et al., 1997)). Whilst previous studies have tested the effects of XA and mosquito –derived GAFs on ookinete density *in vivo* (Arai et al., 2001, Bhattacharyya and Kumar, 2001), there has been no direct connection between XA induced exflagellation and reproductive success (ookinete yield:

ookinetes / exflagellation) in environmentally controlled conditions (*in vitro)*. Furthermore, testing the effect of increasing the concentration of other tryptophan metabolites (from 10^{-3}M to 10^{-1}M) on exflagellation and ookinete yield is key in determining their relative toxicity, or indeed, whether they enhance ookinete development, and the wider implications of this on transmission success.

5.2.3.4 Could the host be the source of XA and other GAFs?

Although a prominent mosquito metabolite (Li and Li, 1997), XA is also present in protozoa (Takeda and Sugiyama, 1993) and uninfected mouse blood (at 0.6 - 2µM (Williams et al., 1984, Truscott and Elderfield, 1995)). In *P. gallinaceum,* the same level of exflagellation was observed in cultures supplemented with chicken serum *vs.* cultures containing 10^{-4}M XA (Arai et al., 2001). In addition, the concentration of all tryptophan meatbolites (e.g. XA, KA and QA) is raised during a malaria infection due to macrophage- mediated activation of the Indoleamine 2,3-dioxygenase (IDO) enzyme, which catalyses tryptophan (figure 5.1) (Testsutani et al., 2007). Furthermore, *Eimeria* (an Apicomplexan parasite related to *Plasmodium*) has been shown to 'co-opt' the tryptophan catabolism pathway of their mouse host to facilitate their own life cycle progression, using IDO (Schmid et al., 2012). The addition of XA to IDO - negative (and therefore XA-negative) mice appears to restore *Eimeria* oocyst growth (Schmid et al., 2012). *Plasmodium* parasites may have evolved to utilise the IDO enzyme in a similar way by co-opting it to increase the concentration of tryptophan metabolites (which could involve XA, KA and QA) to stimulate exflagellation in the bloodmeal. Together, these observations support the hypothesis

of the host blood as an alternative or additional source of XA (and other potential GAFs) in the bloodmeal.

5.2.4 Aims of Chapter

The ambition of this study was perform a comprehensive experiment (i.e., each infection contributing to all treatments of each compound and concentration), to link exflagellation and fertilisation success of *P. berghei* when exposed to a broad concentration range of three key compounds in the tryptophan metabolism pathway: XA, KA and tryptophan (Tryp) itself. By following each independent culture from exflagellation to ookinete yield, the experiment directly connects gametocyte activation and reproductive success (ookinete yield: ookinete density / exflagellation density) for the first time. XA, KA and Tryp may also negatively affect male mating success and offspring viability. Thus, measuring ookinete density per exflagellating male is a better measure of male reproductive success than exflagellation density alone. The experiments also aimed to provide more precise and repeatable exflagellation and ookinete data than previous studies, through the use of haemocytometers (instead of slide and coverslip assays where the densities of cells are unknown). Furthermore, my experiment was performed in slightly different culture conditions: notably, at a lower pH of 7.3 (*vs* 7.4 - 7.5 in previous studies) which is more representative of *P. berghei* infected blood (Chang et al., 2001). To test for any drop off in exflagellation and possible toxic effects of the compounds at high concentrations, concentration peak was increased from 10^{-3}M, used in previous studies (table 5.1) to 10^{-1} M. Testing the effect of Tryp on exflagellation was also novel. Tryp was intended as a negative control for the addition of a compound to the

culture media because it is the least likely member of the tryptophan pathway to be biologically active due to its comparatively large structure which is dissimilar to XA and KA. The study also examined whether three subspecies of *P. yoelii* (*P. yoelii yoelii, P. yoelii nigeriensis and P. yoelii subspecies*) were sensitive to the same GAFs as *P. berghei*. The subspecies' were used as a proxy to test for genetic variation in GAF sensitivity and corresponding fertilisation success.

5.3 Methods

5.3.1 General protocols

For both experiments ('experiment 1' and 'experiment 2'), infections were initiated in male MF1 mice (8-10 weeks old, from an in house supplier, The University of Edinburgh), with 10^7 parasitized RBCs from donor mice infected with cryopreserved parasites (from The University of Edinburgh's malaria reagent repository http://malariaresearch.eu/), as detailed below. For experiment 1, 11 *P. berghei* WT ANKA high producer (*P. berghei*) infections were initiated, and for experiment 2, 5 *P. yoelii nigeriensis* N67 (Pyy), 3 *P. yoelii* subspecies IV (Pys), and 3 *P. yoelii yoelii* 17X (Pyy) were initiated. The mice had been pre-treated with phenylhydrazine at 125 mg/kg (2 days before infection) to enhance the production of gametocytes (Reece et al., 2008).

5.3.1.1 Pre-assay checks

To check that infections were suitable for setting up the main experiments, on days 4 and 5 post infection tests were performed to verify that there was a sufficient density

of mature gametocytes in the infected blood for assaying exflagellation and ookinete development. These tests involved culturing 2µl of tail blood in 100µl fresh ookinete media (RPMI + 10% foetal calf serum, pH 8) at 20^{o}C and vortexing to stimulate exflagellation (Janse et al., 1985). Ten minutes post initiation, 8µl of this culture was placed under the cover slip of a haemocytometer and the number of exflagellation events observed in 1/9 of the haemocytometer grid (100nl culture) was recorded. An exflagellation event was defined as a haphazard, rapidly moving parasite extruding flagella; often forming clumps (exflagellation centres) with nearby RBCs. When more than 20 exflagellation events were counted in 1/9 of a haemocytometer, these infections were considered suitable for assaying. As a control, 2µl of tail blood was also cultured in 100µl fresh stasis media (RPMI + 10% foetal calf serum, pH 7.3) at 20^{o}C and vortexed to confirm that exflagellation was not initiated (or was at a very low level (i.e. less than 5 exflagellation events in a whole haemocytometer grid) at this pH.

5.3.1.2 General protocols for the main experiments

Once infections had been assessed as suitable for assaying, the main experimental cultures (RPMI + 10% foetal calf serum) were set up containing XA, KA or Tryp at concentrations specified in sections 5.3.3 and 5.3.4). The pH of each culture was adjusted to 7.3 after the addition of compounds to eliminate any variation in pH confounding results. Negative (pH 7.3) and positive (pH 8) control cultures were also set up from the same infections. 2 µl tail blood was collected from infected mice, added to the pre-prepared cultures, and vortexed. After 14 minutes, exflagellation was recorded using a haemocytometer (as described in 5.3.1.1). If fewer than 30

exflagellation events were observed in 1/9 of the haemocytometer area, then exflagellation density was counted over 1/3 of the haemocytometer area. All cultures were then placed in an incubator (at 20^{o}C for experiment 1 and 24^{o}C for experiment 2) for 18-21 hours, to allow for fertilisation and ookinete maturation. Following this incubation period, cultures were removed from the incubator and vortexed for at least 20 seconds to eliminate clumping of ookinetes and female gametocytes, and ookinete density was assayed over at least 2/3 of the total haemocytometer area. RBC density counts were taken of the infected blood on the day of culture, to monitor anaemia of the mice and to allow calculations of exflagellation and ookinete densities.

5.3.2 Protocol optimisation

Before the main experiments were carried out, routine culture protocols were optimised to account for the comparatively large scale assays in these experiments (5.3.2.1) and to identify the optimal baseline pH to use (5.3.2.2).

5.3.2.1 Culture optimisation

Whilst the routine, large scale culturing of *Plasmodium* gametocytes (50- 75µl heart blood in 5000µl culture media) is well tested and known to produce high ookinete yields, the two main experiments described below required a large number of culture conditions (so each infection had to contribute to many different treatments), with the added complication of assaying exflagellation within a limited time window (2 minutes). This meant using tail blood rather than heart blood and reducing the routine culture volumes to 2µl blood in 200µl culture media, whilst keeping the concentration within the same range (1.0 ± 0.5% blood). Trials concluded that there

was no difference in the optimal culture conditions for ookinete yield between tail and heart blood initiated cultures (at pH8, with heparin and 10% foetal calf serum) and that there was no difference between culturing in centrifuge tubes (and vortexing to mix blood on culture initiation) *vs.* culture plates (and pipetting to mix blood) (data not shown). Therefore, all assays in experiment 1 used routine culture conditions (RPMI + 10% foetal calf serum, pH 7.3, at 20^{o}C), but at a reduced volume of 200µl, and in centrifuge tubes as this method was more practical and economical than using culture plates.

An additional pilot experiment was carried out to test the time point after culture initiation at which the highest level of exflagellation was recorded. Tests concluded that there was no difference in exflagellation density between 14 minutes and 19 minutes post initiation across all cultures assayed (pH8, XA, KA and Tryp, all at 10^{-4}M) (data not shown). Therefore, in the interests of efficiency, exflagellation counts for all experiments were initiated at 14 minutes post initiation.

5.3.2.2 Baseline pH

As the ambition of the main experiment was to test the effects of compounds on exflagellation and ookinete yield, it was necessary to ensure that no additional culture characteristics would confound any compound effect; namely the pH of the culture. Pilot work showed that there was a significant effect of treatment on the exflagellation level of *P. yoelii yoelii,* strain 17X (relative to the total exflagellation across all treatments applied to that infection) between cultures at pH8, pH 7.3, pH

7.4, and 10^{-4}M XA (treatment: $\chi^2_{3,6}$= 33.791, p<0.001) (figure 5.2). Post hoc tukey tests revealed a significant difference between 10^{-4}M XA (0.209 ± 0.088) and pH 7.3 (0.0096 ± 0.006) (z ratio = 4.39, p<0.001), but no significant difference between 10^{-4}M XA (0.209 ± 0.088) and pH 7.4 (0.0918 ± 0.040) (z ratio = 2.373, p =0.0823). Although this was not quantified for *P. berghei*, I observed pH 7.4 inducing exflagellation for these parasites too. This implies that previous studies which have used pH 7.4 – 7.5 as a baseline for testing XA activity may be confounded. To avoid this problem here, all cultures in the main experiments were set to a baseline of pH 7.3.

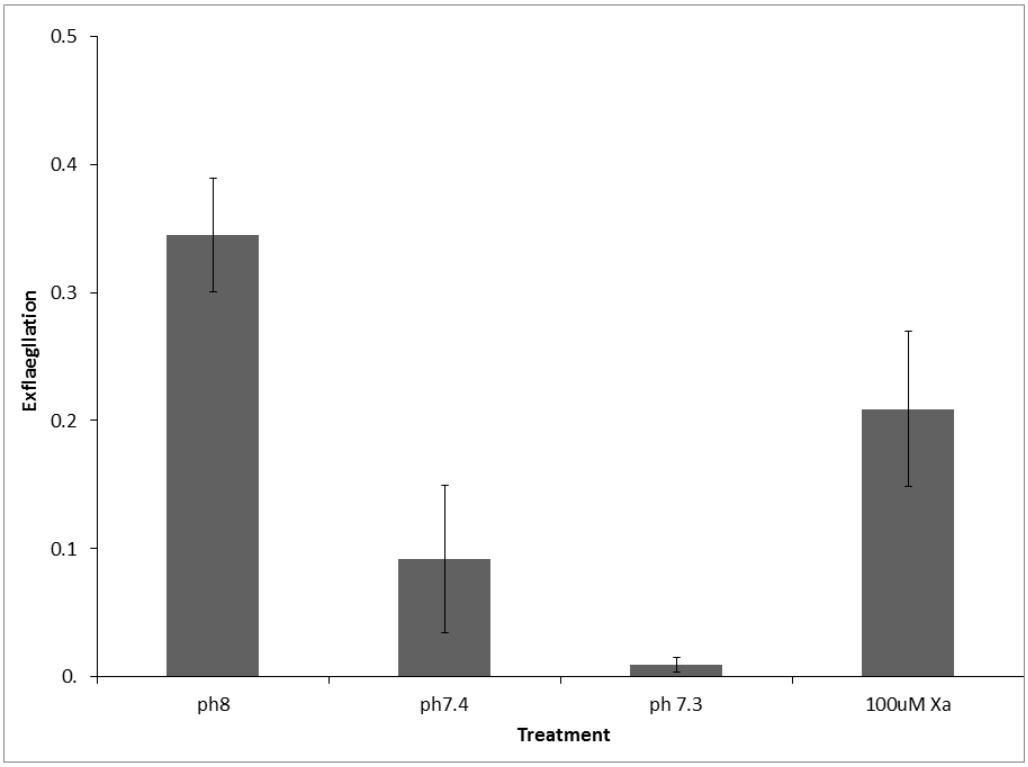

Figure 5.2 Exflagellation at different pH values. Exflagellation relative to the total exflagellation for each treatment for a given infection of *P. yoelii yoelii,* when exposed to pH 8, pH 7.4, pH 7.3 and 10^{-4}M) XA. Mean ± SEM. Number of independent infections, N=3.

5.3.3 Experiment 1: Exflagellation and ookinete yield of *P. berghei* when exposed to 10^{-6} to 10^{-1}M X xanthurenic acid, kynurenic acid, or tryptophan

Experiment 1 was designed to test the role of XA, KA and Tryp in inducing exflagellation and their consequences on ookinete yield over a wider range of compound concentrations than previously tested. For each infection, 18 x 200µl cultures (containing RPMI + 10% foetal calf serum, pH 7.3, at 20^{o}C) were set up containing XA, KA or Tryp each at the following concentrations: 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}M, with 2 µl tail blood.

5.3.4 Experiment 2: Exflagellation and ookinete yield of three subspecies of *P. yoelii* when exposed to 10^{-4}M xanthurenic acid, kynurenic acid, or tryptophan.

The ambition of experiment 2 was to test for any variation in exflagellation and ookinete yields between three subspecies of *P. yoelii:* (*P. yoelii nigeriensis* N67 (Pyy), *P. yoelii* subspecies IV (Pys), and *P. yoelii yoelii* 17X (Pyy) when cultured in 10^{-4}M XA, KA or Tryp. Cultures were set up according to the protocols outlined in section 5.3.2; except that 2µl infected tail blood was cultured in 100µl ookinete media instead of 200µl. This was because gametocyte density for *P. yoelii* infections was on average 10-fold lower than for *P. berghei* (1.35 x 10^7 gametocytes / ml blood for Pyy, Pyn and Pys vs 1.74x 10^8 gametocytes / ml blood for *P. berghei*). Increasing the gametocyte density of cultures enabled faster and more efficient exflagellation assays, and increasing the density of blood in the culture by only 1%, was not expected to interfere with fertilisation or ookinete maturation, as the overall density of blood was still low (2%) (see section 4.4.4.1).

5.3.5 Analysis

After accounting for dilution factors and for the RBC density of each infection contributing to cultures, exflagellation density (exflagellation / ml blood) was calculated. Ookinete yields were also calculated (ookinete density / exflagellation density). All response variables (which were relative to the pH8 control) were transformed to conform to the assumptions of normality and to allow maximum use of the whole data set which was zero inflated. Linear mixed effects models (LME) were fitted to all data using infection as a random effect to account for pseudoreplication arising from repeated measurements of each infection. Models were minimised following stepwise deletion of the least significant term and using log likelihood ratio (χ^2) tests to evaluate the change in model deviance until only significant terms remained in the model.

For experiment 1, when compound concentration = 10^{-1}M, all response values were 0 and so were removed from the analysis to reduce the issue of zero inflation skewing the distribution. For experiment 2, because ookinete yield was consistently zero for KA and Tryp across all strains, these were removed from the analysis. Raw ookinete yields were reported for this analysis because pH8 did not induce exflagellation in Pys (even though XA did).

5.4 Results

5.4.1 Experiment 1

5.4.1.1 Exflagellation

The effect of each compound on exflagellation was dependent on its concentration (treatment*concentration: $\chi^2_{2,8} = 29.489$, $p<0.0001$) (figure 5.3A). XA consistently induced the highest exflagellation followed by KA, then Tryp (except at high concentrations (10^{-2} M) where KA induced the highest exflagellation and XA and Tryp were close to zero). At the highest concentration of 10^{-1}M, exflagellation was 0 for all three compounds tested. At intermediate concentrations (10^{-3}M to 10^{-5}M), XA- induced exflagellation was consistently higher than that observed for pH8 cultures (10^{-3}M: 1. 18 ± 0.53, 10^{-4}M: 1.17 ± 0.65, and 10^{-5} M: 1.70, ± 1.16), and was ~10 fold higher than KA and Tryp –induced exflagellation. Exflagellation for KA (peak at 10^{-2}M: 0.33± 0.17) or Tryp (peak at 10^{-4}M: 0.11 ± 0.07) never reached even half of the pH8 exflagellation level, regardless of concentration. At the lowest concentration of 10^{-6}M, exflagellation between the three compounds was very similar (XA: 0.19 ± 0.10, KA: 0.10 ± 0.03, Tryp: 0.14 ± 0.10).

5.4.1.2 Ookinete yield

Raw ookinete densities followed a very similar pattern to exflagellation. For example, at intermediate densities XA ookinete density was ~10-fold higher than that for KA and Tryp (data not shown). To test for any variation in post-exflagellation effects (on the mating success of microgametes or female gametes, or ookinete development) between compounds and across the concentration range, ookinete yields were analysed (figure 5.3B). The effect of each compound on ookinete yield

relative to pH8 depended on its concentration (treatment*concentration: $\chi^2_{2,6}$ = 10.441, p=0.005) (Figure 5.3B). Ookinete yield increased (with the relative yield for each compound at 10^{-2}M being Tryp>KA>XA), from zero for all compounds at the highest concentration (10^{-1}M) to the highest yield for all compounds at an intermediate concentration (10^{-3}M) (XA: 1.19 ± 0.14, KA: 1.11 ± 0.14, Tryp: 1.46 ± 0.15). At lower concentrations (10^{-4} to 10^{-5}M), ookinete yields for XA, KA and Tryp were similar (10^{-5}M: XA: 0.98 ± 0.14, KA: 0.84 ± 0.13, Tryp: 0.53 ± 0.11) except at the lowest concentration where KA yields were slightly higher than XA and Tryp.

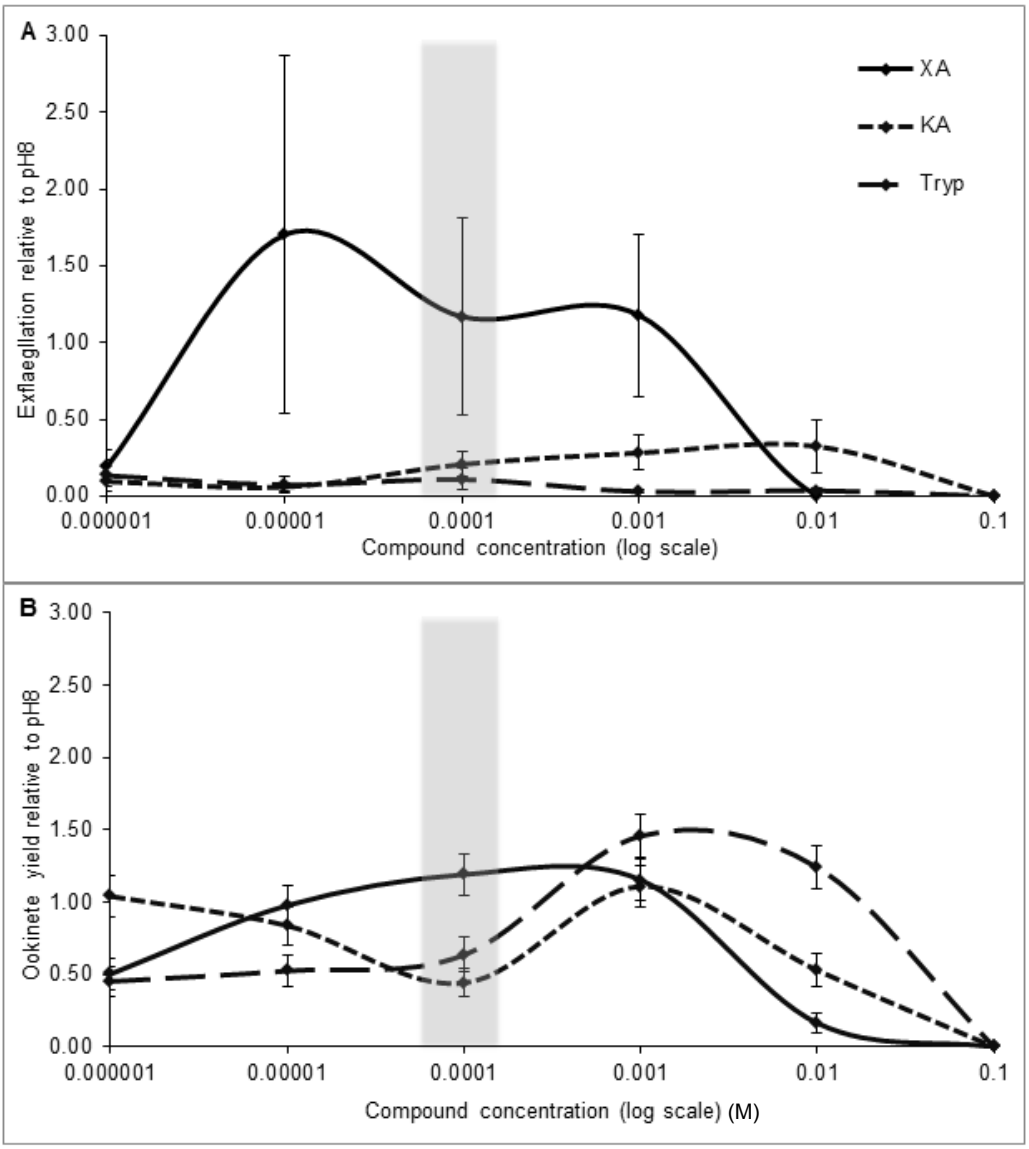

Figure 5.3 Exflagellation and ookinete yields of *P. berghei*. Exflagellation (A) and ookinete yield (ookinete/exflagellation) (B) relative to the pH8 positive control of *P. berghei* cultures when exposed to XA, KA and Tryp ranging from 10^{-6} to 10^{-1}M. Mean ± SEM. N =11 independent infections. Shaded bar highlights 10^{-4}M which is the assumed optimal concentration for XA-induced exflagellation from previous work (Billker et al., 1998).

5.4.2 Experiment 2

The effect of each compound on exflagellation was not dependent on the subspecies of *P. yoelii* tested (compound*subspecies: $\chi^2_{4,7}$= 4.906, p= 0.297) (figure 5.4A).

However, there were main effects of subspecies on exflagellation (subspecies: $\chi^2_{2,6}$= 15.19, p<0.001) and compound identity (treatment: $\chi^2_{2,5}$= 13.256, p=0.001). Across all compounds, exflagellation was highest for Pyn, followed by Pys, and Pyy had the lowest exflagellation. Exflagellation for all subspecies was the highest in pH8 cultures, followed by XA, then KA and finally Tryp, where exflagellation was the lowest for all subspecies (except for Pyy, where KA induced 0 exflagellation).

No ookinetes were observed in any cultures containing KA or Tryp across all subspecies, so these values were removed from statistical analysis. Comparing the ookinete yields in XA and pH8 cultures revealed that yields from each subspecies' were not significantly different from each other (subspecies: $\chi^2_{2,4}$= 2.628, p= 0.268) and not influenced by treatment (10^{-4}M XA *vs* pH8; treatment*subspecies: $\chi^2_{2,6}$= 1.701, p= 0.427 and treatment: $\chi^2_{1,3}$= 0.763, p= 0.382) (figure 5.4B).

