In-Host Ecology and Transmission Dynamics of Malaria Parasites

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I, Andrew R. Wargo, verify that the work contained in this thesis is my own and of my own thought and origin. However, scientific research is of a collaborative nature and therefore I would like to recognize those involved in the experiments described within each chapter.

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ABBREVIATIONS

DNA – Deoxyribonucleic acid

RNA – Ribonucleic acid

mRNA – Messenger RNA

PCR – Polymerase chain reaction

RT-PCR – Reverse transcription polymerase chain reaction

cDNA – DNA generated from reverse transcription of RNA

qPCR – Quantitative real time PCR

qRT-PCR – Quantitative reverse transcription real time PCR

PABA - Para-amino benzoic acid

Epo – Erythropoietin

GLM – General linear model

QT-NASBA – Real time nucleic acid sequence based amplification

ABSTRACT

Malaria, one of the world's most deadly and infamous diseases, has a dynamic in-host ecology which heavily shapes its evolution. However, determining the epidemiological relevance of malaria in-host ecology has been hindered by an incomplete understanding of how it relates to the essential life history ingredient: reproduction and between-host transmission. This lack of knowledge has potentially contributed to the ongoing failure to eradicate the disease. In this doctoral thesis I attempted to begin unraveling the mysterious relationship between malaria in-host ecology and transmission to provide insights into effective disease control strategies.

My research was conducted using the rodent malaria parasite *Plasmodium chabaudi*, which proves to be a highly malleable experimental and laboratory model for its human counterpart, *Plasmodium falciparum*. The initial phases of the work required the development of new methods to more accurately investigate malaria transmission. I harnessed the wealth of growing malaria molecular information, and developed a novel quantitative reverse transcription PCR technique (qRT-PCR) for clone specific assessment of *P. chabaudi* transmission stage production. I combined the qRT-PCR technique with current qPCR methods for quantification of in-host total parasite numbers, to examine in-host replication and transmission investment strategies in relation to infection stage, conspecific interactions, and drug resistance evolution.

This detailed life history investigation revealed that the majority of malaria total parasite and transmission stage production occurs within the initial phase of infection. However, many individuals develop very low level long lasting chronic infections which are transmissible to vectors and likely to heavily impact the epidemiology of the parasite, confirming current findings in the field.

During the high parasite density stage at the start of the infection, in-host dynamics and conspecific competition were found to play a critical role in shaping the fitness of malaria genotypes co-infecting the same host. In response to conspecific competition, a small degree of transmission investment plasticity was observed, but this did not affect the competitive outcome.

In-host competition dynamics were greatly influenced by drug treatment, when drug resistant and sensitive genotypes co-infected the same host. In this case, it was observed that strong drug dosage, which completely cleared the drug sensitive clone, released the resistant clone from competition, resulting in its enhanced growth and increased transmission potential. Reducing drug dosage to levels that did not completely clear the sensitive genotype, still enabled the resistant clone to be released from competition, but prevented its enhanced growth. Thus, drug dosages, which just alleviate clinical symptoms but do not completely clear parasites, will preserve within host competition and may reduce the rate at which drug resistance spreads. This contradicts current medical thinking that incomplete drug treatment regimes will accelerate drug resistance evolution.

In conclusion, this doctoral thesis reinforces that a deeper understanding of the relationship between in-host ecology and transmission will enhance our ability to unravel the evolutionary dynamics of malaria and conquer the summit of global disease eradication.

1. GENERAL INTRODUCTION

Prologue: Forgotten transmission

Darwin revealed that those individuals which are the most reproductive and produce the greatest number of viable offspring will pass on their traits, thereby obtaining the highest levels of fitness, thus driving evolution (Darwin 1859). Those who fail to reproduce, in essence, reach the end of their family tree, leaving their genes behind to be forgotten for the rest of evolutionary time.

"...individuals having any advantage, however slight, over others, would have the best chance of surviving and of procreating their kind? On the other hand, we may feel sure that any variation in the least degree injurious would be rigidly destroyed. This preservation of favourable variations and the rejection of injurious variations, I call Natural Selection..." (Darwin 1859).

Like all organisms, parasite fitness and evolution is driven by reproduction. In the parasite's case, reproduction is closely intertwined with transmission from one host to the next. Although a parasite may spend a lifetime reeking havoc in the one traveler who mistakenly 'thought it was ok to drink the water', eventually that traveler will die, and ultimately the parasite's fitness depends on sending its progeny out into the world to find a new host.

Amazingly, despite all its conceptual simplicity and importance, the reproduction and transmission of many parasites remains sparsely investigated and poorly understood. In fact, the majority of parasite research has focused on more cellular functional biology type questions, like 'what genes do what' and 'how do they get turned off or on'. This approach has clearly been hugely rewarding in the field of drug discovery and vaccine research, as well as exposed a wealth of important parasite biology. However, it cannot explain the evolutionary ecological reason genes are maintained and passed on. In reality, parasites can be particularly shy when revealing their reproductive transmission habits. Here, I attempted to 'pull back the curtains' a bit to dissect the important role reproduction plays in shaping the evolution of one of the world's most deadly parasites, malaria.

1.1. Malaria epidemiology: a global problem

Malaria is one of the ten deadliest global diseases currently infecting mankind, killing over a million people each year and causing economic burden and morbidity for an estimated half a billion more (Breman 2001; Snow *et al.* 2005). Approximately one third of the world's population is at risk of malaria infection, with the disease being most prevalent in Africa, South America, and South East Asia, where inadequate health care, high poverty, and optimal vector breeding conditions all contribute to the persistence of the parasite (Lopez *et al.* 2006). The majority of clinical malaria cases and deaths occur in children, who are believed to be most susceptible to the disease due to a lack of developed immunity (Githeko *et al.* 1992; Kolk *et al.* 2003). Acquired immunity to malaria is typically relatively weak and the illness is very common in adults living in endemic malaria regions, who often develop asymptomatic infections; however, fever, coma, and death also regularly occur (Drakeley *et al.* 2000; Breman 2001).

The regional burden of malaria fluctuates substantially both on a temporal and spatial scale (Hay *et al.* 2000; Snow *et al.* 2005), with many areas having regular seasonal patterns of parasite prevalence and transmission (James 1931; Roper *et al.* 1998; Kolk *et al.* 2003; Shililu *et al.* 2003; Nacher *et al.* 2004; Paul *et al.* 2004a). This seasonality is primarily driven by rainfall which regulates the mosquito vector population (Grover-Kopec *et al.* 2006). The African continent is particularly well known for annual cycles of rainy seasons with high mosquito densities, separated by dry periods with low vector abundance. Just prior to the onset of the rainy season parasite prevalence and parasitemia of infected individuals, are typically very low (Hamad *et al.* 2002). As the rain falls, vectors become more populous and parasitemia rises, due to novel infections or stimulated parasite production from mosquito biting. This ultimately results in increased transmission and elevated disease prevalence (Paul *et al.* 2004a). In areas where transmission seasons are separated by very lengthy dry seasons, individuals can develop very low level infections lasting for several months (Babiker *et al.* 1998; Hamad *et al.* 2000). It is believed that in some cases, long transmission free periods result in a degree of immunity loss in the population, generating small epidemics when the rainy season arrives (Druilhe and Perignon 1997; Worrall *et al.* 2004).

1.2. Control failure

Malaria control efforts have been under way for well over a century and originally culminated in high success with the eradication of the disease from parts of Europe and the Americas in the early 1900's, mainly driven by environmental management (Keiser *et al.* 2005). In recent years control has proven more difficult, primarily due to the evolution of drug resistance by the parasite and insecticide resistance in vectors, resulting in a resurgence of the disease after the implementation of chemical treatment strategies (Campbell 1997; Sachs 2002; Guinovart *et al.* 2006).

Drug treatment of malaria formally began sometime in the 17th century with the advent of quinine, the active ingredient of the *chinchona* plant which had been used by South Americans for centuries before. By the 20th century resistance of malaria to the drug was documented which, compounded with the high cost and undesirable side effects of quinine, led to the design of new safer and more effective compounds, such as chloroquine, towards the end of the Second World War. Resistance to chloroquine, spread within almost a decade, leading to the development of a host of new drug treatments, to almost all of which resistance rapidly evolved (Hyde 2005).

To counteract the effects of anti-malarial drugs, parasites have evolved unique genetic resistance mechanisms (Reed *et al.* 2000). Resistance to chloroquine for example, is believed to be due to a mutation in the chloroquine resistance transporter gene (*pfcrt*), which allows the parasite to use a pump mechanism to clear the drug (Fidock *et al.* 2000; Sidhu *et al.* 2002). Similarly, resistance to the common anti-malarial antifolate treatment pyrimethamine, is believed to be incurred by mutations in dihydrofolate reductase (*dhfr*), and mutations in the dihydropteroate synthetase (*dhps*) gene can lead to further resistance to other antifolates like sulfadoxine. Both the *dhfr* and *dhps* mutations are believed to incur resistance by preventing the drug from binding to its appropriate receptor (Peterson *et al.* 1990; Sibley *et al.* 2001).