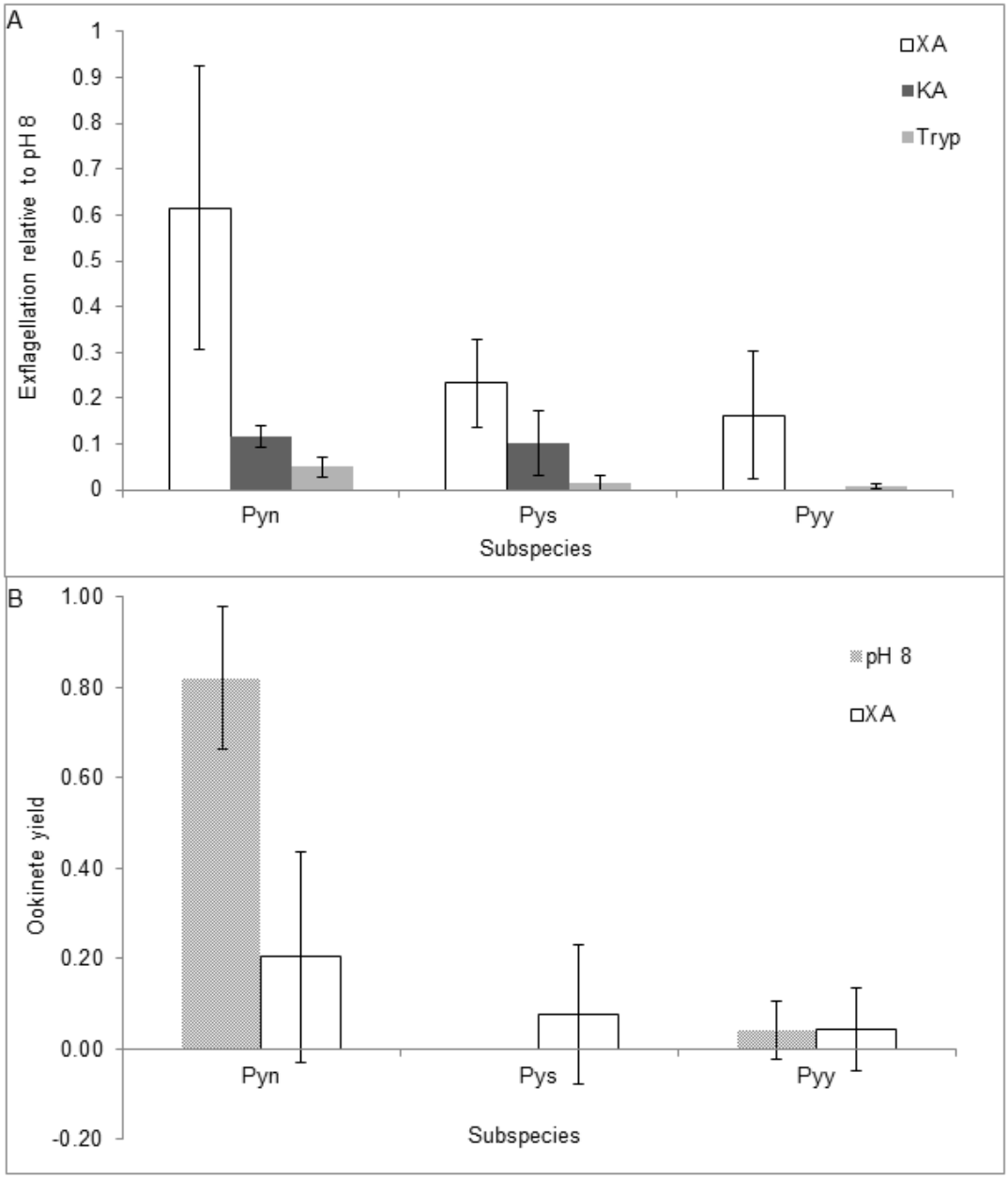

Figure 5.4 Exflagellation and ookinete yields of *P. yoelii yoelii*, *P. yoelii nigeriensis* and *P. yoelii* subspecies. (A) Exflagellation (relative to pH8) of Pyn: *P. yoelii nigeriensis,* Pyy*: P. yoelii yoelii* and Pys: *P. yoelii subspecies,* when exposed to 10^{-4}M XA: xanthurenic acid, KA: kynurenic acid, and Tryp: tryptophan. (B) Ookinete yield (ookinete/exflagellation) when exposed to pH8 or 10^{-4}M XA. Mean ± SEM. N ranges from 3 to 5 infections that contributed to each treatment.

5.5 Discussion

The data show that 1) *P. berghei* exflagellation and ookinete yields vary depending on the presence and concentration of XA, KA and Tryp *in vitro*. 2) Exflagellation is significantly different between three subspecies of *P. yoelii* (Pyn, Pyy and Pys), and XA, KA and Tryp induce significantly different levels of exflagellation for *P. yoelii* subspecies. 3) Tryp - and KA - induced exflagellation failed to yield any ookinetes for any *P. yoelii* subspecies, and there was no significant difference between XA and pH8 induced ookinete yields for Pyy, Pyn, or Pys.

5.5.1 Linking exflagellation and ookinete yields

Ookinete yield (ookinete density / exflagellation density) is a more appropriate measure of transmission success than simply exflagellation or ookinete density alone, because XA, KA and Tryp have unknown post-exflagellation consequences for the success of microgametes, female gametes and ookinete development. These experiments directly connected XA, KA and Tryp induced exflagellation and reproductive success for the first time. If, for example, all microgametes resulting from the exflagellation levels observed in figure 5.3A were equally able to mate and / or produce viable offspring, then ookinete density would be equivalent to exflagellation across all treatments and this would result in an ookinete yield of '1' (i.e., a single flat line with an intercept at 1 would have been observed in figure 5.3B). Any significant deviation from this pattern indicates that the treatments have differing post-exflagellation effects compared to pH8 cultures.

5.5.1.1 *P. berghei* and dose responses

5.5.1.1.1 Intermediate concentrations: 10^{-4}M XA is the optimal GAF, *in vitro*

For *P. berghei*, exflagellation patterns relative to pH8 were largely as predicted (XA>KA>Tryp) at all but the highest concentrations (KA didn't ever reach the same level of exflagellation as pH8, as previously observed) (Billker et al., 1998). For intermediate concentrations (10^{-5} to 10^{-3}M), XA clearly induced the highest exflagellation relative to pH8, while KA and Tryp induced similarly low levels of exflagellation to each other (~10 fold lower than XA). The same ~10 fold difference between XA, and KA / Tryp at intermediate densities was observed for ookinete density relative to pH8 (data not shown).

The general patterns observed in figure 5.3B suggest two main findings. First, ookinete yields were similar across the compounds at intermediate concentrations. Second, ookinete yields for all compounds at low and intermediate concentrations fluctuate around 1. Taken together, these results suggest that the compounds (at intermediate concentrations) have no significant post-exflagellation inhibition or enhancement of reproductive capacity. Therefore, the data support the identity of intermediate concentrations of XA (specifically 10^{-4}M XA, which is widely used in exflagellation studies (Arai et al., 2001)) as the GAF that induces most exflagellation and ookinetes. However, further work is required to verify whether KA induces a biologically relevant increase in ookinete yield at the very low concentration of 10^{-6}M.

5.5.1.1.2 Variable ookinete yields at high concentrations (10^{-2}M)

At a high compound concentration (i.e., 10^{-2}M) ookinete yields for XA and KA started to drop but ookinete yields for Tryp remained at roughly the same level as at 10^{-3}M (where ookinete yields were similar for all compounds) (5.5.1.1). This result suggests that the compounds (XA, KA and Tryp) differentially affected the ability of (one or more of) the microgametes / female gametes / zygotes to produce ookinetes. Non-mutually exclusive explanations for this include the following.

First, high Tryp concentrations could enhance post exflagellation reproduction. For example, the host--derived IDO enzyme (which is activated by malaria infections (Testsutani et al., 2007)) could catalyse tryptophan metabolism (increasing XA, KA and QA concentrations in culture), which could trigger delayed exflagellation. Data from the related Apicomplexan parasite, *Eimeria* supports this hypothesis, in principal (Schmid et al., 2012). However, if activation of males took much longer than could have been observed in the assays (at 14mins), then immature "retort"-shaped ookinetes are likely to have been observed in the cultures.

Alternatively, Tryp itself could provide an additional resource for developing ookinetes *in vitro* (tryptophan is already one component of the RPMI 1640 culture media). However, this may not be relevant to transmission success in natural infections, because previous *in vivo* experiments found a detrimental effect of synthetic tryptophan-supplemented sugar meals on oocyst numbers (Okech et al., 2006).

Finally, instead of Tryp enhancing ookinete development, XA and KA may have inhibited post exflagellation development. This hypothesis is supported by the observation that the ookinete yields for XA and KA cultures are much reduced compared to Tryp at a high concentrations (10^{-2}M) (Figure 5.3).

5.5.1.2 *P. yoelii* and genetic differences

For *P. yoelii,* the difference in exflagellation between subspecies (Pyn>Pys≥Pyy) indicates genetic variation in exflagellation responses. Furthermore, variation in the sensitivity of subspecies to different GAFs was observed. XA induced the highest level of exflagellation, but this was lower than the level induced by pH8, for all subspecies. Tryp induced the lowest exflagellation for Pyn and Pys, but not for Pyy, where KA failed to induce any exflagellation (figure 5.4A). Zero ookinete yields for KA and Tryp may be due to post exflagellation inhibitory effects on the microgametes, female gametes, and/or zygotes (as suggested for *P. berghei* when compound concentration is high: 5.5.1.1.2) that reduced ookinete yield below a detectable level.

Different exflagellation responses between subspecies provide evidence for variation on which selection can act. Furthermore, different selective forces (e.g. exposure to XA / KA/ Tryp) may be shaping evolutionary responses in different subspecies. For example, exposure of a given subspecies to an environment where XA is the predominant GAF is likely to select for higher sensitivity to XA relative to KA and Tryp. For example, if the natural GAF is a mosquito-derived factor and its identity varies across mosquito species, then this could be one reason for adaptation to a

specific vector species. Further work characterising the extent of genetic variation in sensitivity to GAFs (as well as subsequent ookinete yields) could improve our understanding of the selective forces shaping mating success.

5.5.2 Relevance to previous data and the importance of pH

It was not possible to make quantitative comparisons between the responses of *P. berghei* and *P. yoelii* under different culture conditions because they were two separate experiments and *P. yoelii* responses varied across subspecies. However, figures 5.3 and 5.4 suggest that *P. yoelii yoelii* is less sensitive to all compounds at an intermediate concentration than *P. berghei* (e.g., exflagellation relative to pH8 for 10^{-4}M XA was 0.16 *vs.* 1.18 respectively). This contradicts previous studies which suggest that *P.yoelii yoelii* and *P. berghei* exflagellation is maximal (100%) at 10^{-4}M XA (Arai et al., 2001) (table 5.1). Furthermore, previous work suggested that KA induces higher exflagellation than was observed in this experiment, (e.g., while 10^{-3}M: KA induced only 0.29 of *P. berghei* maximal exflagellation here, maximum exflagellation was recorded at the same concentration in Billker et al (1998) (table 5.1, figures 5.3 and 5.4).

Differences between studies may be due to differences in culture set up; in particular, the lower pH used here. The common use of pH 7.4 – 7.5 as a control and baseline may have confounded and artificially increased exflagellation data in previous experiments. Indeed, pilot work for this experiment showed that there was no significant difference in *P. yoelii yoelii* exflagellation between pH 7.4 and 10^{-4}M XA cultures at pH7.3 (figure 5.2). There is no comparable data for *P. berghei*, but it

is likely that the same occurs for *P. berghei.* Furthermore, because pH 7.4 induces exflagellation *in vitro* (to a similar level of 10^{-4}M XA), and the bloodmeal could reach pH7.4 quickly (Billker et al., 2000), it is possible that no additional GAFs are necessary for inducing exflagellation *in vivo*.

Going forward, one key question is whether pH interacts with tryptophan metabolites to cause different effects on exflagellation and crucially, ookinete yields. If increasing pH just shifts the reaction curve for exflagellation to the left (higher exflagellation at lower compound concentrations; but the pattern is the same), as implied by (Billker et al., 1998), there is no cause for concern, but this needs to be explicitly tested with a fully cross factored experiment varying pH and all compound concentrations simultaneously.

5.5.3 Summary and future directions

The data clearly support the identity of XA as the optimal GAF at intermediate concentrations. Results also highlight significant differences in exflagellation and ookinete yields between species and subspecies, as well as the importance of pH in exflagellation and ookinete assays. Furthermore, the data raise complex questions about the post exflagellation effects on free microgametes, female gametes, zygotes or ookinete development, and how this relates to ookinete yields of compounds at high concentrations. To disentangle the effects between gametes and ookinetes, experiments could involve washing cultures and replacing with fresh pH 7.3 culture media post fertilisation. This would remove any compound- or pH- effect on ookinete development. In addition, comparing the results of cultures set up in the

AM vs PM could indicate a role of the circadian activity of tryptophan metabolism (which is highest in the morning) (Rapoport and Beisel, 1968). This may help to reveal whether parasites are using compounds derived from the host as a trigger for exflagellation upon ingestion by the mosquito. For example, melatonin is also a product of the tryptophan metabolism pathway (figure 5.1) and is a well- known regulator of circadian rhythms (Hotta, 2000). Testing whether melatonin and XA production are linked would help to clarify whether circadian rhythms and transmission patterns are linked, as proposed by the "Hawking hypothesis": that reproductive effort is coordinated so that gametocytes reach maximum infectiousness at the time of mosquito feeding (Hawking et al., 1966). Finally, further work is also required to identify the receptor for XA, why pH 8 induces the same downstream signalling pathways as XA (Billker et al., 2004), and the mechanism by which GAFs reach the bloodmeal.

6 General Discussion

The ambition of this work was to integrate evolutionary theory, ecology, cell and molecular biology, parasitology and biophysics to develop a greater understanding of the reproductive strategies of protozoan parasites within the host and vector. Specifically, in this thesis, taking a novel and challenging approach, I have used malaria parasites to i) examine why parasites adjust their investment in gametocytes according to environmental variation (using an evolutionary ecology framework); ii) identify factors that parasites use to detect environmental changes, demonstrated by subsequent changes in their reproductive strategies (integrating parasitology and molecular biology techniques); iii) characterise the swimming dynamics and mating strategies of male malaria gametes (using methods derived from biophysics); and iv) quantify variation in the mating success of parasites when exposed to different gametocyte activating factors and RBC densities (integrating cell and parasitology methods). Throughout the thesis, I have recommended future experiments that could help to solve some of the remaining questions regarding parasite biology and behaviour, and explained how they can be combined with mechanistic approaches. In this section, I summarise my results, explain how they enhance our understanding of the reproductive strategies of malaria parasites and related Apicomplexans.

6.1 Variable reproductive strategies: implications and future work

Using the evolutionary ecology framework introduced in chapter 2, experimental manipulations in chapter 3 revealed a significant reduction in gametocyte upon exposure to lysed parasite material and a borderline significant increase in sex ratio when exposed to lysed red blood cells (both uninfected and infected). Demonstrating

that variation in gametocyte investment and sex ratio observed during infections are a result of parasite strategies (rather than the footprint of host physiology), provides a foundation on which to investigate the fitness consequences of plasticity. Furthermore, the results provide a base to explore whether drugs could be developed to trick parasites into making the wrong decisions for a given infection (in the absence of any *real* environmental variation). This is a significant challenge, because it is likely that the situation is complex; with multiple genes and pathways involved in sensing the environment, processing the information, making reproductive decisions and producing the phenotype (Carter et al., 2013) (chapter 2). Nevertheless, if precise cues can be isolated, it not only facilitates quantification of the costs and benefits of plastic parasite reproductive strategies (which is a longstanding challenge for the study of phenotypic plasticity (Auld et al., 1999), it also paves the way for the development of a novel intervention in the form of an "ecological-trap"; which would trick parasites into investing in gametocytes at the expense of asexual replication (in a vector-free environment) and thus reduce harm to the host. Isolating the precise cues could also aid further characterisation of the numerous molecular pathways implicated in the commitment and differentiation of gametocytes and therefore facilitate the development of novel drugs and vaccines. Comparing the reproductive strategies of wild type parasites to gametocytogenesis –associated knock-out lines (e.g., genes such as *pfgig, pfmdv-1, pfpuf2, npt1, ppm2, ap2-g* (Miao et al., 2010, Guttery et al., 2014, Kafsack et al., 2014, Sinha et al., 2014)) in variable environmental conditions could help to clarify the exact cues used and the time point at which cells commit to developing as a gametocyte.

6.2 Mating within the vector: implications and future directions

Integrating parasitology and biophysics, data collected in chapter 4 shows that *P. berghei* microgametes i) have a complex swimming pattern despite being a simple flagella with no accessory structures, ii) have a reduced reproductive success rate when in culture with a high concentration of microparticles (which were intended to mimic RBCs within a bloodmeal), and iii) may be attracted to *P. berghei* female gamete material. Furthermore, the results of chapter 5 suggest that gametocyte activation and subsequent reproductive success is significantly dependant on the identity and concentration of the factor initiating gametogenesis (gametocyte activation factor; GAF).

Understanding the species-specificity of this mating behaviour (as described above) is necessary to confirm the relevance of rodent malaria parasites as an effective model for the study of human malaria. For example, is the microgamete size, structure, motility and speed of other *Plasmodium* species the same as for *P. berghei*? This should be relatively easy to quantify once the holographic reconstruction of microgametes can be automated (Wilson et al., 2013). Furthermore, high resolution video microscopy coupled with fluorescent labelling of the microgametes may be able to determine whether the microgamete has a single locus for attachment to an RBC or female gamete (building on the reports of nanotube filaments connecting gametes (Rupp et al., 2011)). Finally, expanding assays to test the effect of *P. berghei* female gamete material (or glucose, or Ca^{2+}) on *P. yoelii* microgamete motility would help to reveal whether chemotaxis is involved in reproductive isolation. Mixed species infections are very common in the field, but

what prevents hybridisation between species is unclear (Ramiro et al., 2012). Comparing the swimming characteristics and mating ability of microgametes of other *Plasmodium* species may help to resolve this.

If the mating behaviours observed in chapters 4 and 5 are conserved, further work could help characterise weak points during mating that could aid the development of novel transmission blocking interventions. For example, disrupting chemotaxis, as discussed in 4.5.4 and mechanisms of increasing microgamete interactions with RBCs could be developed to inhibit fertilisation. Integrating this work with molecular studies could also benefit current transmission blocking developments. To date, the genes known to be involved in gametogenesis are: *mdv-1/peg3, pfg377* and exflagellation specifically: *ppm1, srpk, cdpk4, map2, actin2;* male gamete functionality*: hap2/gcs1, pf16, p48/45, p230*; female gamete functionality: *p230, p47, pkg;* zygote formation: *cith, hmpg2, dozi;* and ookinete differentiation: *nek2, nek4, pk7, gak, ppkl, ppm2, ap2-o* (van Dijk et al., 2001, Liu et al., 2008, De Koning-Ward et al., 2008, Ponzi et al., 2009, Guttery et al., 2012, Guttery et al., 2014). Comparing the motility parameters, effect of RBC density and chemotaxis assays of wild type parasites to those with these knock-out lines could help to clarify the exact stage at which each gene is expressed as well as their relative importance for parasite fitness.

6.3 Bridging scales

In order to fully appreciate the epidemiological impact of variation in traits, the relative importance of host- and vector- derived interactions on parasite behaviour, and how these vary over the course of an infection and between genotypes must be carefully considered (Poulin, 2007, Matthews, 2011, Mideo and Reece, 2011). Furthermore, cross-scale (within- and between-host) trade-offs are very rarely studied. For example, the extent of host anaemia (where there is a high density of lysed cells, but low density of whole RBC compared to healthy blood) could have important implications for a range of within- and between- host traits and behaviours that have been tested throughout this thesis. First, low RBC density could trigger an increase in gametocyte production (at the expense of asexual replication) (Reece et al., 2005). Second, the investment in male *vs*. female gametocytes is predicted to increase in response to low RBC density (Reece et al., 2005) and exposure to lysed RBC material (lysed cell material appears to act as a cue for adverse conditions which results in an increase in sex ratio) (Carter et al., 2014, chapter 3). Finally, mating success in the vector appears to be higher when RBC density is lower (section 4.4). Combining these observations; on the face of it, anaemic hosts would have the cumulative effect of increasing fertilisation success. Future experiments that disentangle how each of these factors interacts and trade-off against each other to affect overall parasite fitness are necessary.

For laboratory experiments, quantifying the variation in parasite strategies over the course of an infection in mice and mosquitoes is reasonably straightforward, but achieving this is natural infections is much more challenging. Current data on human malaria infections generally consists of snapshots in time, due to ethical and

logistical constraints in data collection (Färnert, 2008). However, with increasingly sophisticated statistical and modelling techniques, it could be possible to detect patterns of transmission in natural infections across populations to inform the development of interventions (reviewed in (Bousema and Drakeley, 2011, Churcher et al., 2013. The added complications of plasticity in reproductive strategies in response to the availability of insect vectors (Cornet et al., 2014), and vector evolution in response to environmental variation and human interventions must also be carefully considered in future modelling exercises and analysis of field data (Lefèvre et al., 2013, Sternberg and Thomas, 2014). For example, how vector control programmes would affect parasite evolution remains unclear (Gatton et al., 2013). Furthermore, with climate change inducing shifts in parasite host range, (Lafferty, 2009) unless the phenotypic range of the parasite, and the specific trade-offs that parasites face are quantified, it is impossible to predict how changing vectors, and therefore their mating environment, could affect parasite evolution and the spread of disease. To fully understand and predict the epidemiological consequences of variation, linking the within-host and within-vector scales is essential (Mideo and Day, 2008).

6.4 Summary

The work presented in this thesis provides evidence of remarkable variation in malaria parasite reproductive strategies and fertilisation success when exposed to environmental changes, both within the host and the vector, respectively. The observations can be used to help guide future interdisciplinary experimental

approaches to characterise the behaviour and evolution of malaria parasites and related protozoans (e.g. trypanosomes face a similar trade-off between within-host replication and between-host transmission (Pollitt et al., 2011a, Carter et al., 2013, Carter et al., 2014) (chapters 2 and 3), and *Eimeria* and *Toxoplasma* have similar processes of gametogenesis and mating (chapters 4 and 5) (Walker et al., 2013)). Ultimately, by understanding the parasite reproductive strategies which underlie disease epidemiology, we can inform the development of novel strategies to reduce transmission and better understand the potential for parasite evolution to undermine interventions.

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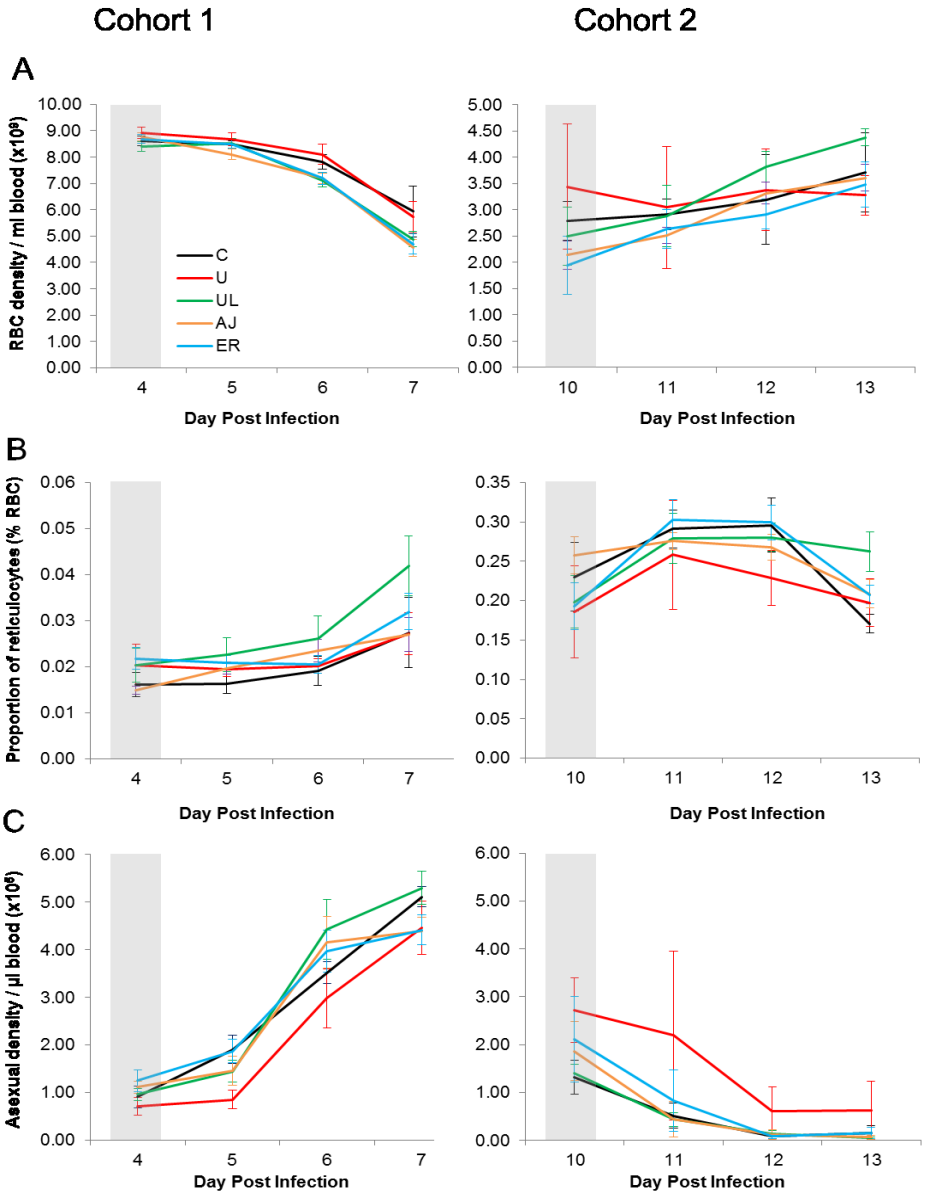
Appendices

Appendix figures and equations



Appendix Figure 1. *Plasmodium chabaudi* AJ infection dynamics: mean (± SEM) for each cue treatment (C: control, U: uninfected RBCs, UL: uninfected lysed RBCs, AJ: AJ-infected lysed RBCs and ER: ER-infected lysed RBCs) administered on day 4 PI for cohort 1 (left) and day 10 PI for cohort 2 (right) (indicated by grey bars). RBC density dynamics (**A**); proportion of RBCs that are reticulocytes (**B**) and asexual density dynamics (**C**). Maximum values for the Y axes differ between cohort 1 and cohort 2 to allow clear visualization of the range of data for each cue treatment group.