Drug resistance did not come freely to the parasite. All drug resistance mutations require the parasite to utilize vital resources for drug clearance rather than growth or reproduction and therefore, they are likely to come at an energetic and fitness cost (Walliker *et al.* 2005). The level of this fitness cost is believed to vary widely

depending on the mechanism of drug activity and the number of mutations the parasite requires for evasion. This can have important consequences for how quickly resistance will evolve (Hastings 2004). For example, chloroquine *pfcrt* resistance requires several mutations and evolved relatively slowly, on the scale of a decade or more. Alternatively *dhfr* resistance only requires a single point mutation, partly explaining its rapid evolution after a few years of first administration in the field (Carlton *et al.* 2001; Hastings and Donnelly 2005). A slew of other epidemiological factors, such as the degree of drug usage in the community, parasite recombination, host immunity, intra-host dynamics, duration of drug efficacy in the host, and transmission intensity, are also all predicted to play a critical role in shaping resistance evolution. Ultimately, this has made it difficult to obtain a complete understanding of the relationship between resistance fitness costs and the rate of evolution (Hastings 1997; Hastings *et al.* 2002; Hastings 2003; Talisuna *et al.* 2003; Hastings and Watkins 2005; Hastings 2006).

Although theoretical literature has provided us with a wealth of ideas, very little is known empirically about the ecological mechanisms and fitness dynamics underlying drug resistance evolution. Furthermore, it is becoming increasingly clear that achieving efficient and effective malaria control requires a deeper understanding of the basic evolutionary ecology of the parasite (De Roode and Read 2003; Paul *et al.* 2003; Molyneux 2006). Several avenues of parasite control have been attempted in the wake of drug resistance, such as insecticide treatment and vaccine development, and they have all met poor degrees of success, in many ways due to an oversight of basic ecological principles (Wellems 2002; Greenwood 2005; Targett 2005). Some of their failures have been, simply put, down right embarrassing, particularly the widespread DDT resistance resulting from the 'global malaria eradication campaign', which could have easily been predicted by evolutionary ecologists. I assert that the newest control strategies which have the most promise are those that have attempted to incorporate parasite ecology into their development (Blanford *et al.* 2005). With the increasing prospect of a malaria vaccine, an understanding of the evolutionary ecology of the malaria is more critical than ever, especially when we consider that some control strategies could do more harm than good (Gandon *et al.* 2001).

1.3. Unravelling malaria evolutionary ecology

1.3.1. A complex life history

At least four species of malaria regularly circulate in humans (*Plasmodium vivax, P. malaria, P. ovale,* and *P. falciparum)* with *P. falciparum* contributing to the majority of clinical cases and mortality (Breman 2001). *Plasmodium* is a protozoan parasite in the phylum Apicomplexa which includes an array of parasitic genera such as *Babesia, Cryptosporidia*, and *Toxoplasma,* all commonly infecting humans. The haemosporidian malarial parasite family of Plasmodiidae is extremely diverse and species are regularly found residing in birds, mammals and reptiles. The human malaria *P. falciparum* and its close relative *P. reichenowi* in chimpanzees appear to be uniquely separated from the other haemosporidian parasites in mammals, suggesting a long evolutionary branching (Perkins and Schall 2002).

Despite their sometimes highly divergent evolutionary past, all *Plasmodium* have a very similar life cycle divided into asexual and sexual replication (Figure 1.1). In the vertebrate host the parasite life cycle begins as a haploid sporozoite transmitted by an insect vector, which in the case of *P. falciparum* is commonly a mosquito in the genus *Anopheles* (Kiszewski et al. 2004). Upon entering the host, sporozoites travel to the liver where they undergo mitosis, releasing thousands of merozoites. These merozoites then infect red blood cells to enter an asexual replication cycle and develop into ring stages, trophozoites and then schizonts. Schizonts burst red bloods cells releasing between 4-20 merozoites, depending on the species, which go on to re-infect other red blood cells and continue the cycle of asexual replication (Vickerman 2005). In many malaria species this asexual cycle is very synchronous, typically resulting in regular fever spikes in patients as red blood cells rupture (James 1931).