Appendix equation 1

To calculate the probability that any potential chemoattractant would diffuse to the 'away' location after 20 minutes, the following equation was used:

$$c(x,t) = \frac{c_L + c_R}{2} + \frac{c_R - c_L}{2} erf\left(\frac{x}{\sqrt{4Dt}}\right)$$

Where:

c = concentration of chemoattractant (1000mM)

x = position

t = time

c_L= concentration at x<0

c_R= concentration at x>0

erf = error function (standard in probability maths)

D = chemoattractant diffusivity = typical molecular diffusivity: 4 x 10^{-9} m^2/s

At t=0 seconds, the interface at x=0 is sharp, with initial concentration cL at x<0 and cR at x>0. The sharp interface blurs out over time to 1200 seconds (20 minutes). Any edge effects are ignored and the distance along the channel is assumed to be the only relevant quantity (i.e. concentration is constant as a function of channel width and height). Estimated values were applied to the equation and plotted in Appendix figure for equation 1 (credit: L. Wilson, Harvard).

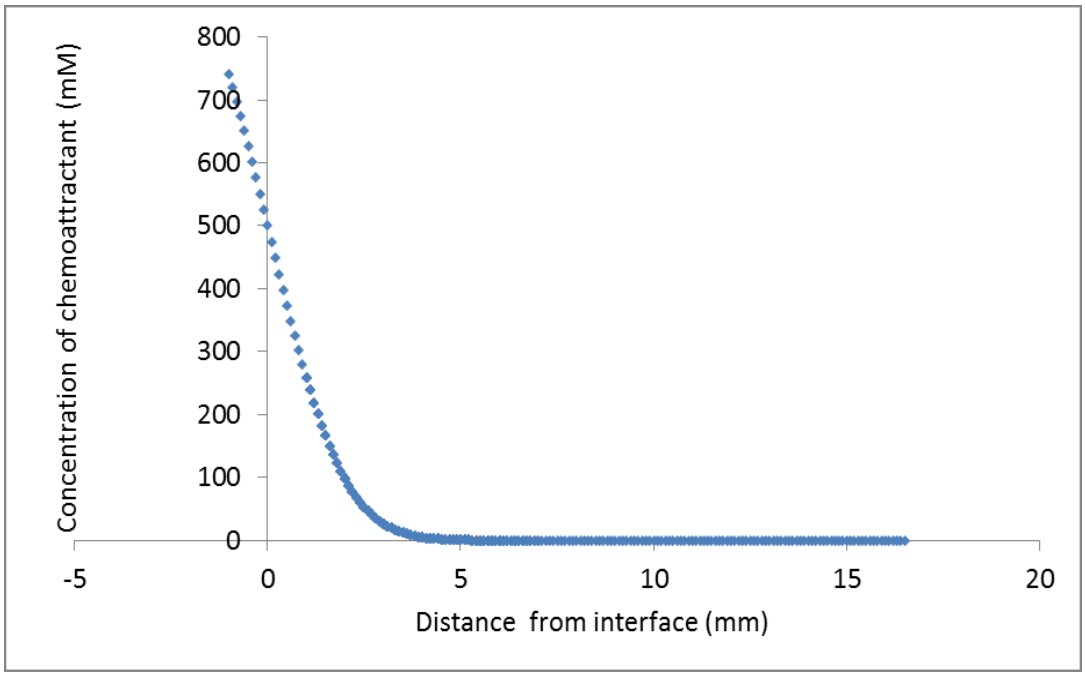
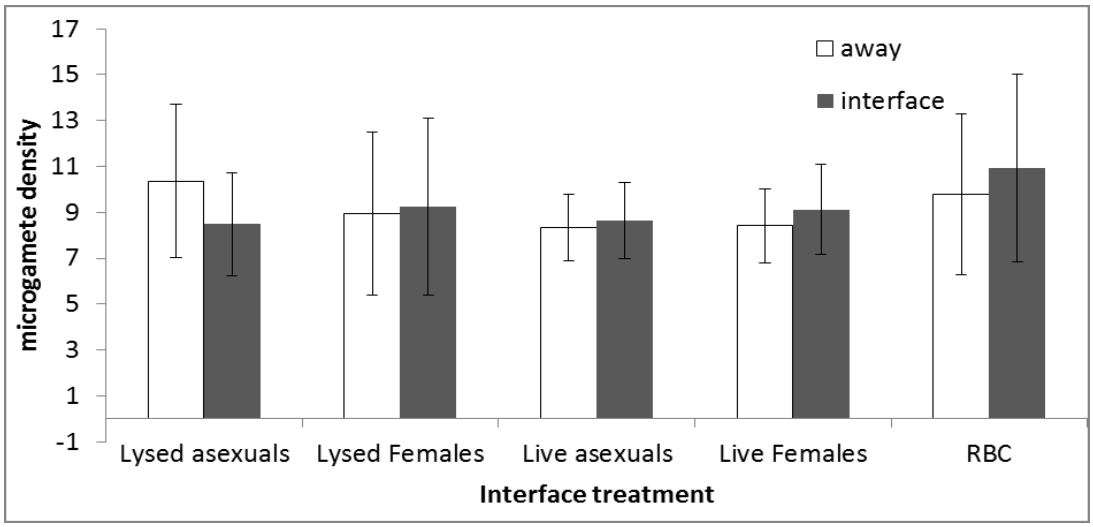


Figure for equation 1. Predicted relationship between the distance from the interface and concentration of chemoattractant after 20 minutes. At 12.5mm

from x (the minimum distance at which microgamete density was counted for the 'away' location), the concentration is 0 after 20 minutes.

Appendix Figure 2



Appendix Figure 2. Microgamete density (microgametes/field) at the interface with the treatment (grey) and away from the interface treatment (white) at the start of the assay (time = 0-6 minutes). Mean (± SEM), n ranges from 9 to 13. Microgametes were evenly distributed around the chamber ($\chi^2_{1,7}$=0.13, p= 0.72), and across treatments ($\chi^2_{4,3}$=7.71, p= 0.10) at the start of the assay.

Papers arising from this thesis

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Information use and plasticity in the reproductive decisions of malaria parasites

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Abstract

Background: Investment in the production of transmissible stages (gametocytes) and their sex ratio are malaria parasite traits that underpin mosquito infectivity and are therefore central to epidemiology. Malaria parasites adjust their levels of investment into gametocytes and sex ratio in response to changes in the in-host environment (including red blood cell resource availability, host immune responses, competition from con-specific genotypes in mixed infections, and drug treatment). This plasticity appears to be adaptive (strategic) because parasites prioritize investment (in sexual *versus* asexual stages and male *versus* female stages) in manners predicted to maximize fitness. However, the information, or 'cues' that parasites use to detect environmental changes and make appropriate decisions about investment into gametocytes and their sex ratio are unknown.

Methods: Single genotype *Plasmodium chabaudi* infections were exposed to 'cue' treatments consisting of intact or lysed uninfected red blood cells, lysed parasitized RBCs of the same clone or an unrelated clone, and an unmanipulated control. Infection dynamics (proportion of reticulocytes, red blood cell and asexual stage parasite densities) were monitored, and changes in gametocyte investment and sex ratio in response to cue treatments, applied either pre- or post-peak of infection were examined.

Results and conclusions: A significant reduction in gametocyte density was observed in response to the presence of lysed parasite material and a borderline significant increase in sex ratio (proportion of male gametocytes) upon exposure to lysed red blood cells (both uninfected and infected) was observed. Furthermore, the changes in gametocyte density and sex ratio in response to these cues depend on the age of infection. Demonstrating that variation in gametocyte investment and sex ratio observed during infections are a result of parasite strategies (rather than the footprint of host physiology), provides a foundation to investigate the fitness consequences of plasticity and explore whether drugs could be developed to trick parasites into making suboptimal decisions.

Keywords: Transmission, Gametocyte investment, Conversion rate, Sex ratio, Host-parasite interactions, Competition, Phenotypic plasticity

Background

Malaria parasites proliferate in the blood through cycles of asexual replication, but every cell cycle a small proportion of progeny commit to developing into male and female gametocytes (which do not replicate in the host) [1-4]. This means that, like all sexually reproducing organisms, malaria parasites face resource allocation trade-offs between survival and reproduction and between producing males and females [5-8]. Specifically, every cell cycle parasites make decisions about how much to invest in gametocytes (which are essential for reproduction and transmission) *versus* asexuals (which are essential for in-host survival) and in males *versus* females. These decisions are sensitive to variation in the in-host environment [9,10].

Extensive variation in gametocyte investment (also known as the 'conversion rate' or 'reproductive effort') and sex allocation (proportion of male gametocytes) of *Plasmodium spp.* has been observed across different species, strains, and during infections [10-18]. Understanding variation in gametocyte investment and sex ratio (collectively referred to as 'reproductive strategies') is important because they are key fitness-determining traits, shaping survival within hosts and the success of

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transmission to new hosts [19-21]. Experiments using rodent malaria parasites *in vivo* and *Plasmodium falciparum in vitro* suggest that parasites alter investment in gametocytes and their sex ratio in response to: changes in red blood cell (RBC) resource availability [12,18,22-24], host derived transmission blocking immune (TBI) responses [25-28], competition from con-specific genotypes in mixed infections [16,25,29,30] and, drug treatment [11,13,14,31-35]. Observational data from natural infections also suggests that *P. falciparum* sex ratios and gametocyte investment differ between single and mixed infections and are altered in response to variation in RBC density [36].

Evolutionary theory offers explanations for why parasites adjust their reproductive strategies in response to the changing environmental conditions encountered in the host [10,20,37-39]. For example, parasites increase gametocyte investment in response to anaemia, reticulocytes and exposure to sub-lethal anti-malarial therapy [11,13,18,23,32,33,40,41]. This has been interpreted as a strategy of 'terminal investment' during extreme stress [42]: investing heavily in gametocytes maximizes transmission potential in a situation likely to be lethal (e.g., before the infection is cleared or the host dies) [11,13,32]. However, recent evolutionary theory predicts that this may be an oversimplification and that less severe stress induces parasites to reduce investment, as a strategy of 'reproductive restraint' [39]. Reproductive restraint is predicted to facilitate in-host survival and therefore future transmission opportunities [39]. Empirical work supports these predictions, revealing that when parasites experience competitive suppression, RBC limitation, and low doses of anti-malarial drugs, they reduce gametocyte investment [14,16,30]. The sex allocation decisions of parasites are sensitive to many of the same factors as gametocyte investment. For example, different sex ratios bring the highest fitness returns in single- *versus* mixed-genotype infections [6,25,43-46] and when hosts are mounting immune responses that differentially affect male and female gametocytes [47]. Experiments with *Plasmodium chabaudi* reveal that sex ratios are precisely allocated according to the number of co-infecting genotypes and their relative representation within a mixed-genotype infection [25]. Therefore, sex ratio data suggest that parasites can determine the genetic diversity of their infections and measure the number (or replication rate) of asexual stages belonging to their genotype [25].

Whilst evolutionary theory can explain why parasites adjust investment into gametocytes and their sex ratio, it does not explain how they do so. Whether parasites identify and respond to individual factors (e.g., RBC density and age structure, the presence of competing parasites and the dose of drugs), or the overall impact the environment has on their proliferation rate (i.e., 'state') is not known [21]. A further complication is that the in-host environment is complex and many factors change simultaneously. For example, both anaemia and immunity develop as parasite number increases [26,48], competition in mixed infections brings RBC limitation and suppresses asexual proliferation [9,49-51], and different drugs kill parasites in dose-dependent ways and can alter anaemia [52]. For the parasite, more accurate information may be obtained from directly measuring individual environmental factors, but measuring changes in overall state may be the most efficient strategy, as it does not require the assimilation of information from multiple environmental variables that could elicit contradictory parasite responses [21].

The experiments presented here investigate the cues that parasites use to make their reproductive decisions by examining whether the gametocyte investment and sex ratio of a single clone infection change in response to material ('cues') derived from uninfected RBCs, RBCs infected with con-generic parasites, and RBCs infected with a con-specific genotype. The experiments were designed to build on previous work [16,25] to more specifically test 'what' parasites sense in their in-host environment. For example, in previous experiments conversion rates [16] and sex allocation [25] were compared in single and mixed genotype infections to ask whether parasites respond to in-host competition. However, numerous factors vary between single and mixed infections (e.g. anaemia, the age structure of RBCs, the concentration and balance of cytokines and the density of parasites) in complex ways. This makes it difficult to pinpoint exactly which factor(s) parasites are responding to. Furthermore, these changes in the in-host environment offer different opportunities and constraints to parasites that could be incorrectly interpreted as a parasite response. For example, parasites may not respond directly to anaemia, but may appear to do so, because a lack of preferred RBCs available for parasites to invade could directly interfere with their replication rate. The experiments presented here were designed to minimize the problem of simultaneously changing multiple aspects of the in-host environment, with the aim of getting closer to identifying the factor(s) which parasites are sensitive to.

Methods

Hosts and parasites

The rodent malaria parasite *P. chabaudi*, genotypes AJ and ER [53] were used. These wild-type clonal genotypes were originally isolated from areas where mixed infections were frequent [54]. Male MF1 mice, between ten and 12 weeks of age (in-house supplier, The University of Edinburgh), were kept in groups of two to five under a 12-hour light/dark cycle, at 21°C and provided *ad*

libitum with food and water containing 0.05% para-aminobenzoic acid (PABA); a growth factor for parasites. Dynamics of the *P. chabaudi* AJ infections were monitored when exposed to treatments consisting of material derived from self, non-self (genotype ER), and RBCs (detailed below and in Table 1). AJ was chosen as the focal genotype, because it has been shown to respond to competition from unrelated strains with large changes in gametocyte investment and sex ratio [16,25]. All procedures were carried out in accordance with the UK Home Office regulations (Animals Scientific Procedures Act 1986) and approved by the ethical review panel at The University of Edinburgh.

Cue treatments

The experiment consisted of five treatment groups that received different cues injected into hosts (Table 1). The cue treatments, and the acronyms they are hereafter referred to as, are: (i) unmanipulated control, 'C'; (ii) uninfected whole RBCs control, 'U'; (iii) uninfected lysed RBCs, 'UL'; (iv) AJ-infected lysed RBCs, 'AJ'; and, (v) ER-infected lysed RBCs, 'ER'. Note that these cues do not include the administration of additional live self (AJ) or competing (ER) parasites, nor do they directly affect the amount of RBC resources available to the focal AJ parasites. This avoids the potential problem of incorrectly interpreting a change in gametocyte investment or sex ratio as a parasite strategy when, for example, competition limits the availability of RBCs for gametocyte development, or induces immunity that increases gametocyte mortality.

The use of lysed *P. chabaudi* infected RBCs was inspired by recent demonstrations that asexual stages contain products that are packaged into 'exosomes' or 'microvesicles' to stimulate sexual differentiation in recipient parasites [55,56]. AJ infected RBCs (AJ) and ER infected RBCs (ER) were chosen to examine whether parasite products can be used to discriminate kin from non-kin (i.e., determine the presence of a con-specific genotype) in mixed infections, as suggested by previous experiments [16,25,30,57]. It is also possible that the high concentration of parasitized material in the AJ and ER cues mimicked a high density infection or high parasite mortality. Lysed, uninfected RBCs (UL) were intended to act as a control for the lysed, parasitized material, to distinguish whether any responses to the AJ and ER cues were due to parasite products or the lysed RBCs themselves. It is also possible that the administration of lysed uninfected RBCs mimics anaemia because many uninfected RBCs are lysed during an infection and gametocyte investment and sex ratio correlate with RBC resource availability [9,18,23]. Cells (RBCs and parasites) and the serum of the blood they were collected in were present in the cues. This was to maximize the chance that the cue material contained all potentially relevant factors, for example molecules released from inside cells, membrane components, or immune factors in the plasma.

To prepare the cue material, eight mice were infected via intraperitoneal (IP) injection with 1×10^6 AJ parasitized RBCs, and eight separate mice with 1×10^6 ER parasitized RBCs; both passaged from donor mice. When these infections reached their peak densities (on day 7 or 8 post infection (PI)), blood (infected with parasites at ring and trophozoite stages) was extracted from anaesthetized mice via cardiac puncture. Total blood volume, RBC density and parasite density were recorded for each mouse. The AJ and ER infected blood was pooled separately. The density of parasites in the pooled blood for each strain was similar; for AJ this was 1.61×10^9 parasitized RBCs/ml of cue and for ER-infected blood this was 1.31×10^9 parasitized RBCs/ml of cue. RBC densities were also similar, with an average RBC density for the AJ cue of 5.14×10^9 RBCs/ml blood and 4.77×10^9 RBCs/ml blood for the ER cue. Blood from naïve mice was collected for the UL cue. The RBC density for blood from naïve mice was much higher (9.06×10^9 RBCs/ml blood) than for the AJ- and ER-infected mice. Therefore, to ensure RBC density was consistent across all cues, the blood for the UL cue was diluted with serum from uninfected mice, to give a final RBC density of 4.53×10^9 RBCs/ml blood. For each of the cue treatment groups requiring lysed material (AJ, ER, UL) the cues went through four cycles of freeze–thaw, to ensure lysis of RBC and parasite membranes. Lysed cues were confirmed not to contain any live parasites capable of initiating an infection prior to the

Table 1 Summary of cue treatment groups, sample sizes, rationales, and classifications

Cue treatment	N	Rationale	Classification		
			Treatment	Lysed parasites	Lysed RBC
Control	5	No-treatment control for the stress of handling and injections	C	NP	NL
Uninfected RBC	5	Control for the stress of handling and injecting the host with blood	U	NP	NL
Uninfected lysed RBC	10	To test for a response to RBC debris	UL	NP	L
AJ-infected lysed RBC	10	Compare AJ to UL to test for a response to high density of self	AJ	P	L
ER-infected lysed RBC	10	Compare ER to AJ to test for a response to non-self	ER	P	L

The analysis involved comparing individual cue treatments and comparing treatments grouped in different ways to test whether parasites respond to lysed parasite material (P vs NP) and/or to lysed RBC material (L vs NL) . N = number of mice that received a particular treatment.

experiment, as follows. Three naïve mice each received 2 × 100 μl IP injections of the AJ cue with a four-hour gap between injections. PCR analysis of blood DNA samples [58] taken from the three mice confirmed that no parasite material was present in the blood 48 hours after injection of the cue and no infections appeared over the subsequent two weeks. Finally, for the U cue treatment group, blood was obtained via cardiac puncture from a naïve mouse immediately before it was injected as a cue.

On treatment days, 2 × 100 μl of cue material was administered to hosts via IP injection, with a four-hour gap between the injections. For the AJ cue, each host received a total of 1.03×10^9 lysed RBCs, of which 3.21×10^8 were parasitized. For the ER cue, each host received a total of 9.53×10^8 RBCs, of which 2.62×10^8 were parasitized. The lysed parasite material that was administered in both the AJ and ER cues was at least at the density that is typically observed at the peak of live AJ infections (assuming some cue material is cleared by innate immune factors before reaching the bloodstream). For example, the mean parasite density at the peak of infection for the control group, in cohort 2, of this experiment was 5.95×10^7 parasites/ml blood. The cue administration regime (2 × 100 μl IP injections), with a four-hour gap between injections was chosen from pilot studies because it results in parasite material being detectable (by PCR) in the blood from 20 minutes and up to 24 hours post administration of the first cue; ensuring that cues are present in the bloodstream during the ring and trophozoite stages of the asexual cycle. Exposing a large proportion of the asexual cycle to cue treatments was necessary, because it is not known which stage is responsible for detecting the environmental signals that influence gametocyte investment and sex ratio decisions.

Experimental design

Two cohorts, each containing 40 mice, were used to compare the effect of the cues administered during the pre-peak phase (day 4 PI; cohort 1) and post-peak phase (day 10 PI; cohort 2) of AJ focal infections (Table 1). Whilst transmission can occur throughout *P. chabaudi* infections, these time-points were chosen specifically because previous studies have revealed that this is when the largest effects of mixed-genotype infections on gametocyte investment and sex ratio have been observed [16,25]. On day 0, all mice were infected with 1×10^6 AJ parasitized RBCs via IP injection, and mice were randomly allocated to the cohorts and cue treatment groups. Gametocyte density and sex ratio were examined on the days of cue administration to verify that there was no significant variation across treatment groups that could confound the detection of parasite responses. For *P. chabaudi*, it is thought that committed parasites differentiate into gametocytes in the cycle following the detection of a cue, that gametocytes require approximately 48 hours to reach maturity, and gametocytes remain infectious for a further 24 hours [33]. Therefore, to cover the period over which the focal AJ parasites could detect cues, adjust their reproductive strategies in response, and for the resulting gametocyte investment and sex ratios phenotypes to be detected, infections were monitored over the three days (i.e., three asexual cycles) following cue administration. To check whether aspects of the in-host environment (known to influence reproductive strategies, which could confound parasite responses to the cues given) varied across the treatment groups, the densities of RBCs, asexual stages and the proportion of RBCs that were reticulocytes were also monitored for three days post cue administration. The experiment was designed so that the responses to all cues could be compared to each other, and so that some cues could be combined to test for general responses to lysed parasites and/or lysed RBCs by grouping cue treatments into those containing parasite material ('P') or not ('NP'), and those containing lysed RBC material ('L') or not ('NL'), (Table 1).

Data collection and analysis

Blood samples (taken from tail snips) were collected for thin smears (to count reticulocyte proportion), to measure RBC densities (using flow cytometry, Beckmann Coulter counter), and for DNA and RNA to quantify parasites, gametocytes and sex ratios. Samples were collected daily, from day 2 to day 15 PI for both cohorts, but analyses were restricted to day 4 to day 7 PI for cohort 1, and day 10 to day 14 PI for cohort 2. Mouse weight was monitored every other day for both cohorts. All samples were obtained in the morning when parasites were at ring stage, before DNA replication for the production of daughter progeny had occurred. The density of reticulocytes was calculated from examination of blood smears and coulter count readings. DNA and RNA were extracted from blood samples using the ABI Prism 6100 Nucleic Acid PrepStation and the Bloodprep chemistry (for DNA, Life Technologies) or total RNA chemistry system (RNA, LifeTechnologies) as described in [58]. cDNA was generated from RNA and quantitative PCR was used to quantify DNA or cDNA, according to the protocols outlined in [58]. Real-time PCR was performed a) on DNA using CG2 primer pairs [30] to quantify asexual parasites, b) on cDNA using CG2 primer pairs to quantify total gametocytes, and, c) on cDNA using MG8 primer pairs to quantify male gametocytes, according to the protocols outlined in [58]. Sex ratios were calculated by dividing the number of male gametocytes by the total number of gametocytes in any given sample.

Data were analysed using R version 3.0.2 [59]. Response variables were log transformed (gametocyte density) or arcsine square root transformed (sex ratio) to meet the assumptions of normality. ANOVAs were performed to compare RBC densities, reticulocyte densities and asexual densities across cue treatment groups. Comparisons were made on the day of cue administration before cues were given, and for the following three days. The cumulative gametocyte densities for three days post cue administration were used to compare gametocyte investment decisions across treatments. In this case, it was appropriate to use gametocyte density as a measure of gametocyte investment because asexual densities did not vary significantly across the treatment groups before cue administration (see Table 2). This means that any observed differences in gametocyte density must result from different levels of gametocyte investment (i.e., given that all else is equal, variation in gametocyte densities can only result from variation in investment in response to cues). This approach also avoids the difficulties of accurately calculating gametocyte investment [21], especially when the time period between parasites detecting cues and their response being measurable is uncertain. Similarly, for sex ratio, the time between parasites detecting cues and their response being measurable is uncertain, so the mean sex ratio for the three days post cue administration was compared across groups. Finally, Welch's T test was used to compare the effects of parasitized *versus* non-parasitized cues and lysed *versus* non-lysed cues on cumulative gametocyte densities and mean sex ratios for both cohorts 1 and 2. The number of samples analysed varied between tests because (a) some mice died during the experiment, and (b) total and male gametocyte densities below the lower limits of detection for the PCR were excluded, because quantification was unreliable.