During the asexual replication cycle a small percentage of merozoites become committed to development into sexual stage gametocytes (Talman *et al.* 2004). Gametocytes in *P. falciparum* usually comprise less than 5% of the in-host parasite population (Taylor and Read 1997); however, they are of vital importance to the parasite as they are the only stage transmitted to the mosquito vector during a blood meal. Once

inside the mosquito midgut, male gametocytes exflagellate and release up to 8 microgametes, which then fertilize the female macrogamete. During this diploid stage, recombination can occur as the parasite develops into an ookinete, which penetrates the mosquito gut wall and develops into an oocyst. The oocyst takes approximately 14 days to mature during which time it undergoes meiosis and several rounds of mitosis, to release tens of thousands of haploid sporozoites that travel into the mosquito salivary glands for transmission to a new host during blood feeding (Garnham 1966).

The development from one life stage to the next in *Plasmodium* is often characterized by the up and down regulation of specific genes. Many of these genes are uniquely expressed in only one life stage, particularly those found in gametocytes (Cui *et al.* 2001; van Dijk *et al.* 2001; Kongkasuriyachai *et al.* 2004; Gardiner *et al.* 2005; Hall *et al.* 2005; Silvestrini *et al.* 2005; Young *et al.* 2005). Much of this information regarding gene specificity has been elucidated only recently, coming to light after the completion of the genome-wide malaria sequence (Carlton *et al.* 2002; Gardner *et al.* 2002). The knowledge of unique life stage gene expression can now be utilized to identify, detect, and even purify individual life stages, by targeting specific RNA or protein transcripts (Florens *et al.* 2002; Lasonder *et al.* 2002; Khan *et al.* 2005). This has led to huge advances in the field, resulting not only in a deeper understanding of the fundamental biology of the parasite, but also the ability to create new drug and vaccine candidates (Kongkasuriyachai and Kumar 2002). From an ecological prospective, it can provide us with powerful tools for studies of parasite life history strategies and their epidemiological impact.