Results

Asexual densities and in-host environmental variables

Asexual density, RBC density, and the proportion of RBCs that are reticulocytes all correlate with reproductive decisions and so variation in these parameters across treatment groups could confound any responses to the cue treatments. However, there was no significant variation in these parameters across treatment groups, either before cue administration, or over the subsequent three-day period, for either cohort (Table 2, Additional file 1: Figure S1).

Gametocyte investment

Gametocyte densities were not significantly different between treatment groups either pre peak of infection (cohort 1) or post peak (cohort 2) on the days of cue administration (Figure 1A and Table 2). This result, together with the validation that asexual densities and in-host environmental variables were not significantly different prior to cue administration means that, in this study: gametocyte density is synonymous with gametocyte investment. For the three days following cue administration, there were no significant differences in cumulative gametocyte densities between the five cue treatment groups in either cohort 1 or cohort 2 (Figure 1A and Table 2). When treatments were grouped to compare the effect of cues containing parasitized (P) *versus* non-parasitized (NP) material, there were no significant differences in gametocyte densities in cohort 1 ($t(35.8) = 0.83$, $p = 0.41$) (Figure 1B). However, in cohort 2, gametocyte density was significantly 50% lower in infections that received parasitized cues (378 ± 75 gametocytes/μl blood), compared to those that received non-parasitized cues (753 ± 125 gametocytes/μl blood), ($t(22.9) = -2.19$, $p = 0.04$) (Figure 1B). Finally, when treatments were grouped to compare cues containing lysed (L) or non-lysed (NL) material, there were no significant differences for cohort 1 ($t(12.8) = 0.12$, $p = 0.91$) or cohort 2 ($t(6.6) = -1.47$, $p = 0.19$) (Figure 1C).

Sex ratio

Sex ratios (proportion of male gametocytes; Figure 2A) were not significantly different between cue treatment groups for cohort 1 or cohort 2 on the days of cue administration (Table 2). Therefore, as for gametocyte density, there was no pre-existing significant variation in

Table 2 Summary of ANOVA analyses

	Cohort 1		Cohort 2	
	Prior: day 4	**Post: days 5-7**	**Prior: day 10**	**Post: days 11-13**
Asexual density	$F_{4, 34} = 1.13$, $p = 0.36$	$F_{4, 34} = 0.79$, $p = 0.54$	$F_{4, 25} = 0.59$, $p = 0.68$	$F_{4, 24} = 0.14$, $p = 0.97$
RBC density	$F_{4, 34} = 1.00$, $p = 0.42$	$F_{4, 34} = 1.70$, $p = 0.17$	$F_{4, 28} = 1.62$, $p = 0.20$	$F_{4, 24} = 0.45$, $p = 0.77$
Reticulocyte proportion	$F_{4, 34} = 1.05$, $p = 0.40$	$F_{4, 34} = 0.32$, $p = 0.86$	$F_{4, 28} = 0.77$, $p = 0.56$	$F_{4, 24} = 1.53$, $p = 0.23$
Gametocyte density	$F_{4, 34} = 0.17$, $p = 0.95$	$F_{4, 34} = 0.39$, $p = 0.81$	$F_{4, 28} = 1.60$, $p = 0.20$	$F_{4, 20} = 1.73$, $p = 0.18$
Sex ratio	$F_{4, 31} = 1.27$, $p = 0.30$	$F_{4, 34} = 0.60$, $p = 0.67$	$F_{4, 28} = 0.63$, $p = 0.64$	$F_{4, 26} = 0.22$, $p = 0.93$

Asexual density and the in-host environmental parameters of RBC density and proportion of reticulocytes did not vary significantly across the treatment groups - either prior to, or post cue administration, in either cohort. Furthermore, gametocyte density and sex ratio did not vary significantly prior to cue administration. This means that the effects of the cue treatments were not confounded by unintended variation in the in-host environment or pre-existing variation in gametocyte density and sex ratio (see also Additional file 1: Figure S1).

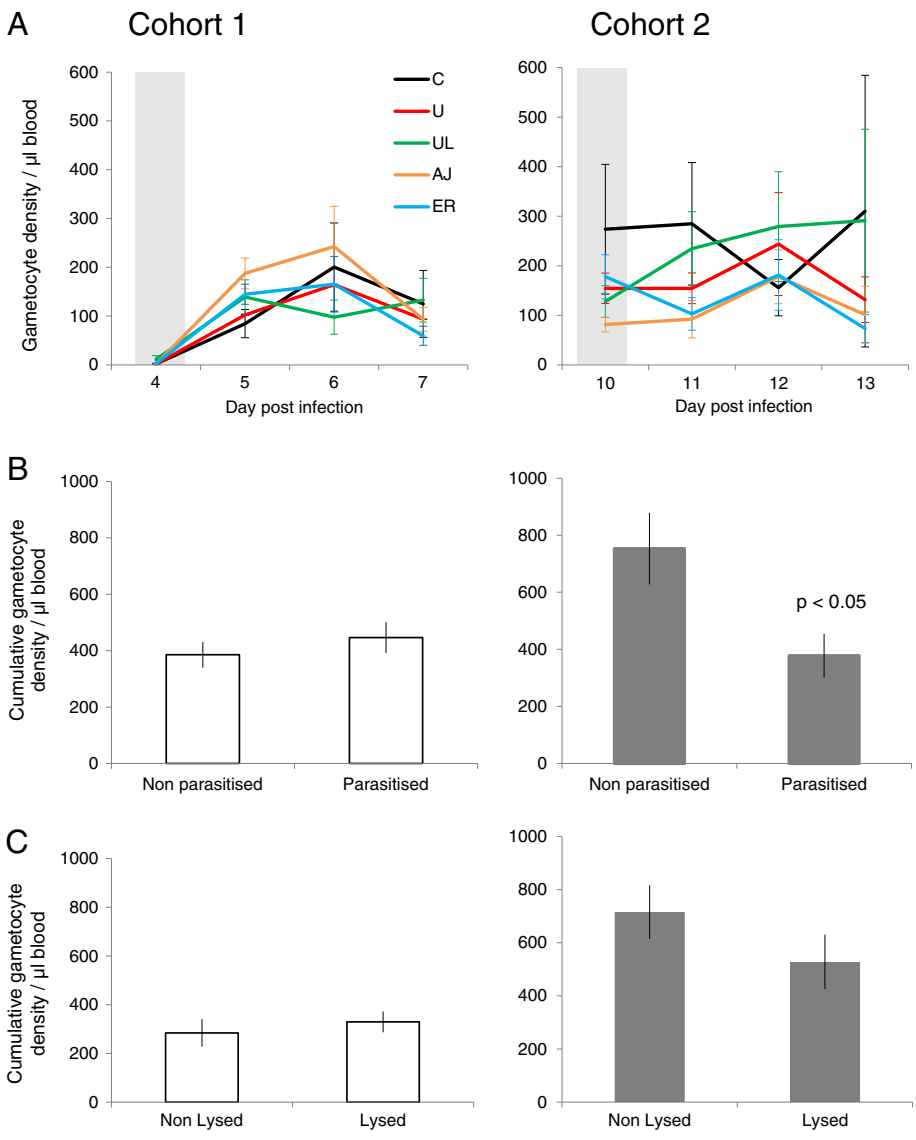

Figure 1 ***Plasmodium chabaudi*** **AJ gametocyte density dynamics.** (± SEM) from the day of administration of five cue treatments: C: control, U: uninfected RBCs, UL: uninfected lysed RBCs, AJ: AJ infected lysed RBCs and ER: ER infected lysed RBCs). Grey bars indicate the days when cues were administered - on day 4 PI for cohort 1 (left) and day 10 PI for cohort 2 (right) **(A)**; cumulative gametocyte densities (± SEM) for three days post treatment with cues containing parasitized material (P: AJ, ER) or non-parasitized material (NP: C, U, UL) for cohort 1 (left) and for cohort 2 (right: where gametocyte density was significantly lower in the P group than NP group) **(B)**; cumulative gametocyte densities (± SEM) for three days post treatment with either lysed RBC material (L: UL, AJ, ER) or non-lysed material (NL: C, U) for cohort 1 (left) and cohort 2 (right) **(C)**.

sex ratios that could have confounded any changes in sex ratio following the cue treatments. For the three days following cue administration there were no significant differences in mean sex ratios between the five treatment groups in cohort 1 or cohort 2 (Figure 2A and Table 2). When cue treatments were grouped to compare the effect of parasitized (P) *versus* non-parasitized (NP) material, there were no significant differences in mean sex ratio in cohort 1 ($t(36.7) = 0.66$, $p = 0.51$), or in cohort 2 ($t(27.8) = -0.35$, $p = 0.73$) (Figure 2B). However, when treatments were grouped to compare the effects of cues containing lysed (L) or non-lysed (NL) material, there was a borderline significant increase in sex ratio (of 45%) in infections that received lysed material (0.11 ± 0.02), compared to those that received non-lysed cues (0.06 ± 0.01) in cohort 1 ($t(27.0) = 2.04$, $p = 0.05$), but not in cohort 2 ($t(9.87) = -0.13$, $p = 0.90$) (Figure 2C).

Discussion

The experiments presented here reveal that: (i) gametocyte investment is reduced by 50% in response to lysed material containing parasites (P) compared to material

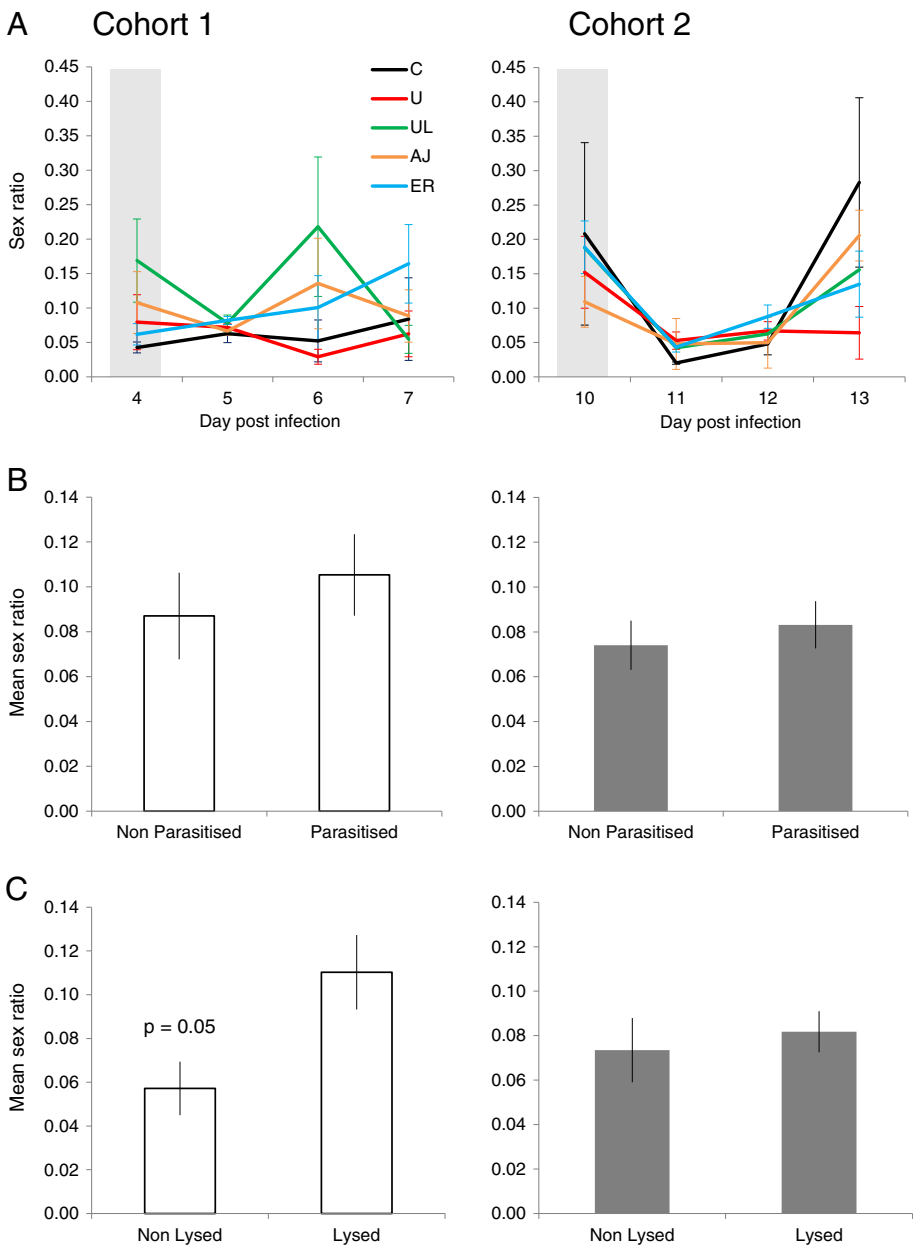

Figure 2 ***Plasmodium chabaudi*** **AJ sex ratio (proportion of male gametocytes) dynamics.** (± SEM) from the day of administration of five cue treatments: C: control, U: uninfected RBCs, UL: uninfected lysed RBCs, AJ: AJ-infected lysed RBCs and ER: ER-infected lysed RBCs. Grey bars indicate the days when cues were administered - on day 4 PI for cohort 1 (left) and day 10 PI for cohort 2 (right) **(A)**; mean sex ratio (± SEM) for three days post treatment with cues containing parasitized material (P: AJ, ER) or non-parasitized material (NP: C, U, UL) for cohort 1 (left) and cohort 2 (right) **(B)**; mean sex ratio (± SEM) for three days post treatment with either lysed RBC material (L: UL, AJ, ER) or non-lysed material (NL: C, U) for cohort 1 (left: where sex ratio was significantly (borderline) higher in the lysed group), and for cohort 2 (right) **(C)**.

without parasites (NP); (ii) the change in gametocyte investment in response to parasitized material occurs post peak of infections, but not during the growth phase; (iii) there was a borderline significant increase (45%) in the proportion of male gametocytes in infections given lysed (L) compared to non-lysed (NL) material; and, (iv) the potential sex ratio adjustment in response to lysed material only occurred in the growth phase of infections. The following paragraphs discuss how these results compare to studies of human and rodent infections that report changes in sex ratio and gametocyte investment in response to variation in RBC resource availability, drugs, competition, and parasite density [11-16,18,23-25,29,31-34].

In the post-peak phase of infections, why do parasites make different gametocyte investment decisions when

exposed to material derived from non-parasitized (NP) blood compared to parasitized blood (P, Figure 1B)? Gametocyte investment is lower in the P group compared to NP group which suggests that either the parasites in the P group are adopting reproductive restraint (i.e., actively reducing investment) or the parasites in the NP group are making a terminal investment (i.e., actively increasing investment). The former scenario is the most likely for the following reasons. When parasites are faced with adverse, but not lethal, circumstances either due to resource limitation or death rates that do not exceed the capacity for replication, they are predicted to adopt a strategy of reproductive restraint [19,21,39]. Lysed parasite material in the P group could signal that many parasites are being killed (e.g., due to immune attack or drugs) and reproductive restraint enables the replication rate to exceed the death rate. The ability to predict future scenarios may seem highly sophisticated for parasites, but this is one of the main evolutionary drivers of adaptive phenotypic plasticity [60,61]. Preparing for environmental change in advance avoids fitness costs incurred by delays involved in waiting for the environment to change and then reacting, or not reacting to environmental change at all [62]. Second, the gametocyte investment of parasites in the NP group appears too low to be explained by terminal investment. This is because the NP group includes the unmanipulated control group and most studies use such infections as a baseline to demonstrate that increased investment (i.e., terminal investment) occurs in response to drugs. In summary, gametocyte investment appears to be reduced in response to material from parasitized blood, which is consistent with parasites adopting reproductive restraint to maximize survival during stressful, but not lethal, challenges during infections [14,16,30].

Instead of parasites actively adjusting gametocyte investment, could differential immune responses in the P and NP groups explain the observed differences in gametocyte investment? It is possible that the administration of lysed parasitized material induced the host to produce the pro-inflammatory cytokines interferon gamma (IFN-gamma) and tumour necrosis factor (TNF), which are known to be involved in killing gametocytes [63-65]. However, data from *in vitro* studies suggest this would be unlikely, as the induction of TNF and IFN-gamma is much reduced when exposed to lysed parasitized RBCs, compared with exposure to live intact parasitized RBCs [66-69]. Furthermore, the induction of TNF by lysed parasites in culture is negligible when the parasitized erythrocytes were harvested and lysed at ring and/or trophozoite stages (compared to lysis at schizont stage) [68]. As such, the P group (a lysed mixture of ring and trophozoite infected erythrocytes) is unlikely to have induced TNF to a level that was sufficient to clear gametocytes. Furthermore, the gametocytocidal activity of TNF is rapid [63] and would, therefore, have produced a sharp drop in the P group on day 11 only, which was not observed. Finally, the cue treatments were the same in cohort 1 and 2 and so should elicit the same immune responses. If these responses killed gametocytes then fewer gametocytes would have been observed in the P group of cohort 1 as well, but this was not the case.

The question of why parasites only adopted reproductive restraint in response to parasite material in the post-peak phase (i.e., in cohort 2) of infections requires further work. This timing is consistent with previous studies showing that the difference in gametocyte investment between parasites in control and sub-lethal conditions increases over time [14,16]. Furthermore, the timing suggests a biologically significant difference in phenotype with real epidemiological relevance, as it is at this later stage of *P. chabaudi* infections where transmission is typically most successful in laboratory studies [70]. Furthermore, a twofold reduction in gametocyte density in *P. falciparum* infections can have a significant impact on the proportion of mosquitoes infected [71]. The lack of any effect in the pre peak phase of the infection may be due to the difficulty in detecting small effects at low parasite densities (as is the case early in infections), or because parasites become increasingly able to detect, or respond to, environmental changes as infections progress. The latter is perhaps the most parsimonious explanation because cumulative gametocyte densities are very similar between all of cohort 1 and the P group of cohort 2 (Figure 1B; $(t\ (41.5) = -1.02,\ p = 0.31)$. This may reflect a necessity to maintain a baseline level of gametocyte production to ensure no transmission opportunity is wasted, even during reproductive restraint.

Why might parasites make different sex ratio decisions when exposed to material derived from lysed cells (L; parasites and RBCs), and why is this only observed in the growth phase of infections? Further work is required to confirm whether parasites do produce a less female-biased sex ratio when exposed to lysed cues (because significance was borderline), but this pattern is predicted by evolutionary theory and consistent with other data [25,46,72]. Lysed material could either represent host anaemia, or the material could have stimulated innate host immune responses that reduce the fertility of males more than females. In these situations, males become a limiting resource for fertilization and so parasites are predicted to partially compensate by increasing their investment in male relative to female gametocytes [25,46,47,72-74]. That extra males are required to ensure females are fertilized when transmission blocking immune factors have more severe effects on males is intuitive, but why are more males required when hosts are anaemic? Each male gametocyte can produce up to eight gametes, but each female only produces one gamete, which means that the number of parasite progeny is maximized at a ratio of eight female

gametocytes to one male gametocyte [6,44]. However, when there are eight-fold fewer male gametocytes circulating in the host and gametocyte density is very low, or hosts are anaemic, there is a stochastic risk that blood meals do not contain enough males to ensure the females are fertilized [46,72]. Therefore, if lysed material represents anaemia and/or immune factors, parasites will be most sensitive to these scenarios when gametocyte density is low (i.e., in cohort 1; Figure 1C). In summary, similarly to the gametocyte investment results, the sex ratio data suggest lysed cell material (parasitized and non-parasitized) is interpreted as a cue for adverse conditions.

Based on previous observations of mixed genotype infections and evolutionary theory [6,15-17,25,39,44,75], parasites were predicted to adopt different reproductive strategies when exposed to cue material derived from self (AJ) *versus* a non-self, con-specific genotype (ER). However, there were no significant differences either in gametocyte investment (Figure 1A) or sex ratio (Figure 2A) when parasites were exposed to AJ *versus* ER cue material, in either cohort. This could be due to a number of (non-mutually exclusive) reasons. First, there may not have been a high enough concentration of lysed ER parasite material in the bloodstream in the ER group for live AJ parasites to discriminate kin from non-kin. Alternatively, the cue to discriminate kin may be something that is only actively secreted by live parasites in direct response to competitors (which were not present in the cue-generating infections), or degraded in the freeze-thaw process. For example, malaria parasites could employ a similar quorum-sensing strategy to that observed in bacteria [76,77] and use microvesicles [56] or exosome-like vesicles [55] derived from infected RBCs as a carrier for the cue. However, microvesicle or exosome structures may have been destroyed during cue preparation lysis. The cue treatments were designed simply to test whether parasite responses could be elicited, rather than to identify precisely what they are detecting, so it is possible that the live AJ parasites could discriminate kin, but the AJ and ER cues also represented other scenarios (e.g., a high death rate), that provided a stronger stimulus and resulted in the responses detected.

Conclusions

Despite decades of investigating gametocytes, how the genes and molecular pathways underpinning commitment to gametocytes and sexual differentiation interact with environmental sensing has proved elusive [2,3,78], although recent characterization of the ApiAP2 gene in *P. falciparum* [UniProt:PFL1085w/PF3D7_1222600] and *Plasmodium berghei* [PlasmoDB: PBANKA_143750] is promising [79,80]. The difficulty may be partly due to different genes and pathways being involved in: (a) sensing environmental cues relevant to decisions about reproductive strategies; (b) processing information and making decisions; and, (c) producing the gametocyte investment and sex ratio phenotypes resulting from the decisions made [21]. Breaking down treatments to isolate the molecule(s) used as a cue(s) within the morass of lysed cells and serum used in this study could facilitate further characterization of molecular mechanisms underpinning commitment and differentiation into gametocytes. Repeating the experiments presented here *in vitro,* to expose synchronous parasites at different time points within the cell cycle could reveal which developmental stages are responsible for sensing and responding to changes in the in-host environment. More broadly, it may be possible to harness cues to 'trick' parasites in an infection into producing gametocytes instead of asexuals, or only producing gametocytes of a single sex [21,81]. The former strategy could be useful for treating returned travellers in hospital (without malaria vectors) because the virulence of infections will be reduced, and the latter strategy would prevent fertilization and subsequent transmission. Finally, precisely identifying the cues that parasites use to make reproductive decisions is required to quantify the costs and benefits (fitness consequences) of their strategies, which is central to understanding their evolution.

Additional file

Additional file 1: Figure S1. *Plasmodium chabaudi* AJ infection dynamics: mean (± SEM) for each cue treatment (C: control, U: uninfected RBCs, UL: uninfected lysed RBCs, AJ: AJ-infected lysed RBCs and ER: ER-infected lysed RBCs) administered on day 4 PI for cohort 1 (left) and day 10 PI for cohort 2 (right) (indicated by grey bars). RBC density dynamics (A); proportion of RBCs that are reticulocytes (B) and asexual density dynamics (C). Maximum values for the Y axes differ between cohort 1 and cohort 2 to allow clear visualization of the range of data for each cue treatment group.

Abbreviations

RBC: Red blood cell; TBI: Transmission blocking immunity; PI: Post infection; IP: Intraperitoneal; C: Control cue treatment; U: Uninfected whole RBC cue treatment; UL: Uninfected lysed RBC cue treatment; AJ: Lysed AJ-infected cue treatment; ER: Lysed ER-infected cue treatment; P: Parasitized material group; NP: Non-parasitized material group; L: Lysed material group; NL: Non-lysed material group.

Competing interests

The authors have declared that they have no competing interests.

Authors' contributions

LMC and SER designed the experiments, LMC and PS performed the experiments, LMC and SER analysed the data and all authors contributed to the manuscript. All authors read and approved the final manuscript.

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High-speed holographic microscopy of malaria parasites reveals ambidextrous flagellar waveforms

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Axonemes form the core of eukaryotic flagella and cilia, performing tasks ranging from transporting fluid in developing embryos to the propulsion of sperm. Despite their abundance across the eukaryotic domain, the mechanisms that regulate the beating action of axonemes remain unknown. The flagellar waveforms are 3D in general, but current understanding of how axoneme components interact stems from 2D data; comprehensive measurements of flagellar shape are beyond conventional microscopy. Moreover, current flagellar model systems (e.g., sea urchin, human sperm) contain accessory structures that impose mechanical constraints on movement, obscuring the "native" axoneme behavior. We address both problems by developing a high-speed holographic imaging scheme and applying it to the (male) microgametes of malaria (*Plasmodium*) parasites. These isolated flagella are a unique, mathematically tractable model system for the physics of microswimmers. We reveal the 3D flagellar waveforms of these microorganisms and map the differential shear between microtubules in their axonemes. Furthermore, we overturn claims that chirality in the structure of the axoneme governs the beat pattern [Hirokawa N, et al. (2009) Ann Rev Fluid Mech 41:53–72], because microgametes display a left- or right-handed character on alternate beats. This breaks the link between structural chirality in the axoneme and larger scale symmetry breaking (e.g., in developing embryos), leading us to conclude that accessory structures play a critical role in shaping the flagellar beat.

digital holographic microscopy | low Reynolds number | ciliary and flagellar motion | malaria transmission

Flagella and cilia are ubiquitous across the eukaryotic domain. They perform critical roles such as the propulsion of microorganisms and sperm, sensory detection, and transport of fluids in the brain (1–3). Although the appearance of motile cilia and flagella can vary in different organisms, it is based on an underlying structural motif: a cylinder of nine microtubule doublets that move lengthwise relative to each other under the action of dynein molecules. The peripheral doublets in the axoneme often surround a central pair of singlet microtubules; the whole structure is then referred to as a "9+2" axoneme. Interestingly, motility does not seem to be contingent on the central microtubules. Motile flagella with three, one, or zero central microtubules (4, 5) have been reported. Dynein molecules are distributed along the length of each of the peripheral doublets asymmetrically. Viewed from the axoneme's basal end, the dyneins are permanently anchored to one doublet and face its clockwise neighbor, where they can attach and move longitudinally. This structural chirality has been invoked as the underlying cause of symmetry breaking in developing embryos (6). Certain "nodal" cilia present in the early stages of development have been shown to rotate consistently in the same direction, counterclockwise, viewed from the basal end. The collective effect from many such cilia is unidirectional fluid circulation within the embryo, which has been posited to carry certain signaling molecules to their receptors (7). This symmetry breaking has been shown to have far-reaching consequences. Among other things, it leads to the familiar left-right patterning of organs in the human body (e.g., heart to the left, liver to the right). Although the link between functional cilia and symmetry breaking has been discussed for some time (8, 9), the proposed link between the axoneme's structure and its motion is more speculative. We note that these nodal cilia do not have a central pair of microtubules (they are "9+0" axonemes), but the chirality in the dynein configuration is the same.

Indeed, although the structure and components of axonemes are fairly well known, the way in which these parts interact to produce beating action remains an open question. A range of medical conditions can arise from cilia and flagella malfunctioning. Understanding how beat patterns arise in correctly functioning axonemes is a critical step in understanding these "ciliopathies" (e.g., hydrocephalus, male and female infertility). Recent theoretical investigations into the hydrodynamics of beating flagella and cilia (10–12) have made progress in understanding the mechanical constraints governing these structures, how they interact, and what their optimal configurations might be. Several competing hypotheses have been advanced to describe the operating principles of a single flagellum (13–15), but these theories have only been tested by data from conventional (2D) videomicroscopy.

Another factor complicating analysis of experimental data arises from the choice of specimen, in that most experimental studies have used sperm in their model systems. However, sperm vary in morphology and demonstrate a wide variety of swimming patterns, even among the standard models. For example, human sperm tails beat in a quasiplanar fashion in typical physiological

Significance

Cilia and flagella are ubiquitous in eukaryotes, enabling cells to move and pump fluid. Understanding how flagella beat is critical in understanding medical "ciliopathies" (e.g., hydrocephalus, male and female infertility). We introduce a unique flagellar model system: the male microgametes of the malaria parasite *Plasmodium berghei*. These microorganisms are isolated swimming flagella, unencumbered by large structures such as a cell body (as in *Chlamydomonas* algae). We measure their 3D beating patterns using high-speed holographic microscopy and find a surprising diversity of waveforms. Structural chirality (handedness) in the flagellum is not manifested in the beat patterns. This interrupts the link between the molecular structure of the flagellum and its dynamics, which was believed to break left-right symmetry in developing embryos.

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conditions (2); sea urchin sperm can adopt helical or planar beating patterns depending on external conditions (16), or more complex patterns in proximity to a surface (17); and quail sperm exhibit a self-similar hierarchy of meandering structures (18). The underlying "engine" is a 9+2 axoneme in all cases, but each has a particular set of accessory structures, such as the fibrous sheath found in human sperm (2). These structures alter the shape (and therefore the hydrodynamics) and introduce passive mechanical constraints to the axoneme, complicating modeling efforts. The biflagellated alga *Chlamydomonas reinhardtii* is another model system for eukaryotic flagella, for which a number of mutant strains are available. However, in the case of *C. reinhardtii*, the large cell body (diameter of ∼10 μm) is coupled hydrodynamically to the flagella, influencing their motion (19, 20).

We have overcome these problems by identifying a unique model system to give uncomplicated access to the beat pattern of a 9+2 axoneme. In recent years, proteomic surveys have revealed that the (male) microgametes of the rodent malaria parasite *Plasmodium berghei* may be a suitable candidate (21). In malaria and related Apicomplexan parasites, male and female cells (gametocytes) are taken up into the midgut when an insect vector, typically a mosquito, takes a blood meal from an infected host. In the midgut, gametes are rapidly generated (each male gametocyte produces up to eight microgametes within 10–20 min), and microgametes must find and fertilize female gametes within 30–60 min for the parasites to reach the next stage in their life cycle (22) (*SI Text*). The microgametes are assembled in the cytoplasm of the male cells and have no intraflagellar transport apparatus, such as that found in the alga *C. reinhardtii* (23). In fact, they are structurally simple microorganisms (sketches in Fig. 1 *A*–*C*). Wass et al. (21) record that the microgamete contains just four "compartments": the nucleus, the axoneme, the cell membrane, and the cytoplasm. It therefore represents a type of "sperm" stripped down to a bare minimum of functioning components. Mitochondria, accessory structures, the intraflagellar transport apparatus, and a large accompanying "cell body" are all absent, making it an excellent limiting-case model system for understanding the axoneme. This is of particular interest in light of recent experiments where beating action was obtained from "artificial axonemes" composed of a small set of components, either robotic (24) or those containing just three key ingredients (microtubules, motors, and cross-linkers) plus an energy source (3, 25). The microgametes have a basal body composed of nine microtubule singlets (26), but unlike most sperm, there is no clearly defined "head" structure. The ultrastructure of the microgametes uncovered by cryo-EM studies (27) shows nuclear material distributed along the axoneme over a length of 1–2 μm. This reduces the effective cross-section of the microgamete, which may be an adaptation to facilitate easy movement between tightly packed RBCs in the mosquito midgut (*SI Text*).

To identify the mechanical processes underlying the beat pattern and the resulting large-scale swimming dynamics, accurate data on the shape and motion of the flagellum are required. Unfortunately, the beat frequency (typically 10–100 Hz) and the 3D nature of the waveform have proved too challenging for conventional approaches. Digital holographic microscopy (28,

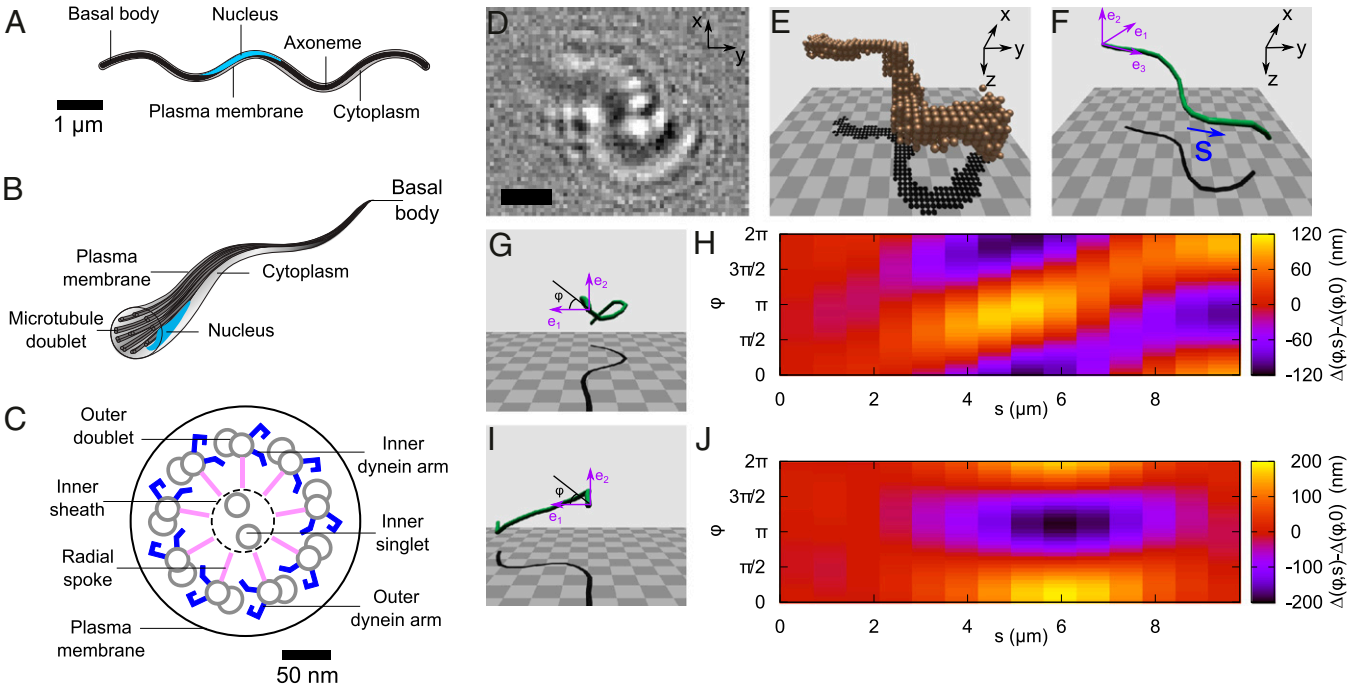

Fig. 1. Longitudinal (*A*) and angled (*B*) cross-sectional illustrations show the simplicity of a typical *P. berghei* microgamete. The sketches are based on electron micrographs in studies by Straschil et al. (27) and Sinden et al. (26), and are labeled to show the key features of the flagellum. (*C*) Cross-sectional schematic diagram of a microgamete detailing the elements common to a typical 9+2 axoneme. The flagellar waveform is driven by microtubule doublets, which, in turn, are driven by the shearing force generated by the dynein arms. (*D*) Raw holographic data of a *P. berghei* microgamete. (Scale bar = 3 μm.) (*E*) Reconstructed volume pixels (voxels), derived from data in *D*, encompassing the volume occupied by the microgamete. The *z* axis is the illumination direction. (*F*) Segmented contour fitted to the voxel data in *E*. The contour length *s* increases from tail to head, and the purple axes show the material reference frame, with $\mathbf{e}_3$ lying along the gamete center line, in each segment. (*G*) Quasihelical waveform. At each joint, the material reference frame rotates about a line in the $\mathbf{e}_1\mathbf{e}_2$ plane, at an angle φ to the $\mathbf{e}_1$ axis. The $\mathbf{e}_3$ vector then points along the next segment. The material reference frame is thus fixed to the underlying structure of the gamete (the microtubules), which allows us to extract the differential shear at each joint. (*H*) Differential shear map corresponds to the waveform in *G*. In the absence of twist, the microtubules would lie parallel to the horizontal axis, at an unknown φ offset. The diagonal feature indicates that a wave of sliding has passed circumferentially around the gamete. (*I*) Example of a quasiplanar waveform. (*J*) Differential shear in the flagellum (analyzed in the same way as for *H* corresponding to the waveform in *I*).

29) allows 3D imaging at frame rates limited by the imaging device, which is a complementary metal oxide semiconductor (CMOS) camera in our case. As a consequence of their "minimal" construction, the microgametes are relatively small objects, ~10 μm in length (L) and 200 nm in diameter (a) (27). Their refractive index is close to that of their surroundings, which places them within the Rayleigh–Gans (weak) scattering regime. In this regime, extended objects may be modeled as a superposition of scattering centers lying within the object's volume (30). We used the Rayleigh–Sommerfeld back-propagation method to reconstruct the optical field away from the focal plane (31) and the Gouy phase anomaly method (32) to localize the microgamete in three dimensions (details are provided in *Materials and Methods* and *SI Text*). Unlike previous holographic studies that have tracked the average positions of microorganisms in three dimensions (28, 29, 33), our approach allows us to measure the position and configuration of the subjects so as to study the swimming strokes in detail.

The goal of this study was to measure and analyze the 3D dynamics of the model flagellar microswimmer *P. berghei*. We have developed a high-speed holographic microscope that we use to characterize swimming behavior. Using this instrument, we map the differential shear between microtubules in the flagellum in planar and helical waveforms. We also measure dynamic quantities, such as the beat frequency, beat wavelength, and wave speed, and overturn the hypothesis that chirality in the axoneme structure results in chiral flagellar waveforms.

Results and Discussion

Differential Shear Displacement. By examining the instantaneous 3D geometry of a flagellar waveform, we measure the local differential shear displacement (2) between opposite sides of the axoneme, which can be used to estimate the underlying pattern of microtubule sliding. Fig. 1 *D–F* shows the results of reconstruction based on a single frame of raw data (Fig. 1*D*). A volume of interest (VOI) is extracted from the reconstructed optical field (Fig. 1*E*), and a contour is fitted through the center of mass of the VOI (Fig. 1*F*). This contour takes the form of joints (j) connected by segments ($\mathbf{T}_j$) of a constant length $\Delta s = 0.7$ μm (more details and an error analysis are provided in *SI Text*). To infer the relative sliding of the underlying microtubules, we need to make some assumptions about the axoneme. First, we make a typical assumption that the energetic cost of twisting a straightened axoneme about its length is much higher than the cost of bending the same axoneme (11, 34). Second, we assume that the relative sliding is minimal (zero) at the basal body of the axoneme. We assign a "head" and "tail" to the axoneme and locate the basal body at the "tail", based on observations of the release of microgametes, the swimming direction, and the shape of the microorganism; this is described in further detail in the section on dynamics. We use the variable s to denote position along the fitted contour, ranging from $s = 0$ (passive, "tail") to $s = L$ (active, "head"). Third, we assume that only the lowest wavenumber azimuthal modes are allowed (35). In other words, when the axoneme bends, doublets on the inside of the bend slide forward relative to the centerline and those on the outside slide backward; the others vary smoothly between these extrema. With these assumptions in place, we define a material reference frame (36) that maps to the underlying microtubules, specified by the unit vectors ($\mathbf{e}_1$, $\mathbf{e}_2$, $\mathbf{e}_3$) (Fig. 1*F*). To infer structural deformations, we examine how this material frame is transformed as we pass along the contour, from $s = 0 \rightarrow L$. At each joint, the material frame is rotated about a vector located in the $\mathbf{e}_1\mathbf{e}_2$ plane; this vector is oriented at an angle φ to the $\mathbf{e}_1$ axis (Fig. 1 *G* and *I*). The material reference frame is rotated about this line, through an angle θ, so that after the rotation, the $\mathbf{e}_3$ axis points along the next segment of the contour.

Given this 3D representation of the microgamete, we can map the differential shear (2, 37), $\Delta(s,\varphi) - \Delta(0,\varphi)$. This quantity describes the relative sliding of microtubules that produces a particular waveform, in the absence of shearing at the basal body [denoted $\Delta(0,\varphi)$] and twist. This highlights the regions where peripheral microtubules would be displaced relative to the centerline, as a function of s and position around the axoneme circumference φ. Fig. 1 *G* and *H* shows a reconstructed frame with a largely helical configuration, with its corresponding pattern of differential shear displacement, and Fig. 1 *I* and *J* shows a reconstructed frame with a largely planar configuration, with its corresponding pattern of differential shear displacement. Both of these frames were taken from the same gamete and occurred 2 s apart in a video sequence. This change in the flagellar waveform between two relatively closely spaced times is quite remarkable, given that waveforms are usually classified as either planar or helical. The absence of mechanical accessory structures in this microgamete allows a broad variety of waveforms, showing the versatility of the bare axoneme.

Dynamics. By measuring the geometry of flagellar waveforms with high temporal resolution (500 Hz to 1 kHz), we can examine how flagellar beats initiate and propagate (Movies S1 and S2). As previously observed elsewhere, the microgametes swim in two distinctive modes, fast and slow (38). These swimming modes appear to transport the microgamete in opposite directions, and the forward (or fast) mode was the most prevalent. Fig. 2*A* shows a spatiotemporal map of flagellar curvature during fast beating. Vector manipulation gives the external bending angle between consecutive segments, $\mathbf{T}_j$ and $\mathbf{T}_{j+1}$, as a function of s and time. Waves of curvature clearly propagate from head to tail as the microgamete swims, demonstrated by light-colored bands inclined from the upper right to lower left. Based on the swimming direction of the axoneme, we can infer the position of the basal

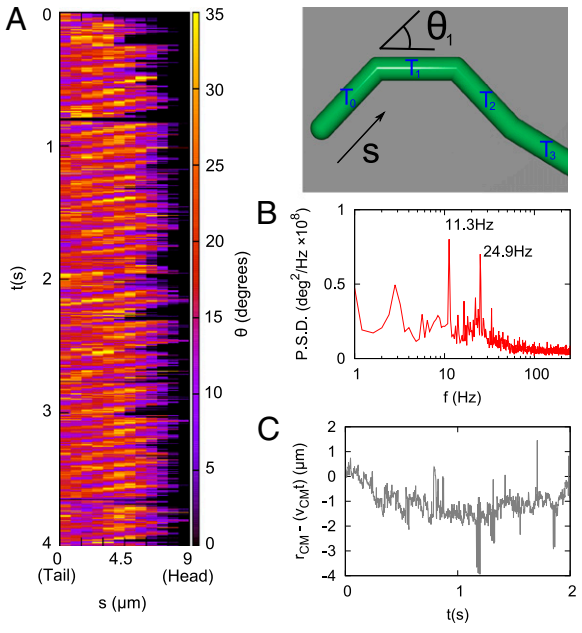

Fig. 2. (*A*) Exterior angle θ (*Left*), as indicated (*Right*), between two adjacent segments as a function of contour length s (micrometers) and time (seconds). The bright bands inclined from the upper right to the lower left show waves of curvature passing along the gamete from the "head" end ($s = L$) to the "tail" end ($s = 0$) of the gamete. (*B*) Power spectrum of curvature fluctuations, with harmonic components as indicated. P.S.D., power spectral density. (*C*) Deviation from linear swimming speed. A straight-line fit was performed to 4 s of center-of-mass displacement data to find a straight line speed of 6.2 μm/s. This graph of residuals ($r_{CM} - v_{CM}t$) shows no evidence of the beat frequency in the center-of-mass displacement.

body: When the microgametes are released (exflagellate) from the gametocyte, they are initially anchored within the cell by the basal body from which they are constructed. As they swim away, the leading "head" end of the microgamete is noticeably more active than the trailing end. More evidence of this is given in the section on average motility parameters, where we find that the basal region shows a smaller average curvature (implying a higher bending stiffness or resistance to sliding).

A curious aspect of fast beating is that the waves of curvature propagate toward the basal body rather than away from it. To our knowledge, this is unique among sperm documented in the literature [although it has been observed in other microorganisms, such as trypanosomes (39)]. This beat pattern may facilitate the microgamete's exploration of a convoluted substrate (e.g., close-packed RBCs) when searching for female gametes in the blood meal (*SI Text* and Fig. S1). It is energetically efficient to explore an environment and look for paths of least resistance to travel along, and a recent investigation into the motility of the parasite *Trypanosoma brucei* (40) has shown that swimming speed can be enhanced by the presence of a microstructured substrate (in that case, a 2D array of micropillars).

Fig. 2*B* shows the power spectrum of these curvature fluctuations (averaged over all s values) with dominant harmonic components at 11.3 Hz and 24.9 Hz. Although the beating is clearly periodic, the displacement of the microgamete's center of mass does not share this periodicity and is fairly constant as a function of time. Fig. 2*C* shows the deviation from a constant swimming speed. A microgamete's center of mass was calculated in each frame over a period of 4 s, yielding a straight-line swimming speed of 6.2 μm/s. The graph shows the deviation of the center-of-mass position from the predicted displacement; no periodic fluctuation of the displacement is apparent.

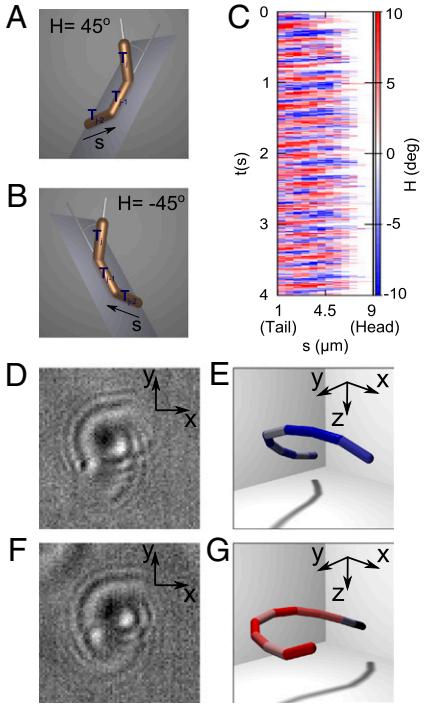

Fig. 3. (*A* and *B*) Demonstration of how we define local chirality using three contiguous segments of the contour. H is the angle between the third segment and the plane of the first two segments. The sign is determined as positive (right-handed) if $\mathbf{T}_j$ lies in the same half-space as the cross-product $\mathbf{T}_{j-2} \wedge \mathbf{T}_{j-1}$ and as negative (left-handed) if not. (*C*) Temporal pattern of local chirality as a function of contour length s and time. The bands correspond to those in Fig. 2*A*, but waves alternate in time between left- and right-handed character. deg, degrees. (*D*) Raw data correspond to a left-handed waveform with holographic reconstruction in *E*. (*F* and *G*) Raw and reconstructed data for a right-handed structure. Note the superficial similarity of *D* and *F*; the fundamental difference in the waveform is revealed only with holographic reconstruction. The photos (*D* and *F*) are 17 μm on a side.

Waveform Chirality. The 3D nature of our data allows us to obtain quantities that are inaccessible to standard 2D microscopy. We define a local chirality, H, as the angle between a segment ($\mathbf{T}_j$) and the plane formed by the two previous segments (defined by $\mathbf{T}_{j-2} \wedge \mathbf{T}_{j-1}$; Fig. 3 *A* and *B*). Fig. 3*C* shows a spatiotemporal map of chirality derived from the same data as Fig. 2. The map shows propagating waves of alternating handedness, indicated by the sequential red and blue bands (Fig. 3*C*); these waves are of the same frequency and phase as the bending waves shown in Fig. 2*A*. The ability to change chirality seems to be a generic feature of microgamete motion because we observed this in every individual in our dataset (n = 24), both in fast- and slow-beating modes. The periodic reversal is somewhat unexpected in light of the axoneme's structural chirality. In contrast to recent theories and experiments suggesting that symmetry breaking in developing embryos occurs because chirality is hard-wired into the axoneme structure (6, 7), we find no evidence of fixed chirality in this mechanically simple axonemal flagellum. We therefore suggest that, in general, mechanical accessory structures are responsible for symmetry breaking. Previous studies of more complex cells, such as sea urchin sperm (17) and trypanosomes (39), have inferred chiral properties from differential interference contrast (DIC) and dark-field images. However, the beat pattern of *P. berghei* microgametes is more complex, with no discernible beat plane and varying amounts of planarity in successive beats. Movie S3 has been arranged to demonstrate this aspect of motility. The contour in Movie S3 has been translated and rotated so that the head and tail points of the microgamete overlap on a straight line pointing away from the observer. Successive beats are seen as prominences that intersect this point, appearing as loops of varying area, according to how helical the particular wave is. Curiously, we find no discernible pattern in the orientation or shape of these waves; although successive waves have opposite chirality, their shapes are dissimilar.

To clarify the notion of handedness in flagellar waveforms further, Fig. 3*D* shows an example of a single frame of data with a left-handed character; segments in the reconstruction in Fig. 3*E* have been colored according to the scheme in Fig. 3*C* to indicate local chirality. Fig. 3 *F* and *G* shows raw and reconstructed data for a purely right-handed waveform. The handedness of the waveform is not easily attainable from the raw data but may be quantified using the holographic reconstruction. The two segments nearest the (x, y) origin in each reconstruction (obscured in Fig. 3*E*) are colored black, because two preceding segments are required to establish chirality.