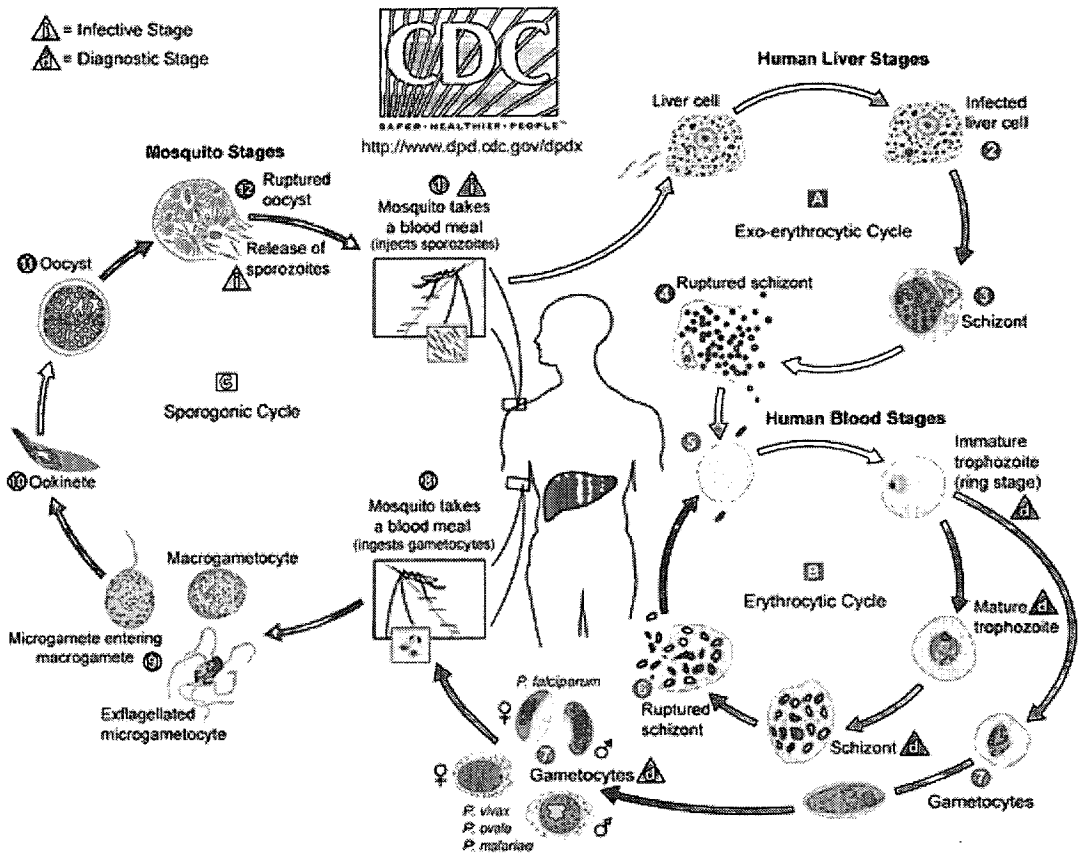


Figure 1.1. Malaria life-cycle in mammals, in this case humans, reprinted from Center for Disease Control website (CDC 2006).

1.3.2. An undetected pest

Malaria parasite densities in the host usually peak within the first week or two after transmission, in what is known as the acute phase, and then trail off as the host controls the infection (Garnham 1954). Subsequently, recrudescent waves in parasite density can occur, with the infection often persisting for several months to years, in what is termed the chronic phase (James 1931; Jeffery and Eyles 1955; Druilhe and Perignon 1997; Hamad *et al.* 2000). During intervals between recrudescent peaks, parasites are frequently undetectable in the blood stream and are believed to be sequestered in host organs such as the liver (Landau *et al.* 1999; Vardo *et al.* 2005; Wargo *et al.* 2006). This sequestering phenomenon is common in many mammalian malaria species including *P. falciparum.*

The classical approach for diagnosis of malaria infection has been microscopic scanning of thick blood smears to determine both species and infection status. Due to the low parasite densities which commonly occur in malaria infections, individuals have often been misdiagnosed as uninfected (Roper *et al.* 1996; Babiker *et al.* 1999a; Awadalla *et al.* 2001). As a result, new more sensitive approaches for parasite detection were developed. The most widely implemented approach has been PCR amplification of parasite DNA. This method has many benefits for field research, most notably, its ease of use and low cost. Due to the very recent advent of sensitive molecular detection techniques, the importance of low level infections in the host has been largely uninvestigated. However, it is becoming increasingly clear that they may play a critical role in the epidemiology of the parasite, particularly in areas of long lasting low level infections (Bottius *et al.* 1996; Hamad *et al.* 2002; Bousema *et al.* 2006; Schneider *et al.* 2006) (see Chapter 2 and 3). We now have the ability to not only detect, but also begin quantifying transmission from these types of low level infections.

1.3.3. Hidden diversity

Another major advancement provided with molecular technologies was the ability to infer parasite genetic diversity. Although genetic diversity work began many years before using enzyme markers (Walliker *et al.* 1972; Carter and McGregor 1973), it was greatly enhanced by the implementation of PCR. These studies ultimately confirmed that malaria infections are commonly highly genetically diverse, especially *P. falciparum*, with individual hosts typically harboring several distinct alleles, *i.e.* multi-locus genotypes (Babiker and Walliker 1997; Druilhe *et al.* 1998; Anderson *et al.* 2000; Farnert *et al.* 2001; Magesa *et al.* 2002; Jafari *et al.* 2004). Due to the haploid and clonal asexual replicating nature of the parasite within the vertebrate host, these genotypes are often referred to as clones. Multiplicity of infection (clones/host) appears to be highest in areas of intense transmission, but is recorded world wide (Arnot 1998; Babiker *et al.* 1999b). This is especially true when considering that PCR underestimates the degree of genetic diversity, due to limits in the types of detectable genetic variation and the tendency to only amplify the most abundant genotypes (Hill *et al.* 1995; Babiker *et al.* 1997; Druilhe *et al.* 1998; Ofosu-Okyere *et al.* 2001; Abdel-Muhsin *et al.* 2002).

The limitations of PCR are likely to be circumvented by the development of new quantitative PCR technologies, which make it possible not only to differentiate clones based on as little as two or three point mutations in genetic material, but also provide an accurate method to quantify the parasitemia of individual clones in mixed infections (Bell *et al.* In Press). Quantitative PCR technologies are now being further combined with the new information on life cycle variation in gene expression, to develop in-host stage specific quantification (Babiker *et al.* 1999a; Bruna-Romero *et al.* 2001; Witney *et al.* 2001; Schneider *et al.* 2004; Wargo *et al.* 2006) (see Chapter 2). These technologies have finally made it possible to examine the interactions and dynamics of individual clones co-infecting a single host, which previously remained sparsely investigated due to the high labor intensity of the mono-clonal antibody techniques required (Taylor *et al.* 1997b; Bell and Ranford-Cartwright 2002).

1.3.4. The ecology of mixed infections: competition has its hay-day

The discovery of high genetic diversity in malaria and the new genotype specific quantification molecular technologies rapidly brought to question what role genetic diversity plays in shaping the epidemiology of the parasite (Awadalla *et al.* 2001; Read and Taylor 2001). Most notably, how clones interact in the host is likely to heavily influence the evolution of the disease. For instance, epidemiological studies from the field suggest that in-host ecology and competition between clones, are the probable driving forces behind the displacement of genotypes from certain geographical areas (Daubersies *et al.* 1996; Mercereau-Puijalon 1996; Arnot 1998). These findings have been further supported in the last ten years by the strong competition dynamics observed in mixed clone infections of the rodent malaria *Plasmodium chabaudi*, the research of which was greatly enhanced by clone specific parasite density quantification technologies (Taylor *et al.* 1997a; Taylor *et al.* 1997b; Taylor *et al.* 1998; Taylor and Read 1998; De Roode *et al.* 2003; De Roode *et al.* 2004a; De Roode *et al.* 2004b; De Roode *et al.* 2005a; Bell *et al.* In Press).

Due to its likely high prevalence in the field, conspecific competition is now believed to heavily shape the degree of disease severity the parasite causes to its host, i.e. virulence. For many years theoretical work has ascertained that in-host competition should select for rapidly replicating genotypes, which will have higher transmission success and fitness than their competitors. Since in-host replication is also believed to be correlated with parasite virulence, the evolution of higher virulence is predicted as a by product of competition (van Baalen and Sabelis 1995; Frank 1996; Mosquera and Adler 1998; Amarasekare and Nisbet 2001; Brown *et al.* 2002; Mackinnon *et al.* 2002a; Paul *et al.* 2004b). Intra-specific competition experiments with *P. chabaudi* have recently demonstrated that a positive relationship between virulence and competitive ability is indeed present (De Roode *et al.* 2005b; Bell *et al.* In Press).

The growing experimental support for conspecific competition has also had a large impact on theories of drug resistance evolution, which is now thought to be heavily shaped by intra-host dynamics (Hastings and D'Alessandro 2000). The high genetic diversity observed in malaria provides strong evidence that drug resistant and sensitive

genotypes will regularly circulate in the same host. In the face of in-host conspecific competition, resistant genotypes are believed to be competitively inferior in the absence of drug treatment, due to the fitness cost that resistance incurs, and there is some experimental evidence that this is true (De Roode *et al.* 2004a; Walliker *et al.* 2005). The level to which drug treatment alleviates the resistance fitness cost, and the degree of competitive superiority the resistant genotype obtains, will play a particularly critical role in shaping the rate of drug resistance evolution (Hastings 2006). Unraveling this dynamic has been cited as one of the most important factors in understanding the evolution of drug resistance (Hastings and D'Alessandro 2000). Experimental investigation into this subject is, to my knowledge very limited, especially when considering competition dynamics in response to differing drug treatment strategies (see Chapter 5).

1.3.5. The missing link: transmission

The importance of malaria ecology in shaping the evolution of the parasite is becoming increasingly clear. In particular, in-host ecology is now believed to be a key player in parasite epidemiology (Daubersies *et al.* 1996; Mercereau-Puijalon 1996; Arnot 1998; Read and Taylor 2001; Paul *et al.* 2004b; De Roode *et al.* 2005b; Bell *et al.* In Press). However, one very important piece to the puzzle is still missing: transmission. Surprisingly, this is also the one piece with the most relevance. From a purely Darwinian population biology perspective, it is the frequency of alleles in the population that really matter, and transmission to new hosts that will ultimately shape lifetime fitness and evolution. This does of course bring to mind the obvious question: can transmission be predicted from observed in-host dynamics? Simply put, we do not have enough evidence to conclude either way.