Average Motility Parameters. The reconstruction of a segmented contour enables unequivocal measurements of motility parameters. In Fig. 4, we show average values of some parameters obtained from our set of 24 individuals, moving in forward (*F*) and reverse (*R*) directions. The frequency, wave speed, and center-of-mass velocity are markedly dissimilar in these different modes (Fig. 4). Interestingly, the characteristic length of curvature fluctuations ("wavelength") is similar in both cases (averaging 5.6 μm in forward and 4.5 μm in reverse), which matches the diameter of murine RBCs (4–7 μm) (41). Again, this is suggestive that microgametes may have evolved an adaptation in which swimming speed is enhanced by friction generated from mechanical interactions between cells and obstacles in the environment (40) (more details are provided in *SI Text*).

Fig. 4*E* shows the average curvature $\langle\theta\rangle$ as a function of contour length. We only show data from forward swimmers because the number of reverse swimmers is smaller (n = 5

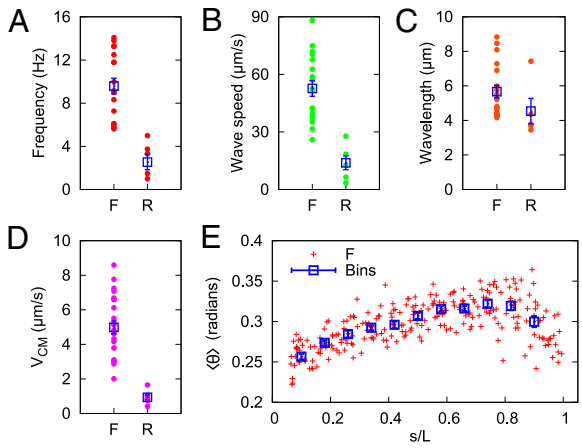

Fig. 4. (*A*) Principle beat frequency component of forward (F) and reverse (R) microgamete waveforms. (*B*) Speed at which waves of curvature propagate along the microgamete (*Materials and Methods*). (*C*) Resulting characteristic wavelength, found by dividing wave speed by frequency. (*D*) Speed of the microgamete's center of mass over a linear trajectory of around 20 μm. (*E*) Average curvature of forward-swimming gametes as a function of the normalized contour length (contour length *s*/microgamete total length *L*). In *A*–*E*, each point represents data from a different microgamete (from five independent infections). The boxes in *A*–*D* represent a mean value (± SEM) of forward and reverse swimming directions. The boxes in *E* represent data binned in increments of $\Delta s/L = 0.08$ (±SEM).

compared with $n = 19$ for the forward swimmers). The smaller sample size and lower beat frequency lead to inconclusive $\langle\theta\rangle$ data in reverse swimmers. There is a clear head/tail asymmetry, where the average curvature increases toward the "head" ($s = L$) end of the microgamete and is independent of the actual physical length. This adds extra support to our claim that the relative sliding of microtubules is smaller at the trailing end of the gamete (which contains the basal body). The curvature data are normalized by the total microgamete length L because there is considerable variation (14%) in the length of microgametes ($L = 8.4 \pm 1.4$ μm) that cannot be explained by measurement error (estimated at ±100 nm). This may be a result of variation in the number of microtubules available, or accuracy possible, in the short time available for microgamete synthesis.

Conclusions

We have used high-speed digital holographic microscopy to investigate the 3D dynamics of a eukaryotic flagellum. This method allows us to characterize the waveforms and swimming behavior of the microorganism, as well as giving insight into the action of the underlying microtubules. In presenting our results, we also introduce *P. berghei* microgametes as model microswimmers. To our knowledge, they are the simplest naturally occurring example of a swimming axoneme (in mechanical terms at least). As a result, they do not suffer from the shortcomings of other models, for example, sea urchin sperm cells or the alga *C. reinhardtii*, where flagella are attached to a large, hydrodynamically important cell body (19, 20). Furthermore, the microgametes lack mechanical accessory structures that may help to guide the flagellar beat, instead allowing the axoneme to behave in a less constrained fashion. Last, in contrast to prior assertions regarding a closely related structure, we find that the chirality of the 9+2 axoneme structure does not directly transfer to the overall beating motion. Although both left- and right-handed waveforms have been observed in other species, the use of a mechanically unconstrained flagellum demonstrates that there is no inherent bias in chirality coming from the axoneme. This result, along with the general irregularity of the beat pattern, suggests that mechanical accessory structures play crucial but overlooked roles in determining the dynamics of flagella. This has implications not only for analyzing swimming behavior but for understanding the root causes of symmetry breaking in developing embryos.

Materials and Methods

Microgamete Preparation. We used the rodent malaria parasite *P. berghei*, line 820cl1m1cl1 (42). Infections were initiated in male MF1 mice (8–10 wk old), which had been pretreated with phenylhydrazine at 120 mg/kg (2 d before infection) to enhance the production of gametocytes (43). Five independent infections were initiated with 10^7 parasitized RBCs. Infected blood was collected by tail snip when gametocytes reached maturity (day 4 or 5 postinfection). To stimulate the differentiation of gametocytes into microgametes for each sample, 2 μL of infected blood was added to 1 mL of complete ookinete culture media [900ul RPMI + 100ul FCS (pH 8)] and incubated at 21 °C (44). All of the work involving mice was carried according to the Animals (Scientific Procedures) Act, 1986 and approved by Edinburgh University.

Microscope and Optical Setup. Experiments were performed on a Nikon Ti inverted microscope, with a 60× magnification water immersion objective lens, as described elsewhere (32). Illumination was provided by a Thorlabs M660L2 high-power light-emitting diode, with a peak emission wavelength of $\lambda \approx 660$ nm and an FWHM bandwidth of ~40 nm.

Data Acquisition. A Mikrotron MC-1362 monochrome CMOS camera was used to record video images; the camera was connected to a frame grabber card with 1 GB of onboard random-access memory. Video data were initially acquired at 1 kHz with an exposure time of 0.994 ms, but this appeared to be a significant oversampling of the motion of the microgametes. In further experiments, we decreased the frame rate to 500 Hz (1.994-ms exposure time), which was entirely sufficient to capture the dynamics of the beating pattern. No distinction was observed between data taken at different frame rates. Imaging a standard reference chart allowed us to calibrate our image sampling frequency (pixel spacing) as 4.29 pixels per micrometer.

Preliminary Image Processing. Video recordings were edited by hand to extract regions of ~30 μm on a side with microgametes in them. An example region is shown in Movie S4. For each reduced video recording, a background frame was obtained by averaging a series of images of the same subregion from a period when the microgamete was not present in the images. Pixel values in frames containing microgametes were then normalized (divided) by their values in the background frames to remove the static background contribution in the image and improve the overall signal-to-noise ratio.

Holographic Reconstruction. From each frame, a stack of reconstructed image planes was generated, spaced $\Delta z = 0.233$ μm apart. This resulted in an image stack with the same sampling frequency (4.29 samples per micrometer) in *x*, *y*, and *z*. An example image stack is shown in Movie S5.

Image Processing. The image stacks were then subjected to several steps of processing. We first applied spatial bandpass filters in the *x*–*y* plane to remove high-frequency pixel noise. We then took the intensity gradient in the *z* direction, $\partial I/\partial z$, as in previous work (32). Next, we performed a Gaussian fit on each column of volume pixels (voxels) in the stack at particular *xy* coordinates [$I_{xy}(z)$] to isolate the point of highest intensity in the *z* direction. This volume containing "bright" pixels, one per *xy* address, was convolved with a 3D Hanning filter. The procedure of fitting and convolution was repeated. The brightest region in the volume was extracted, starting at the voxel with maximum intensity and stepping outward until the intensity dropped to zero. This VOI was then ready for contour fitting.

Contour Fitting. The VOI extracted in the image processing step was reduced and then fitted piecewise with straight line segments. To reduce the size of the VOI, each voxel within it was modeled as the source of a truncated scalar potential, with a radial profile given by

$$V(r) = A\left(1 - \left(\frac{|r|}{r_{max}}\right)^2\right)^2. \quad [1]$$

This potential was evaluated at every point in the stack.

It should be noted that the VOI shown in Fig. 1*E* depicts the locations, but not weights, of the voxels. Voxels near the center of this VOI have a significantly stronger weighting (*A*) than those at the edges. Locations where the potential was greater than a threshold value were set to 1, and those with lower potential were set to zero. The active voxel furthest from the center of mass was used as a starting point, and the VOI was stepped through in increments of $\Delta s = 0.7$ μm, tracing the locus of points that lie inside the VOI, furthest from its surface. This chain of points (not including the starting point) makes up the estimate of the microgamete position in each frame.

Data Analysis. Motility parameters (Fig. 4) were extracted from curvature data similar to the representative set in Fig. 2*A*. To obtain beat frequency, we found curvature as a function of time at each contour joint *j* and took the power spectrum. A single data point in Fig. 4*A* represents the mean power spectra over all *j* values for one microgamete. Wave speed was found by examining the phase of the principal frequency component as a function of *j* for each microgamete. As waves propagate along the flagellum, there is a phase lag between consecutive joints. Thus, the wave speed was calculated from the distance between each joint, the phase at each joint, and the frequency. Wavelength was found by dividing wave speed by frequency. Although the microgamete never truly adopts a sine-wave shape, the wavelength is a valid characteristic length scale.

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Supporting Information

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SI Text

This document serves two purposes. First, it gives the broader context of our measurements, as well as their implications for the mating biology of malaria parasites. We propose how results obtained from biochemical and genetic studies can help to answer long-standing questions in physics by allowing an unprecedented level of control over the structure of the axoneme. Second, given that a new microscopy technique is used in our study, we provide a summary of the method, along with an account of the main sources of error.

Despite over a decade of research since sexual reproduction was discovered as an essential requirement for the transmission of malaria (*Plasmodium*) parasites, important aspects of their reproductive behavior remain unknown (1, 2). Developing drugs and/or vaccines that prevent transmission by disrupting mating are major goals (3), and the microgamete is an attractive target for such interventions. We have accurately measured and quantified several key physical characteristics from more than 800 beat cycles (42,000 video frames at 500 Hz). Here, we explain in biological terms how our results advance understanding of the morphology and motility of microgametes and discuss their implications for the evolution of parasite mating strategies and transmission-blocking interventions.

Sex in *Plasmodium*. To transmit to new vertebrate hosts, malaria parasites must produce specialized sexual stages (gametocytes), which are taken up in the blood meal of the mosquito vector. Gametocytes are produced continuously [but in varying numbers (4)] throughout infections in the vertebrate host and circulate in the blood stream for several days while waiting to be taken up when an insect vector (mosquito) bites the host and takes a blood meal (3, 5). As soon as male and female gametocytes are ingested by a mosquito, they rapidly differentiate into gametes, and the flagellated male gametes (microgametes) must locate and fertilize the nonmotile female gametes within a brief (~30–60 min) time window (6). Although female gametocytes each produce a single gamete, unlike most male organisms, male gametocytes can only produce a maximum of eight microgametes, and it is rare that all of these are viable (2, 7). The mosquito gut is a challenging mating environment because mosquitoes concentrate the blood meal and digestion begins; as soon as parasites leave the relatively protective environment of the RBCs they were living in, they are vulnerable to host immune factors that have also been taken up in the blood meal (8). That fertilization occurs at all, given the low fecundity of male gametocytes, the short window of opportunity, and the hostile environment, is remarkable. Due to the difficulties parasites face during mating, and because fertilization appears to be a significant bottleneck in the parasite life cycle, interventions that target the fertility of microgametes offer the opportunity to stop disease transmission (9–11). However, there is a lack of knowledge about fundamental and diverse aspects of microgamete morphology and behavior. Here, we demonstrate how digital holography can reveal these important characteristics, enabling future experiments (targeting fertility) to be undertaken within a meaningful ecological context.

Morphology. The average length of *Plasmodium berghei* microgametes in our study was 8.4 ± 1.4 μm (SEM, $n = 24$). The large variation in microgamete length could be attributed to a number of factors, not least the potential errors accumulated due to the speed at which the microgametes are assembled and released (2). Variable lengths may also be due to limited availability of resources for producing full-length microtubules at the time of synthesis. The microgametes are 200 nm in diameter and bend into a quasisinusoidal shape with a wavelength around 5 μm. Although microgametes have no defined head, our analysis revealed clear "active" and "passive" ends. The passive tail end is associated with the basal body, where the microgamete detaches from the residual gametocyte (2). This is consistent with other sperm studies, where the basal region is the least active part of the flagellum owing to the increased stiffness of passive accessory structures located there. It is also possible that the passive end exhibits lower flexibility because this is where the microgamete's DNA is located. However, on balance, this seems unlikely; DNA is drawn into the cell through the tail end during the final stages of gametogenesis, but studies have demonstrated that the nucleus is usually distributed along the center of the cell (12–14). We note that previous studies have suggested that 60% of *P. berghei* microgametes are malformed or anucleate, or contain multiple axonemes (2); thus, we have calculated the probability that all the microgametes in our sample were aberrant. Assuming that one-third of the 60% are anucleate, and that it is impossible to distinguish between nucleate and anucleate microgametes visually, the probability of randomly imaging a single anucleate microgamete from a mixed population of swimmers is approximately 33% (anucleate swimmers constitute 20% of the total population, and nucleate swimmers constitute 40% of the total population). It is therefore likely that some (around one-third) of the microgametes in our sample were anucleated. However, we do not find two distinct populations in the data; thus, we conclude either that the presence or absence of nuclear material has little impact on the swimming behavior or that anucleate microgametes are not as common as previously estimated. Finally, we note that it is highly unlikely that all 19 of our forward-swimming microgametes were anucleated; the probability is around one in a billion. The variation in average curvature that we observe is more likely to be due to the presence of a mechanically distinct basal body, or a nonuniform distribution of molecular motors along the length of the flagellum [such as that found in *Chlamydomonas* (15)]. However, further experiments would be necessary to resolve this.

Swimming. In contrast to the conventional direction of sperm motility, the microgamete swims in the direction of the "active end," that is, the end with the higher average curvature. Waves of curvature propagate from the active end to the passive end as the microgamete swims. This is illustrated in Fig. S1. This mode of swimming is analogous to the flagellum "pulling" the cell through the medium, rather than being "pushed" by a greater activity at the tail end. Unlike many other sperm flagella, microgametes do not have a discernible beat plane; beating is complex and irregular.

Speed. Microgametes displayed both fast and slow swimming patterns as previously described (2). The more waves of curvature travel along the microgamete in a given period, the faster it swims. For the majority of the time, active microgametes moved with a fast beat, which we define as "forward swimming" at an average speed of 5.0 ± 0.4 μm/s ($n = 19$) and a mean frequency of 9.6 ± 0.7 Hz. A previous study (from a smaller number of independent infections and microgametes) estimated the same parameters by hand from videos at 16 frames per second, finding a speed of ~9 μm/s and beating at ~6 Hz (1). Although these results are broadly consistent with our findings (and variation may be introduced by differences in sample preparation), our

methods have three important advantages for comparative studies: (*i*) they are completely automated, making estimations substantially less painstaking and less subjective than estimation of parameters by hand; (*ii*) because our data are 3D, and obtained at higher frame rates, out-of-plane motion and motion-blurring artifacts are removed; and (*iii*) because our data are in the form of coordinates that specify microgamete position as a function of time, it is far easier to investigate new motility metrics systematically without recourse to the raw data. Occasionally, microgametes were observed to swim in the opposite direction, which we call "reverse swimming," at a slower speed and lower frequency (up to threefold lower) than the forward, fast-swimming microgametes. However, these measurements are preliminary because they come from a smaller sample of microgametes ($n = 5$, whereas $n = 19$ for forward swimmers).

Discussion. We have characterized the microgamete of the malaria parasite *P. berghei* and made the following observations. Microgametes, on average, are 8.4 ± 1.4 μm long and 200 nm in diameter, and they swim at 5.0 ± 0.4 μm/s, with a mean frequency of 9.6 ± 0.7 Hz. They swim in the direction of the active end, which bends into a quasisinusoidal shape with a wavelength around 5 μm and an irregular beat pattern.

There are several nonmutually exclusive aspects of mating biology that may explain the irregular beat plane of microgametes. For example, they could be affected by immune factors that are also taken up by the mosquito in a blood meal. Even though immune factors are likely to be diluted in our culture conditions, any binding to one part of a microgamete could potentially alter beat plane and affect swimming direction (16). Alternatively, a recent study of trypanosome motility by Heddergott et al. (17) demonstrated that in the absence of any obstacles (similar to the environment of diluted blood in which we imaged microgametes), the reversal of the flagellar beat was random and resulted in slower, irregular waveforms, which could explain the irregular beat pattern that we observe in microgametes. Heddergott et al. (17) also revealed that trypanosome flagella have a wavelength that matches the distance between RBCs in the blood, enabling them to use the friction generated from mechanical interactions with RBCs to swim eightfold faster in blood (up to 40 μm/s) than in a Newtonian fluid (cell culture medium). Interestingly, the wavelength of microgametes (5 μm) matches the average diameter of murine RBCs (4–7 μm) (18), which may represent a similar adaptation to enhance motility using interactions with RBCs in the blood meal.

Swimming in the direction of the active end of the flagellum is rare, if not unique, among sperm. However, in a blood meal environment with tightly packed RBCs, having the active end at the front may enable microgametes to probe the environment more efficiently to find spaces to pass between cells. Furthermore, it is not known how microgametes locate females, but if chemotaxis cues are involved, traveling in the direction of the active end may maximize the likelihood of detecting a chemotaxis gradient. Similarly, the ability to swim in reverse may also be useful for finding pathways between RBCs and/or tracking chemotaxis gradients. There is no evidence for chemotaxis, but our calculations below suggest it is unlikely that microgametes randomly encounter female gametes.

The approximate lifetime of a microgamete is 30 min, and the flagellar beats are randomly oriented with an amplitude (peak to trough) of roughly 5 μm. The swimming speed may increase in a blood meal, but the microgametes swim in viscous-dominated environments (i.e., at a low Reynolds number). This means that microgametes cannot swim faster than the speed at which waves propagate along the flagellum, around 50 μm/s, regardless of whether interactions with RBCs enhance speed. If the microgamete swims at the speed we measure for 30 min (1,800 s), it sweeps out a cylindrical volume measuring

$$V_{gam} \sim \pi \cdot \left(2.5 \times 10^{-6}\right)^2 \cdot 1,800 \cdot \left(5 \times 10^{-6}\right) \sim 2 \times 10^{-13}\mathrm{m}^3.$$

The volume explored will be 10-fold larger if the maximum (50 μm/s) swimming speed is used. Assuming a blood meal size of 2 μL ($2 \times 10^{-9}\mathrm{m}^3$), this equates to between 1/1,000 and 1/10,000 of a blood meal in 30 min. It would take more than 1 mo for the microgamete to explore an entire blood meal at the speed we have measured (5.0 ± 0.4 μm/s), assuming it never retraces its steps. However, blood meals contain multiple gametocytes and assuming a gametocyte density of 10^5 gametocytes per microliter of blood and a sex ratio of 30% males, ∼400,000 microgametes will make it into the blood meal. In this case, it is likely that at least 1 microgamete visits everywhere in the blood meal in 30 min. These calculations are a "best case scenario" estimate based on the gametocyte density of *P. berghei* infections. Gametocyte densities of human malaria (e.g., *Plasmodium falciparum*) in natural infections are variable but generally much lower [e.g., 500 gametocytes per microliter (including males and females)], which translates to only ∼1,800 microgametes in the blood meal. An additional limiting factor here is the ratio of male to female gametocytes present in the blood meal. The gametocyte sex ratio is variable and dependent on numerous environmental factors (19). The resulting tradeoff is between the area of the blood meal that the microgametes can cover (increased when the proportion of males is high) vs. the density of female gametocytes available for the microgametes to locate (decreased when the proportion of males is high). Our estimates also do not account for the negative effects of transmission-blocking immune factors (20) and the high failure rate in the production of viable microgametes (2). Given the increasing appreciation that transmission to mosquitoes occurs readily from submicroscopic gametocyte densities [<5 per microliter (21)], our results suggest that the evolution of mechanisms to facilitate encounters between male and female gametes would be favored by natural selection. These could include (*i*) the use of interactions with RBCs to increase swimming speed, (*ii*) males locating females nonrandomly by a mechanism such as chemotaxis or nanotubes (22), and (*iii*) gametocyte aggregation in the circulation of the vertebrate host maximizing the densities of gametocytes in blood meals of vectors that become infected (23). Even if microgametes swim at the maximum speed of 50 μm/s, successful mating in the absence of such mechanisms would seem unlikely for submicroscopic gametocyte densities. For example, if 5 gametocytes enter the mosquito midgut, even if 4 of these are males, this would result in a maximum of only 32 microgametes, with each exploring 1/1,000 of the blood meal in the 30-min window.

Given the drive to develop transmission-blocking interventions and that interfering with the fertility of microgametes is an attractive target, a better understanding of the behavior of microgametes is central to making interventions as "evolution-proof" as possible. Two key questions emerge from our work: (*i*) What role do RBCs have for microgamete swimming speed? (*ii*) Are encounters between males and females nonrandom? An intermediate RBC density appears to be optimal for the motility of trypanosomes (17), and if this is also the case for microgametes, interventions that change the density of RBCs in blood meals will have consequences for malaria transmission. For example, vector control measures may cause parasites to encounter other vector species (as is occurring due to insecticide use) with different diuresis behaviors [up to 55% of the fluid ingested can be excreted (24)]; thus, different packing densities of RBCs could affect mating success. There are several ways to investigate whether RBC density has an impact on microgamete velocity, including the use of beads or pillars in microfluidic chambers to simulate different RBC packing arrangements. Testing whether females produce

chemotaxis gradients that microgametes follow is more challenging but important because it could provide a novel target for transmission blocking. Automated imaging methods like the one we have developed can be extended to track microgamete trajectories over longer distances, with a high throughput. Advances in microscopy and microfabrication, often originating from physics laboratories, are ideally suited to addressing these questions.

Finally, the contribution of biology to physics in this context should not be underestimated. The microgamete turns out to be an ideal model system for understanding the axoneme on a mechanical level. The strikingly small number of components in a microgamete, coupled with genetic control over its structure and assembly (1), allows for a rigorous test of current physical theories. In particular, the ability to disrupt genes responsible for the central pair of microtubules (in other words, producing "9+1" or "9+0" axonemes) offers the chance to resolve a long-standing debate about the role they play in determining flagellar waveforms, and their influence on the flagellar beat (25). For example, an ortholog of the flagellar protein PF16 (first characterized in *Chlamydomonas*) in *P. berghei* (PbPF16) has recently been shown to be crucial for flagellar motility in malaria parasites. The majority of the PbPF16 mutant microgametes lacked at least one central microtubule and were either immotile or had slower swimming speeds (1). Such mutant lines are ideal for identifying the role of the central pair in flagellar motility, compared with WT *P. berghei* microgamete motility.

Holographic reconstruction and feature extraction. Given that the work presented here relies on the use of a novel microscopy technique, we feel that it is appropriate to summarize the method briefly. A more detailed account can be found in an earlier work by one of the authors (26). We implemented inline digital holography, illuminating a sample with partially coherent plane waves that were locally scattered by the sample. The unscattered portion of the plane waves acted as a phase reference, and interfered with the scattered light at the sample plane. This hologram was then recorded with a standard complementary metal oxide semiconductor (CMOS) digital camera. Our reconstruction method was based on the Rayleigh–Sommerfeld (RS) technique described by Lee and Grier (27); this is particularly appropriate for weakly scattering objects like our eukaryotic flagella. An object of refractive index n_{obj} in a medium of refractive index n_{med} is weakly scattering if the relative refractive index $m = n_{obj}/n_{med}$ obeys the following:

$$|m-1| \ll 1,\ kd|m-1| \ll 1, \qquad \textbf{[S1]}$$

where the wavenumber is $k = 2\pi n_m/\lambda$, λ is the illumination wavelength, and d is a characteristic dimension of the object (28).

At heart, the RS technique is an application of Huygens' principle, paraphrased by Born and Wolf (29) as the following: "Each element of a wave-front may be regarded as the centre of a secondary disturbance which gives rise to spherical wavelets; and moreover that the position of the wave-front at any later time is the envelope of such wavelets." The plane reference wave is assumed to have a constant phase across the image plane, so we model the pixels in the image as an array of point sources with equal phase, and with amplitudes set by the individual pixel values. The optical field is numerically propagated to the desired distance, where the optical field is evaluated. In our implementation, a stack of numerically refocused images was generated (typically 100 images) with axial spacing equal to the image pixel spacing, giving a sampling frequency of 4.29 μm^{-1} in each direction.