We do know that genetic variation in transmission life history traits exists (Mackinnon and Read 1999a; Eisen and Schall 2000). There is also strong experimental evidence that transmission dynamics can be shaped by an array of environmental factors including drug treatment, host anemia, and immunity; through alterations in gametocyte production timing, density, and sex-ratio (Gautret *et al.* 1996b; Buckling *et al.* 1999c; Price *et al.* 1999; Paul *et al.* 2000; Buckling and Read 2001; Paul *et al.* 2002a; Paul *et al.* 2003; Paul and Brey 2003; Reece *et al.* 2005). Parasite influence on vector behavior has even been observed (Rossignol *et al.* 1986; Koella *et al.* 2002; Ferguson and Read 2004).

It seems logical to assume that malaria parasites will exhibit similar transmission adaptive responses to competition. Inter-specific competition leading to reduced transmission of the avian malaria *Plasmodium gallinaceum* from hosts co-infected with *Plasmodium juxtanucleare* has been observed experimentally (Paul et al. 2002b). Further evidence from field data also suggests that between species competition is likely to shape the transmission and epidemiology of human malaria, and one study found enhanced *P. falciparum* gametocyte production in competition with *P. malariae* (McKenzie and Bossert 1997; Bruce *et al.* 2000; McKenzie *et al.* 2002; Mayxay *et al.* 2004).

Transmission dynamics of individual clones during conspecific competition are less clear and the interactions are likely to be very different to those occurring between separate parasite species. Local mate competition theory predicts that gametocyte sex-ratio variation should regularly occur during conspecific competition, with greater male gametocyte production as clonal diversity increases (Read *et al.* 1992). Generally the theory holds true for *Plasmodium* (Read *et al.* 1995; West *et al.* 2001), although to my knowledge the sex ratio investment of individual clones during conspecific competition has not been assessed. Ultimately, gametocyte sex ratio is likely to heavily impact transmission success depending on how it affects overall fertility (Schall 2000; West *et al.* 2002).

Mixed genotype infections of *P. chabaudi* have shown some evidence of increased transmission during conspecific competition, although an alternative study found no changes in transmission, this dichotomy likely reflecting the different clones examined (Taylor *et al.* 1997a; De Roode *et al.* 2005b). Neither of these studies examined the transmission response of individual malaria clones explicitly, which to my knowledge remains uninvestigated. Using new quantitative molecular methods it is now possible to analyze clone specific investment towards transmission. This sort of investigation could potentially play a critical role in bridging the gap between in-host dynamics, transmission, and ultimately, epidemiology (Chapter 4).

1.4. A note on rodent malaria as a model system

Addressing the relationship between parasite in-host ecology and transmission ultimately demands detailed experimental investigation, where clonal diversity can be experimentally controlled. Due to the testing and development of new technologies necessary, this line of inquiry proves both ethically and logistically impractical in the natural human host. Therefore an experimental model system is required. Although *in vitro* experimental studies are feasible, the host's effect on dynamics is an important factor for parasite evolution (Gupta and Anderson 1999). All experimental work in this thesis was therefore conducted using the rodent malaria parasite *Plasmodium chabaudi.*

There are many similarities between *P. falciparum* and *P. chabaudi* that make the rodent malaria species a particularly good model system. From a clinical disease perspective, both parasites cause severe anemia and rosetting of host red blood cells which likely contribute to host morbidity (Udomsangpetch *et al.* 1989; Mackinnon *et al.* 2002b). The life cycles of both parasite species are also very congruent, signified by an initial acute phase of high parasitemia followed by long lasting chronic infections with recrudescent waves of parasites (James 1931; Garnham 1954; Jeffery and Eyles 1955; Garnham 1966; Druilhe and Perignon 1997; Hamad *et al.* 2000; Kolk *et al.* 2003; Wargo *et al.* 2006). Evolutionarily, genome similarity between human and rodent malaria species is extensive (Janse *et al.* 1994; Carlton *et al.* 1998; Thompson *et al.* 2001; Carlton *et al.* 2002; Waters 2002). Additionally, both parasites exhibit high levels of genetic diversity in the field with mixed genotype infections regularly occurring in the host (Carter and Walliker 1975; Awadalla *et al.* 2001).

A particular benefit to utilizing *P. chabaudi* as an experimental system is the ability to experimentally induce controlled infections composed of multiple clones. A stockpile of several unique *P. chabaudi* clones is currently available, with each clone having been maintained as a distinct genetic line with a well documented history, since its collection from the natural thicket rat host (*Thamnomys rutilans*) in the field (Beale *et al.* 1978).