To localize the feature of interest within our reconstructed image stack, we appeal to the Gouy phase anomaly, a phenomenon well known in optical tweezers instrumentation (30–32). When a converging spherical wave passes through its geometrical focus point, the wavefront phase is retarded by π radians compared with a plane wave propagating in the same direction. This can be observed in any bright-field microscope; weak phase objects have light centers when they lie on one side of the focal plane, have dark centers when they lie on the other, and are invisible when they lie directly in the focal plane. This phenomenon has been observed before by groups studying cell membrane fluctuations (e.g., ref. 33). The same phenomenon is present in our reconstructed optical stacks; by applying a gradient filter in the axial direction of an image stack, we can isolate a point-like object's position in three dimensions, as the position where the axial intensity gradient is an extremum (its sign depends upon which side of the focal plane the object originally lay).

The interaction of light with weakly scattering objects is described by Rayleigh–Gans scattering theory (28). This states that the scattered field is well approximated by the sum of contributions from an array of point-like sources. We therefore approximate our eukaryotic flagellum as a line of point-like scattering centers and use the optical gradient method to obtain its location.

This method differs from most holographic schemes, which often display a large depth of focus in the reconstructed field. Other authors (34–36) have applied various focusing heuristics to define the focal plane in these cases, which can lead to uncertainty in the axial position up to 10-fold greater than that in the lateral direction. Our method allows us to find the axial coordinate of the scatterer with high precision. Previous work (26) found this method to be accurate to within 150 nm in the axial direction for both single particles and extended, flat aggregates of particles; this limit is set by the reproducibility of our independent reference (the microscope stage-positioning motor). Other measurement and reconstruction errors tend to dominate, and we provide a brief account of these in the next section.

Holographic data reduction and error analysis. The holographic method described in the previous section applies strictly to weakly scattering objects and allows the localization of point-like scatterers to within 150 nm in the axial direction. In the lateral direction, standard video tracking arguments apply (e.g., those in ref. 37), allowing a localization accuracy in principle down to tens of nanometers.

A superposition of point-like scatterers presents different challenges in terms of accuracy, which tends to depend on the object's configuration. This is most clearly illustrated in the pathological case of a rod aligned along the optical axis. Although the rod may scatter weakly (according to Eq. **S1**) if viewed through one of its short axes, this may not be the case when viewed through the long axis. In these conditions, the reconstruction is unreliable and tends to be exaggerated in the vertical direction. Mild examples are shown in Movie S1, at around 22 and 29 s in, when the front end of the microgamete points along the optical axis and effectively disappears. This dominant failure mode is distinctive and leads to a vertically "flared" structure in the reconstructed data and a drastically shortened contour. Data subject to this failure mode are limited to $\lesssim$5% of the total frames.

The greatest position uncertainty arises when fitting a segmented contour of section length Δs to the raw data, as described in the main text. The choice of Δs is not arbitrary, but is set by the optical magnification and the true size of the object. In our system, 0.7 μm corresponds to three pixels, which is just larger than the apparent width of the microgamete in the reconstructed image (which occupies two pixels at most, and with an average position that can be measured with subpixel accuracy). If Δs is much smaller, the reconstruction is susceptible to camera pixel noise, leading to a more jagged fitted contour. If Δs is much larger, we risk "averaging out" some of the curves in the feature of interest. Clearly, the microgamete is not, in general, a multiple of 0.7 μm in length. This is why the right-hand edges of Figs. 2*A* and 3*C* are somewhat "ragged"; the number of segments extracted fluctuates a little from frame to frame (very rarely greater

than ±2), with segments lost at the "head" end. There is also a small measurement error due to how we define the first point in the contour and how subsequent frames are aligned. The "tail" of the microgamete is reliably found by the reconstruction routine, so we can find one end with an accuracy on the order of plus or minus one pixel (note: the tail point can be in an average position between other pixels). This is then the uncertainty in registration between subsequent frames in Movie S1.

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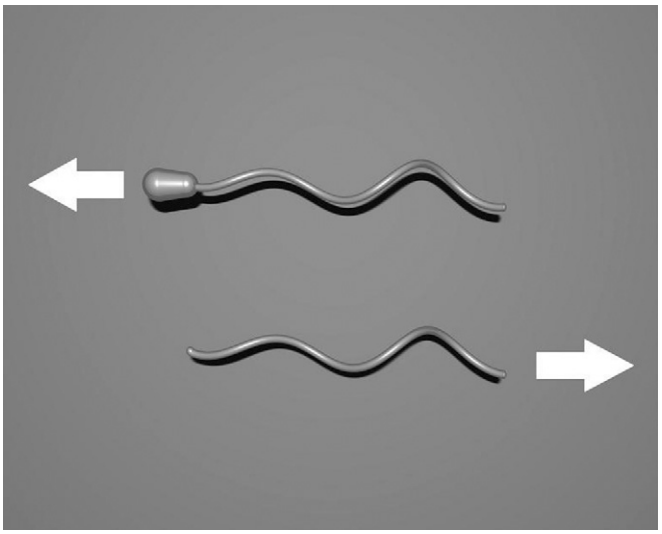
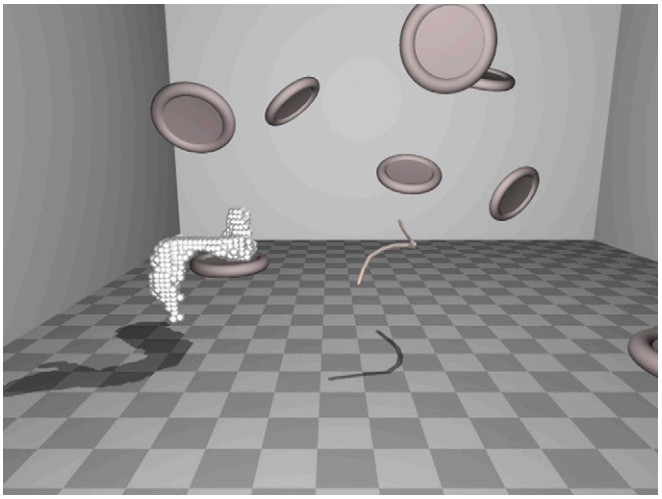
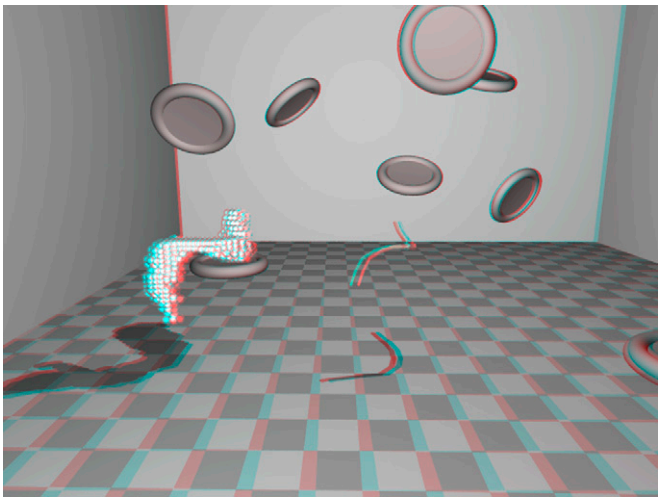


Fig. S1. Swimming directions of a mammalian sperm (*Upper*) and microgamete (*Lower*).



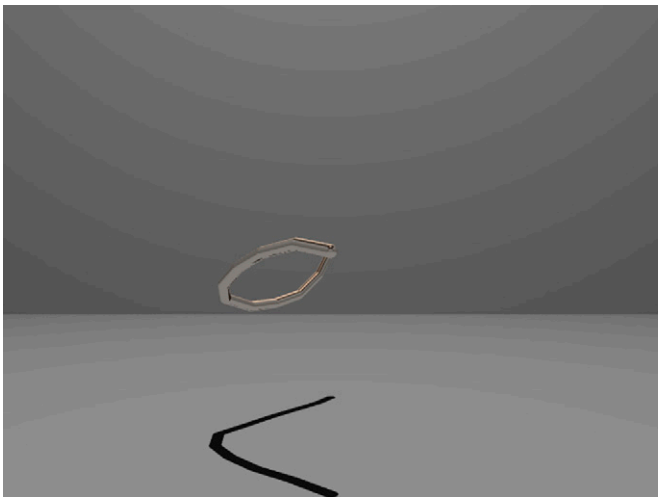
Movie S1. Reconstruction of a swimming microgamete, played back at 1/10 of true speed. The RBCs are to scale, and squares on the floor are 2 μm on a side. The swimming object on the left is the gamete volume of interest (VOI); the object on the right is the fitted contour.

Movie S1



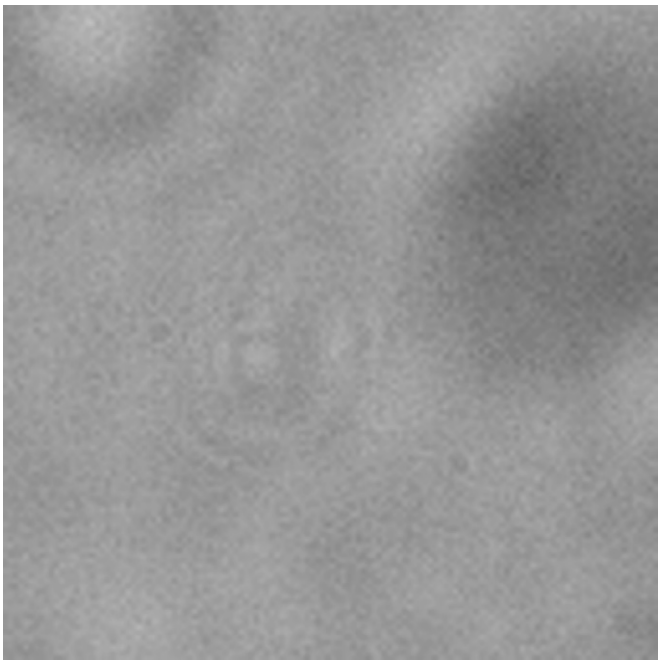
Movie S2. 3D anaglyph movie of a swimming microgamete played back at 1/10 of true speed. The RBCs are to scale, and squares on the floor are 2 μm on a side. The swimming object on the left is the gamete VOI; the object on the right is the fitted contour.

Movie S2



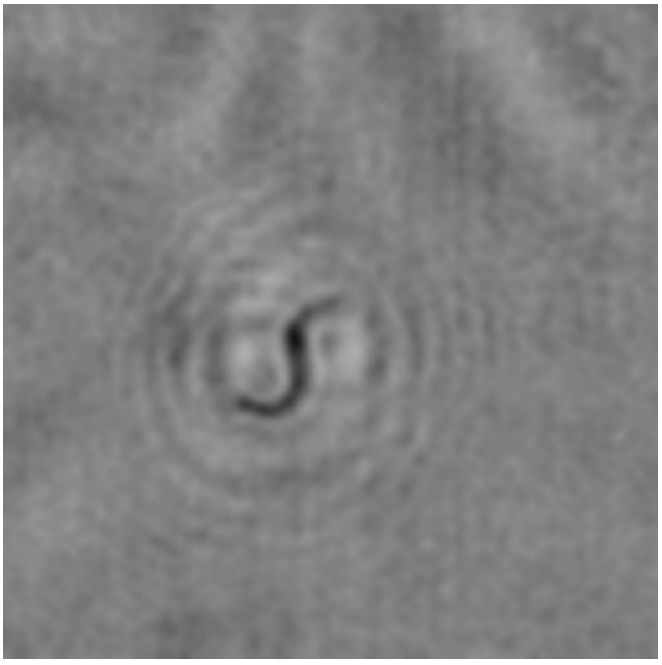
Movie S3. Demonstration of the irregular microgamete waveform. The gamete coordinates used to generate Movies S1 and S2 have been rotated to lie along the observer's line of sight, as described in the main text, to illustrate the variability in the flagellar waveforms. The data are played back at 1/10 of the acquisition speed.

Movie S3



Movie S4. Example of raw holographic data. The movie measures 30 μm on a side and is played back at 1/10 of the acquisition speed.

Movie S4



Movie S5. Example of a numerically refocused image stack. The movie scans through the reconstructed volume (along the optical axis) at a speed of 3.5 μm/s, and the movie measures 30 μm on a side.

Movie S5

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COMMENTARY

EVOLUTION, MEDICINE, & PUBLIC HEALTH

Stress and sex in malaria parasites

Why does commitment vary?

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ABSTRACT

For vector-borne parasites such as malaria, how within- and between-host processes interact to shape transmission is poorly understood. In the host, malaria parasites replicate asexually but for transmission to occur, specialized sexual stages (gametocytes) must be produced. Despite the central role that gametocytes play in disease transmission, explanations of why parasites adjust gametocyte production in response to in-host factors remain controversial. We propose that evolutionary theory developed to explain variation in reproductive effort in multicellular organisms, provides a framework to understand gametocyte investment strategies. We examine why parasites adjust investment in gametocytes according to the impact of changing conditions on their in-host survival. We then outline experiments required to determine whether plasticity in gametocyte investment enables parasites to maintain fitness in a variable environment. Gametocytes are a target for anti-malarial transmission-blocking interventions so understanding plasticity in investment is central to maximizing the success of control measures in the face of parasite evolution.

KEYWORDS: Plasmodium; transmission; commitment; stress; phenotypic plasticity; gametocyte

INTRODUCTION

Plasmodium spp (malaria parasites) and other Apicomplexans are some of the most serious pathogens of humans, livestock and wildlife [1]. Cycles of asexual replication inside host red blood cells (RBCs), lasting from 24 to 72 hours [2], enable parasites to establish and maintain infections. To transmit to new hosts, every cell cycle a proportion of parasites develop into specialized sexual stages called gametocytes, which do not replicate in the host, but are infectious to the mosquito vector

(unlike asexual stages). When taken up by the vector, male and female gametocytes differentiate into gametes and mate. The resulting offspring infect the vector and eventually produce stages infective to new hosts [3].

It is well known that the production of gametocytes varies during infections and across hosts [4–7]. However, the factors that induce commitment to produce gametocytes, and why parasites respond to these factors, are long-standing questions [8–11]. This information is central to understanding severity and transmission of disease, for predicting how disease control strategies will affect infectiousness [12–15], and may also reveal novel ways to target parasites.

Here, we propose that malaria parasites strategically adjust investment into gametocytes (hereafter, the conversion rate) in response to the changeable conditions experienced during infections and that plasticity in the conversion rate enables parasites to optimize their survival and transmission during infections. Our conceptual model stems from the integration of diverse experimental data into an ecological and evolutionary framework, thereby making the predictions of our model and its underlying assumptions explicit and testable. While we focus on malaria parasites, the concepts and approach we outline can be applied more broadly to species for which in-host replication and between-host transmission are achieved by different specialized stages.

CONVERSION RATE: EVOLUTIONARY CONTEXT

Parasites experience rapid and extensive variation in their in-host environment (e.g. in resource availability, competition with other genotypes and species, immune responses, and drug treatment) throughout their infections and while occupying different hosts and vectors. There is mounting evidence that traits underpinning in-host replication and between-host transmission (spanning from immune evasion traits [16, 17] to investment in transmissible forms [4, 18, 19]) are adjusted by parasites during infections. This flexibility in traits is called 'phenotypic plasticity' defined as the ability of a genotype to produce different phenotypes in response to environmental change [20, 21]. Phenotypic plasticity is an important solution to the challenges of life in a changing environment because it enables organisms to maintain fitness by altering their phenotype, through mechanisms such as differential gene expression, to match their circumstances [22].

Every cell cycle malaria parasites face a resource allocation trade-off between how much to invest in asexual stages that are required for in-host survival and in sexual stages that are essential for between-host transmission [23, 24]. This is analogous to the trade-off between survival and reproduction faced by all sexually reproducing organisms [25, 26]. Because reproduction is costly, phenotypic plasticity in the conversion rate influences two key fitness components: in-host survival and between-host transmission [24]. High conversion early in infections increases the potential for transmission, but this strategy risks insufficient investment in asexual stages to maintain the infection within the host, resulting in a short duration for transmission. Conversely, excessive investment in asexual parasite replication reduces the rate of transmission at any given time, but this may be compensated for by longer infection durations and continued opportunities for transmission [24, 27].

The number of gametocytes produced during infections is generally low [9] and it has been suggested that high densities of asexual stages are needed to shield gametocytes from transmission blocking immune responses [28]. However, this hypothesis does not explain why conversion rates vary during infections, between conspecific genotypes, and across species [7, 37, 39] (Fig. 1). The conversion rate is defined as the proportion of asexual stage parasites that commit to producing gametocytes in subsequent cell cycles (Box 1), and is called 'reproductive effort' in evolutionary biology. Therefore the conversion rate is not synonymous with the density or prevalence of gametocytes; variation in gametocyte densities can be generated by the same level of investment from different numbers of asexual stages [6].

In multicellular organisms, reproductive effort decisions are based on multiple extrinsic and intrinsic cues, mortality risk and how these factors vary through an individual's lifetime [25, 26, 29–31]. Evolutionary theory predicts organisms should invest less in reproduction as they age because deterioration in their physiological condition (referred to as 'state') means that more resources need to be allocated to maintenance to ensure continued survival [29–31]. However, when facing an irrecoverable decline in state, or other fatal circumstances, organisms should make a terminal investment to maximize short-term reproduction [29, 32, 33].

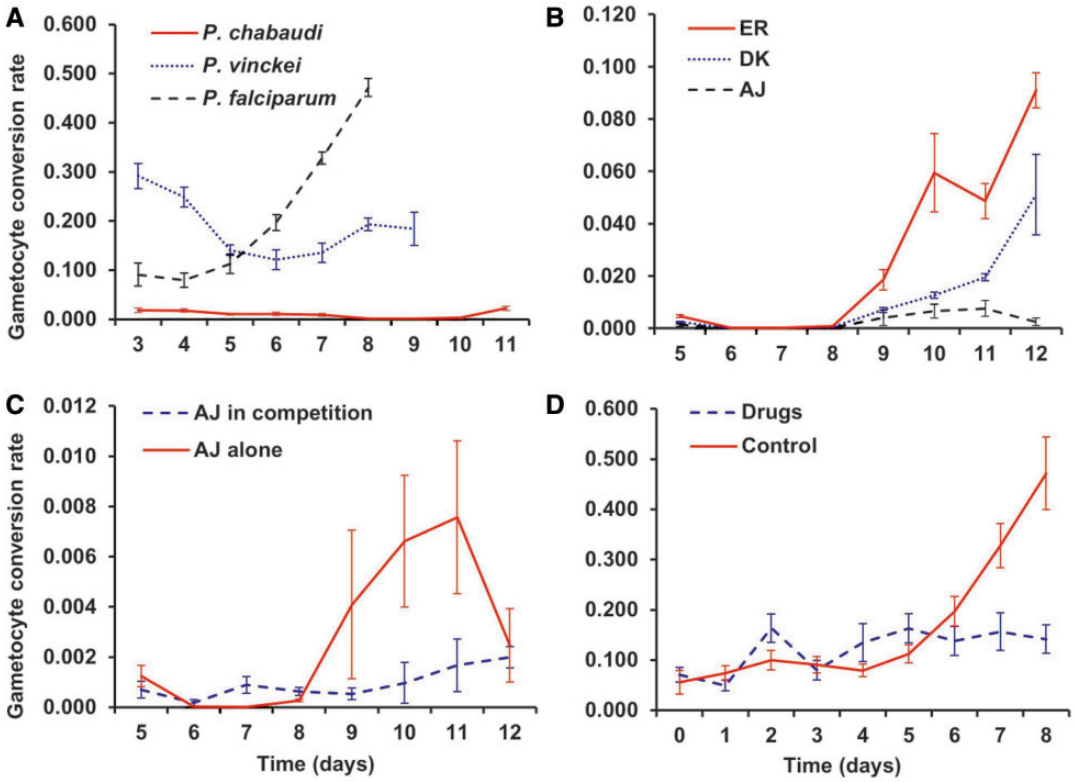

Figure 1. *Plasmodium* conversion rates are variable. The conversion rate (±SEM) represents the proportion of a given cohort of asexual parasites that differentiate into sexual stage gametocytes. Variation in conversion is observed across species and during infections/culture (**A**). *Note*: conversion is calculated differently for rodent malaria parasites (*P. chabaudi, P. yoelli, P. vinckei and P. berghei, in vivo*) and for *P. falciparum* (*in vitro*) (see Box 1). Different conspecific genotypes of *P. chabaudi*, in the same experiment, exhibit different patterns for conversion during infections (**B**). *Plasmodium chabaudi* reduces conversion when experimentally exposed to in-host competition (**C**). The conversion rates of genotype AJ are illustrated; during a single genotype infection (alone), and the mean conversion when in competition with either genotypes ER, AS, or both together (in competition). The reduction in conversion observed when drug sensitive *P. falciparum* isolates are exposed *in vitro* to antimalarial drugs or control conditions (**D**) [7, 37, 39]

When translating this to malaria parasites each genotype within a mixed infection is the target of selection and should behave as a multicellular organism [34]. The density and/or proliferation rate of parasites is analogous to the 'state' of multicellular organisms. During infections, numerous factors, such as competition with unrelated genotypes, other species, drug treatment, immune responses, RBC resource availability and host nutritional status can all change dramatically and impact upon parasite proliferation in the host. Thus, in-host environmental factors that negatively affect proliferation can be considered as 'stressors' which impact on the 'state' of parasites.

STRESS-INDUCED SEX?

Human (*Plasmodium falciparum*) and rodent (*Plasmodium chabaudi*) malaria parasites elevate gametocyte densities in response to high doses of antimalarial drugs [4–6, 35] and an increase in young RBCs (reticulocytes) [36, 37]. However, care must be taken when making comparisons as there are discrepancies between the approaches used to estimate conversion rates in different studies (Box 1). Increasing conversion has been interpreted as a strategy parasites adopt when they experience adverse conditions, enabling them to maximize transmission before the infection is cleared or the host dies [4, 8], a so-called 'terminal investment' [29]. While this makes intuitive sense in the case of drug treatment, it is not clear whether reticulocytes are, or indicate, adverse conditions.

In contrast, recent experiments (using *P. chabaudi* rodent malaria parasites *in vivo* [7, 38], and human *P. falciparum* parasites *in vitro* [39]) reveal that when exposed to competition with other genotypes in the host, RBC resource limitation, or low doses of anti-malarial drugs, parasites reduce conversion rates, adopting 'reproductive restraint' (Fig. 1). Evolutionary theory predicts that reproductive restraint during periods of mild stress improves

BOX 1: CALCULATING CONVERSION RATES

Current protocols for *in vitro* studies of *P. falciparum* calculate the conversion rate on day t as the number of stage II gametocytes observed in 10 000 RBCs on day $t+3$ (the earliest time point when *P. falciparum* gametocytes are distinguishable from asexual blood stages) divided by the number of ring-stage asexual parasites observed in 10 000 RBCs on day t [83].

For *P. chabaudi*, conversion is calculated from *in vivo* measurements according to [6]. The description of the biological process underlying the model in [6] overcomes challenges posed by hard-to-quantify parameters (i.e. parasite death rates in the bloodstream and schizont burst sizes) and takes into account the maturation times of gametocytes and asexual blood stages (48 and 24 hours respectively, for rodent parasites). Although the mathematical formulation assumes gametocytes are counted 24 hours into development, current molecular assays count gametocytes of an unknown age (but are likely to be between 24 and 48 hours old). Ideally we need to know the schedule of development and the precise point at which gametocytes are assayed, since these will determine the exact form of the conversion rate equation. For example, if markers in mature (48 h old) gametocytes are used, then conversion rate, ε, should be quantified as:

$$\varepsilon = \frac{\frac{G_{t+3}}{A_t}}{\sqrt[3]{\frac{A_{t+3}}{A_t}} + \frac{G_{t+3}}{A_t}}$$

where A_i and G_i are asexual and gametocyte densities on day i.

While these tools are easy to implement, the assumptions underpinning them are key to making accurate estimates of conversion rates. These assumptions, and their caveats, include:

(1) The probability of asexual parasites producing gametocytes is constant over the period between gametocyte production and detection. Given the expectation of plasticity in conversion, whereby a different proportion of asexual parasites can commit for every cell cycle, this assumption may often be hard to fulfil.

(2) Both *in vivo* and *in vitro* approaches assume that the death rate of asexual parasites and gametocytes is equal. Whilst *in vitro* culture conditions do not have the problem of sequestration (disappearance from the circulation) or immune factors that could exacerbate differential mortality rates between lifecycle stages [9], for *in vivo* assays these factors could confound conversion estimates [40]. Furthermore, conversion rates can be overestimated if the death rate for asexual parasites is higher than for gametocytes (which could well be the case during drug treatment [39], or underestimated if early stage gametocytes are mistakenly identified as asexual stages. It is possible to develop mathematical models and formulate predictions for how different survival rates need to be if they are the sole driver of observed patterns in conversion rates. For example for the *in vivo P. chabaudi* data in [7], we find that the difference in survival rates between asexual parasites and gametocytes must vary over the course of infections (e.g. immunity sometimes focuses efficiently on killing gametocytes while at other times survival rates across parasite stages are equal) and must vary considerably in different kinds of infection (N. Mideo, unpublished results). In particular, to explain the difference in patterns of conversion observed in Fig. 1C, survival rates of gametocytes (relative to asexual parasites) in mixed infections must be several orders of magnitude lower than in single infections. As yet, there is no known mechanism that could underlie such drastically different patterns of survival between parasite stages, during and across infections. Therefore, we propose that differential survival is unlikely to be the sole cause of variation in patterns of conversion rates. However, developing a better understanding of immune responses and subsequent parasite death rates remains an important goal.

In the literature, there are considerable discrepancies in how conversion rates for *P. falciparum* have been examined, with some studies measuring the gametocyte density in circulation and others presenting gametocyte prevalence (reviewed in [12]). This is, in part, due to the difficulties in calculating conversion rates for natural *P. falciparum* infections since repeated samples—at specific time points—are required to assay the number of asexual parasites in a cohort and the number of gametocytes they produce.

Basing inference simply on gametocyte density can be problematic: for example, observations of elevated gametocyte densities post drug treatment could be due to the release of sequestered gametocytes and/or an increase in conversion rate [9]. Data on the timing of gametocytes appearing in the circulation can resolve this issue, but again, requires

(continued)

BOX 1: CONTINUED

repeated sampling at specific time points. While there are important ethical and logistical considerations when studying natural infections of humans, monitoring infections, with measurements of conversion and in-host variables (e.g. anaemia and genetic diversity) would be extremely useful.

To address the problems outlined in points 1 and 2, ideally, conversion rates for rodent malaria parasites *in vivo* could be calculated in the same way as is now possible for *in vitro* cultures of *P. falciparum* (using GFP-tagged molecular markers of sexually committed schizonts and flow cytometry to sort fluorescent parasites [84]). However, despite the issues raised, measuring conversion rate remains a more desirable approach than simply analysing gametocyte density or prevalence, because changes in the density of gametocytes can be generated from cohorts that simply differ in asexual parasite number, but invest in the same relative number of gametocytes.

the prospects for in-host survival, and therefore the opportunities for future transmission [40]. The experimental data also suggest that parasites respond to the presence of the extrinsic (environmental factors) as well as to their intrinsic effect (impact on state). Moreover, data from monitoring a cohort of infected patients collected in the same area from which the parasites used in Reece *et al.* [39] were isolated provide tentative (*in vivo*) support for the reproductive restraint of *P. falciparum* in response to drug pressure [41].

The contrasting observations of increased and decreased conversion rates in response to environmental variation within the host can be reconciled by considering the severity of stress imposed on parasites by in-host factors. This is illustrated in Fig. 2A in which we propose that parasites adjust their conversion rate according to the impact of conditions on their proliferation (state) or via directly detecting the presence of stressors (Fig. 2B). In low stress conditions (e.g. infections of naïve hosts) parasites can afford to invest in gametocytes, and do so at a rate that maximizes transmission. When in-host conditions deteriorate due to the appearance of stressors (e.g. competition with other genotypes and species, immune responses, drug treatment), parasites are constrained to invest in survival, which they achieve by reducing the conversion rate (reproductive restraint) [23, 42]. By ensuring survival during periods of stress, parasites benefit from the fitness returns of future transmission (i.e. by reducing the rate of transmission in the short term, parasites gain a longer duration for transmission). When faced with attack from immune responses, investing more in replication may also have the added benefit of increasing opportunities for immune evasion via antigenic switching [43]. However, in very poor conditions, when parasites experience severe stress and their death rate exceeds the capacity for proliferation or host mortality is imminent, they should make a terminal investment to maximize short-term transmission by diverting resources to gametocyte production.

The pattern of conversion we predict in Fig. 2A is qualitatively similar to that predicted through a mathematical analysis by Koella and Antia [23]. Their analysis relied on strict assumptions: infections are lethal to the host above a threshold density and conversion rates are adjusted to limit asexual parasite densities to just below this threshold. This work raises the point that all else being equal, increasing investment in gametocytes should lead to decreasing virulence of an infection; a large body of theory predicts how virulence should depend on in-host factors (e.g. [15], reviewed in [44]). However, virulence is only one of the many selective forces acting on conversion rates. As only a small proportion of modern human malaria infections are fatal, we predict parasites more often need to respond to in-host factors that are able to clear infections than to imminent host death. The high prevalence of chronic malaria infections and the increasing appreciation of their contribution to the infectious reservoir [45–47], also suggests that a long duration of transmission matters and producing gametocytes 'few but often' results in the greatest lifetime fitness. Transmission success is also heavily dependent on vector availability. In areas where transmission is seasonal, parasites must survive in the host during the dry season. Indeed, parasites have evolved diverse strategies to facilitate long-term in-host

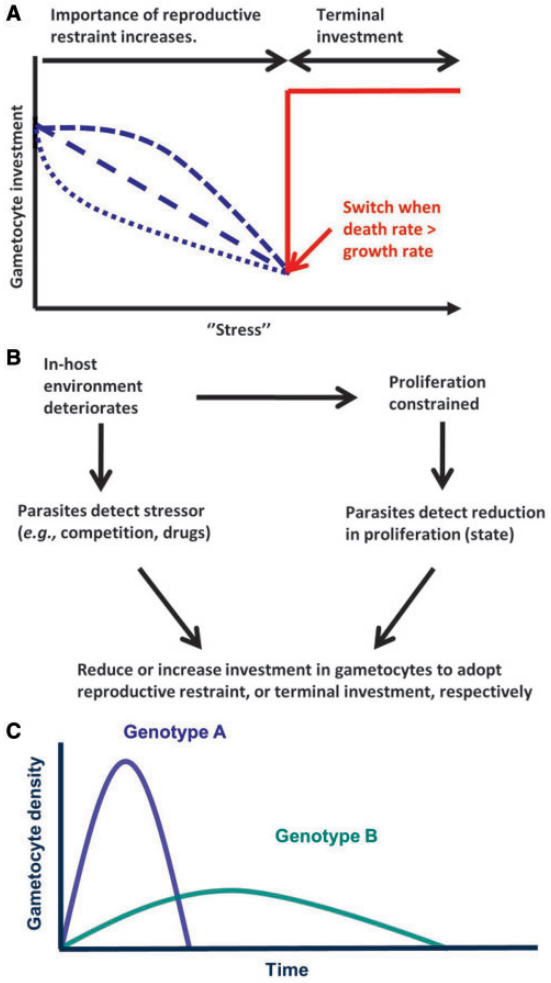

Figure 2. Predicted pattern for conversion. (**A**) Under low 'stress' (e.g. early in infections of naïve hosts) parasites can afford to invest in gametocytes, but if conditions deteriorate and proliferation is constrained (e.g. when parasites face stressors such as anaemia, competition or immune responses) parasites reduce conversion, employing reproductive restraint (blue dashed lines), to ensure in-host survival and the potential for future transmission. The form that reproductive restraint takes could follow any of the patterns illustrated with the dashed blue lines, depending on a number of factors (e.g. the cues parasites respond to, how accurately survival probability is determined, and the value of future versus current transmission). When parasites face circumstances likely to be fatal (e.g. when their death rate exceeds the potential for replication during radical drug treatment) or host death is imminent (e.g. due to severe anaemia), parasites should make a terminal investment by investing remaining resources into gametocytes (red solid line). A switch point and step function between reproductive restraint and terminal investment is predicted because investing all remaining resources is the best option in a situation likely to be fatal. Note: the *x*-axis does not simply translate to 'time since infection' because the severity of different stressors fluctuates during infections. (**B**) Data suggest that parasites can detect and respond directly to individual stressors and also to the effect they have on proliferation rate. Information from the cues parasites use must be fed into the molecular pathways that underpin commitment to effect a gametocyte investment decision. (**C**) The total production of gametocytes (the area under the curve) is equal for both genotypes [14]. However, genotype A invests heavily into transmission early in the infection and therefore achieves higher gametocyte densities over a shorter period of time, whereas B has a lower relative investment in gametocytes at each time point, but achieves a longer period for transmission. The optimal balance between these two extremes is predicted to depend on many factors including the frequency of vector blood meals, and the chances of the host clearing the infection or dying

survival, from immune evasion mechanisms (e.g. antigenic switching in *P. falciparum* [16]); to resisting competition (e.g. rodent malaria parasites prevent incoming, competing parasites from establishing an infection via the host iron regulatory hormone hepcidin [48]). In the majority of parasite species, the success of these strategies depends on maintaining asexual replication at a sufficiently high rate, which can be achieved through reproductive restraint.

TESTING THE THEORY: COMPLICATIONS AND CHALLENGES

The model outlined above provides a foundation to explain variable conversion rates when considered in light of several key questions:

(i) Which cues do parasites use to make conversion rate decisions?
(ii) What are the mechanisms that enable plasticity in conversion rate?
(iii) How finely tuned are conversion rates to the in-host environment and state?
(iv) Does adjusting conversion rates in the manner predicted maximize parasite fitness?

We consider answers to these questions in the following sections and outline the challenges required to evaluate these hypotheses in Box 2.

Cues for conversion decisions

The extent to which parasites respond directly to extrinsic stressors or simply the overall effect those stressors have on state is not known. Experimental data suggest parasites can respond both to state and environmental factors. For example, experiments exposing *P. falciparum* to low doses of

BOX 2: CHALLENGES AND FUTURE DIRECTIONS

While our conceptual model is general, testing it requires examining specific circumstances. Here, we outline the main challenges and outstanding questions involved.

Response to drugs: Data for conversion rates—especially from experiments using drugs—are consistent with the basic prediction of parasites adopting reproductive restraint (*P. falciparum in vitro* [39]), or terminal investment (*P. chabaudi in vivo* [4, 6] and *P. falciparum in vitro* [5]), in response to different levels of stress. However, further work is required to explicitly test the effects of varying dose within the same experiment—both for rodent models and *in vitro* for *P. falciparum*. Furthermore, not all drugs appear to induce changes in conversion rate [6, 35]. This may be because drugs with different modes of action differentially affect the capacity of survivors to detect/respond to changes in state, or the capacity of dying parasites to provide signals.

Response to competition: In-host competition is a stressor with a negative effect on state because the densities of all genotypes (individually and when combined) is reduced in mixed infections compared with single infections. This is due to a mixture of competition for RBC and the action of immune repsonses that are not genotype specific. Competition within the host could occur via a single bite from a mosquito infected with multiple genotypes (to a naive host). Alternatively, competition can be established when a mosquito infected with one genotype bites an individual already infected with a different genotype. The latter example of sequential infection would be less stressfull for the resident genotype than the newcomer, even if it the resident genotype is competitively inferior to the incoming genotype [85]. This is because the incoming genotype will enter a RBC resource depleted environment with cross-reactive immune responses already in place [86]. *In vivo* studies of simultaneous in-host competition using *P. chabaudi* reveal reproductive restraint across several genotypes [7, 87], but there are no reports of increased conversion in response to competition. Adopting reproductive restraint in response to competition might be the only strategy required because in-host competition is never stressful enough to merit terminal investment. Alternatively, this may be an artefact of experimental design in which mixed infections do not result in competitive exclusion, even for the weakest genotypes [7, 88, 89]. Experiments using genotypes that vary in competitive ability, inoculated at different starting doses and times during infections are needed to test whether in-host competition can induce terminal investment. At the host population level, the consequences of different investment strategies would be much harder to test experimentally, but theory demonstrates that there will be feedback from the within- to between-host levels, and vice versa (e.g. [90]). For instance, if mixed infections really do promote reproductive restraint, then this should result in less transmission and, consequently, fewer mixed infections. Some of the variations observed in conversion rates may be a consequence of this sort of dynamic feedback.

Response to reticulocytes: Conversion has been observed to both increase and decrease in response to reticulocytes. For some species (e.g. *P. berghei* and *P. vivax*) that preferentially invade reticulocytes, an increase in conversion upon exposure to reticulocytes is consistent with parasites making use of available resources. However, species able to infect a wide range of RBC ages, such as *P. falciparum* and *P. chabaudi,* also increase conversion in response to reticulocytes [36, 37]. This may be because reticulocytes are also exploitable resources. However, the lifespan of gametocytes in *P. falciparum* is at least five times that of asexual stages, so the longer expected lifespan of reticulocytes may provide a better resource to support the development of gametocytes than mature RBCs. Alternatively, for all species, increased reticulocytaemia could indicate severe anaemia leading to imminent host death, and thus, terminal investment is the best strategy. For example, the poultry malaria parasite *P. gallinaceum* appears to be able to determine whether the host will survive or die from severe anaemia because it produces different sex ratios in these different circumstances [55]. However, an influx of reticulocytes could also indicate the opposite—that the host is generating an appropriate erythropoietic response and will recover from severe anaemia. In this case, reproductive restraint maximizes the potential for the parasites to survive.

When in-host survival does not rely on asexual parasite replication: Parasite species producing dormant stages that persist in the liver (hypnozoites) and dendritic cells, such as the human malaria parasites *P. vivax* and *P. ovale* [91, 92], may not adopt reproductive restraint in response to stress because survival in the host does not depend on blood stage replication. Terminal investment due to imminent clearance will also be unnecessary but may be required to cope with host death. To our knowledge there are no data on the conversion rates of *P. vivax* experiencing different in-host conditions. However, during natural *P. vivax* infections, higher gametocyte densities are correlated with a mixture of seemingly favourable and unfavourable conditions, including younger (immunologically naive) patients, those with higher parasite densities, lower haemoglobin levels, lower platelet counts and an absence of fever (reviewed in [12]). *Plasmodium vivax* gametocyte densities are also generally much higher compared with those recorded for *P. falciparum*, but each gametocyte circulates for a shorter time; a maximum of 3 days (reviewed in [12]). These observations suggest that *P. vivax* may have a non-plastic strategy of a relatively high conversion during the short-lived erythrocytic stage of their infections.

different anti-malarial drugs in culture have included both drug sensitive and resistant genotypes but only sensitive genotypes respond. This suggests that parasites do not directly detect each drug, but instead, respond to the negative effect they have on state [39]. Responding to state seems the more efficient strategy: it avoids the need to integrate information about multiple factors, potentially giving opposing information, to mount an appropriate response. For example, the level of anaemia induced by *P. falciparum* infections varies depending on the type of antimalarial drug administered to patients and whether the parasites are cleared [49]. Because anaemia triggers the formation of reticulocytes, the reproductive strategy employed in response to the presence of drugs may be complicated by the simultaneous change in RBC age structure. Parasites could be responding directly to the drugs, the resulting changes in RBCs, both, or the overall effect that both factors have on the 'state' of the infection [36, 37].

Whether the best measure of state is parasite density *per se* or proliferation (i.e. rate of change in density) is unclear. Data from several *P. chabaudi* genotypes [7, 50] and subsequent modelling [51] suggests that parasites alter their conversion rate according to their density in mixed genotype infections. Density could be determined by quorum sensing [52], markers of RBC lysis from burst parasitized cells [53], immune factors, or metabolic measures such as energy balance or reducing power (e.g. the expression of genes associated with starvation are associated with increased conversion in *P. falciparum* [54]). However, detecting the density of a parasite cohort does not necessarily reveal a change in state (i.e. is parasite density increasing or decreasing?).

Measuring proliferation requires that parasites integrate information on density over consecutive cell cycle cohorts. This information may be more accurate for parasite species with synchronous progression through cell cycles than for species with asynchronous cycles. In this case, if proliferation rate information is unreliable, parasites could respond to individual environmental stress factors; either directly or indirectly, by detecting a co-varying factor. For example, parasites may use the onset of anaemia as a signal for the imminent arrival of antibodies and the development of immune responses [37, 55, 56]. Using proxies in this way may also enable parasites to predict future changes in state and respond preemptively [57]. Alternatively, parasites could measure their death rate; although mechanisms for this are more difficult to envision, they could include monitoring the concentration of immune effectors or the release of SOS signals by dying parasites similar to bacteria and *Chlamydomonas* [58, 59].

Mechanisms underpinning conversion

The mechanisms regulating the switch to gametocyte production remain elusive. Advances in genomics, transcriptomics, proteomics and functional gene targeting studies have identified several markers of early gametocyte development in human and rodent malaria parasites (reviewed in [8, 10, 60–63]). These studies provide further evidence that commitment occurs at or prior to the schizont stage preceding the release of sexually committed merozoites (as has been previously suggested for *P. falciparum* [64, 65]). Studies using GFP reporters with known gametocyte specific promoters also support this developmental pattern (reviewed in [10, 13, 60, 61]). Recently, the gene *P. falciparum* gametocyte development 1: Pfgdv1 (PFI1710w) has been identified as a regulator of gametocyte production (and is associated with an increased expression of genes involved in early gametocytogenesis (Pfge genes) [66]), and work from our group has identified an ApiAP2 DNA binding protein [67] that is required for gametocyte commitment (Kafsack and Llinás, unpublished data).

While identifying molecular markers for commitment is useful for quantifying conversion decisions, the evolution of plasticity in conversion rates is shaped by the nature of the pathways involved in: detecting cues, processing the information, producing a conversion rate phenotype and the maturation of gametocytes. The critical regulators underlying gametocyte conversion may act within a complex network of interactions between different modules involved in information assimilation and integration to produce a conversion rate phenotype. This level of complexity is very challenging to unravel and made more difficult because gene function and changes in expression must be assessed in the context of variation in both the environment and genetic background of the parasites. Furthermore, it is possible that the environmental sensing mechanisms underlying conversion decisions may also feed information into other plastic life history decisions such as sex ratio, cell cycle arrest and var gene switching (which is responsible for antigenic variation to evade

host immune responses), as these traits are sensitive to similar environmental perturbations (reviewed in [24]). As these traits are likely to be linked by genetic correlations (e.g. epistasis/pleiotropy: different traits are shaped by the same genes), understanding the nature of these interactions is central to explaining plasticity in these traits.

Parameterizing patterns of conversion

The shape and switch point(s) of the reaction norm (how a trait varies across an environmental gradient) reveal how fine-tuned parasite responses are to environmental variation, including novel stressors. The extent of genetic variation for reaction norms is a determinant of the potential for evolution. Reaction norms are influenced by many interacting factors. This includes the reliability of cues, costs of maintaining detection and response mechanisms, and how much multiple sources of information affect the risk of making the wrong decision [68–70]. Differences in reaction norms across species, that have different cell-cycle durations, gametocyte development times or RBC age preferences, may reflect how differences in costs and constraints on plasticity shape parasite strategies. As many different factors can independently and simultaneously affect in-host conditions and parasite state, examining the patterns of conversion rates resulting from varying factors individually is useful, but providing cues in different combinations is required to reveal the full picture.

The reaction norm for conversion is predicted to follow a non-linear pattern, with any of the patterns illustrated and at least 1 switch point (reproductive restraint to terminal investment; Fig. 2A) [23, 42]. This switch should occur when the death rate exceeds the proliferation rate. We expect this point will be influenced by species-specific variation in cell-cycle duration and gametocyte development time, and by how quickly the environment and/or state changes. For example, the cell-cycle duration and gametocyte development time of rodent malarias are much shorter than that of *P. falciparum*. While the cell cycle for rodent malaria parasites is 24 hours, and gametocytes reach maturity and are infectious to mosquitoes after 24–48 hours, the cell cycle of the human malaria parasite *P. falciparum* is 48 hours and gametocytes require 10–14 days to reach maturity [10, 11]. Therefore, if *P. falciparum* makes a terminal investment in advance of host death the host is required to survive at least 10–14 days until the investment can pay off (five further asexual cycles), but only 48 hours are required for rodent parasites to produce transmissible gametocytes. As such, *P. falciparum* may 'play it safe' and adopt a more conservative strategy by making a terminal investment in response to lower levels of stress than rodent parasites, whose gametocytes reach maturity within 48 hours (two asexual cycles). If a fast drop in numbers were normally a reliable indicator of a terminal situation, this would explain why increased conversion is observed when parasites are exposed to high, but subcurative, drug doses [39, 41]. Also, if the longer cell-cycle duration of *P. falciparum* compared to rodent malarias makes *P. falciparum* more vulnerable to being cleared by the host, reproductive restraint will be induced at lower stress than for rodent parasites.

As shown in Fig. 2C, the characteristics of populations can also influence the shape of reaction norms. For example, a 'live fast, die young' strategy in which parasites readily switch to terminal investment may bring greater pay offs in an epidemic setting—where there are plenty of naïve hosts to be transmitted to—than in an endemic setting where parasites will be transmitted to hosts containing competitors and with active immune responses [71]. This is because genotypes with a high conversion rate risk being unable to establish infections in new hosts, due to being outcompeted by resident genotypes [15, 40]. Furthermore, Parasites in hypoendemic areas experience lower levels of in-host competition than those from regions with high genetic diversity (hyperendemic) and so may be less responsive to novel stressors such as competition and its effect on state.

Linking variable conversion rates to fitness

A key prediction to test is whether plasticity in conversion rate is adaptive [72]. The extent to which reproductive restraint provides an in-host survival advantage under stress is yet to be determined (e.g. how much does reproductive restraint ameliorate the suppression of a genotype in a mixed infection?). At the between-host level, how different reproductive strategies map to the rate and duration of transmission is hard to assess from data (e.g. gametocyte prevalence) available on natural infections. Therefore, whether (under some conditions) prolonging the duration for transmission enhances fitness, and whether terminal investment benefits

parasites in lethal situations through an increase in short-term transmission, remain unknown.

Testing the fitness consequences of variation in traits is notoriously difficult, but identifying the host and parasite factors that elicit a change in conversion rate and the reaction norms generated by different levels of stress will provide the required foundations. For example, by providing a cue that elicits reproductive restraint in different circumstances (e.g. cues for competition provided in single infections) parasites can be induced ('tricked') into making inappropriate responses for their circumstances. The consequences for in-host survival and transmission for parasites responding to fake cues could then be quantified, and compared to the performance of parasites exposed to cues that accurately reflect their circumstances [73]. This framework also opens up the possibility of developing interventions that co-opt plasticity in conversion rates, by manipulating parasites into making suboptimal decisions for their fitness.

The maintenance of mechanisms required to detect and respond to environmental change requires resources that could be otherwise allocated to different functions [74]. Evolutionary theory predicts that if these costs are sufficiently high then plasticity is selected against and lost if organisms no longer experience variable environments, but evidence for costs of plasticity is scarce [75]. Because gametocytes are costly, selection for in-host replication during long-term culture of *P. falciparum* and serial passage of *P. berghei* result in the loss of gametocyte production [8, 76]. However, whether plasticity is actually lost is unclear because gametocyte production is sometimes recoverable [77].

CONCLUSIONS

That in-host ecology shapes the dynamics of infections [78, 79] and patterns of transmission is well known [12, 80–82]. Despite this, why the density of circulating gametocytes in malaria is generally low [9, 40] has eluded explanation. We provide an evolutionary theory-based model, which predicts that parasites can rarely afford to invest in more because their life history spreads reproduction across multiple attempts over a relatively long time period.

Given renewed interest in transmission blocking interventions, understanding parasite strategies for gametocyte investment is central to making such measures as resilient to parasite counter evolution as possible [12, 15]. For example, inducing all parasites to commit to gametocytes (ideally of the same sex) would reduce the virulence of the infection and could also produce an effective transmission-blocking immune response that acts against future infections. For example, this could be useful for travellers returning to non-malarious countries. Inducing commitment *in vitro* could also generate material to inform the development of other transmission-blocking interventions such as vaccines and drugs with gametocytocidal action.

Finally, it is often not appreciated that plasticity in parasite life history traits can also shape evolutionary responses to environmental change. For example, if plasticity in conversion rate acts as a buffering mechanism to minimize the impact of drug treatment, this may weaken selection for other forms of resistance. This may be favourable from the perspective of maximizing the timespan of efficacy of antimalarial drugs. However, such infections will likely be harder to treat than if malaria parasites exhibited a higher, fixed, conversion rate.

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