Furthermore, drug resistant and sensitive *P. chabaudi* clones are accessible, possessing the same genetic mutations as those occurring in *P. falciparum* (Carlton *et al.* 2001). Therefore, it is possible to examine the fitness of drug resistant clones in relation to drug sensitive clones. Ultimately, *P. chabaudi* has frequently been utilized as a model for *P. falciparum* and its merits as a candidate for this type of comparison are well validated (Mackinnon and Read 2004). However, since it is a model system, certain limitations do apply, depending on the type of questions examined and comparisons made. The specific limitations of the *P. chabaudi* system, as they directly relate to the research and findings of my doctoral thesis, are discussed in more detail in section 6.4.

1.5. Experimental Aims

The overall objective of this thesis was to fill in the gap in our understanding of the relationship between in-host malaria ecology and transmission dynamics. Each chapter was written as an independent unit for publication, with its own introduction placing it in the broad context of malaria evolutionary ecology, and therefore I will avoid reiteration here. In summary, my first aim was to develop an accurate clone differentiating method for selective quantification of malaria parasite transmission stages. I then hoped to implement this technique to assess the impact of critical epidemiological phenomenon, such as long lasting infections, conspecific competition, and drug resistance, on the transmission dynamics and evolution of malaria. Ultimately this research strived to enhance our understanding of malaria evolutionary ecology and provide insights into effective disease control strategies.

Specific questions:

Can low level chronic infections in the host transmit to the mosquito vector, and if so, what is their epidemiological impact (Chapter 2 and 3)?

Does within-host competition influence the transmission dynamics of individual clones and what consequences will this have for the evolution of virulence (Chapter 4)?

How does the competition dynamic between drug resistant and sensitive clones depend on drug treatment regime and what role does this play in shaping the transmission and evolution of drug resistance (Chapter 5)?

2. REVERSE TRANSCRIPTION PCR (RT-PCR) FOR THE DETECTION AND QUANTIFICATION OF TRANSMISSION STAGE MALARIA PARASITES

This is a lightly edited version of the published paper: Wargo, A. R., Randle, N., Chan, B. H. K., Thompson, J., Read, A. F. & Babiker, H. A. 2006 *Plasmodium chabaudi*: Reverse transcription PCR for the detection and quantification of transmission stage malaria parasites. *Experimental Parasitology* 112, 13-20. (See Thesis Appendix)

2.1. Abstract

I have developed two reverse transcription Polymerase Chain Reaction (RT-PCR) techniques to detect and quantify the transmission stages (gametocytes) of *Plasmodium chabaudi* malaria parasites. Both the qualitative and quantitative techniques are based on the amplification of mRNA coding for the *P. chabaudi* protein Pcs230, which is expressed exclusively in gametocytes. The quantitative RT-PCR technique (qRT-PCR) was developed and validated by examining serial dilutions of known gametocyte densities. The method generated a high correlation between calibration curves of blind samples ($R^2 = 0.86$). The technique was found to be specific, reproducible, and time efficient for quantification of both patent and sub-patent gametocytemia, with a sensitivity level 100 - 1000 times greater than microscopy. The qualitative RT-PCR (RT-PCR) technique was used to monitor the persistence and dynamics of *P. chabaudi* gametocytes following acute infection. Mice in two independent experiments were sampled daily for up to 87 days post infection. RT-PCR showed that gametocytes can persist for up to 8 weeks, post infection, whereas microscopy could only detect gametocytes up to 6 weeks. Potential applications of the above techniques for studying the ecology, evolution, and epidemiology of malaria transmission are discussed.

2.2. Introduction

Molecular methods are now becoming widely utilized to study malaria parasites both in field and laboratory settings. The power of these techniques is increasingly clear, particularly for studying the ecology and evolution of parasites. In nature, malaria infections often occur at sub-patent levels where parasites cannot be detected by microscopy. This is very common in areas such as eastern Sudan, where the transmission of *Plasmodium falciparum* is highly seasonal and chronic sub-patent infections are prevalent in the dry season (Babiker and Walliker 1997; Abdel-Wahab *et al.* 2002; Hamad *et al.* 2002).

Numerous studies have been conducted to examine the diversity and dynamics of asexual forms of malaria parasites, but the ecology of gametocytes is still poorly understood. Recently, reverse transcription PCR (RT-PCR) techniques have been used to detect RNA of proteins that are expressed exclusively in gametocyte stages, distinguishing gametocytes from asexual stages of *P. falciparum*, even at sub-patent levels (Babiker *et al.* 1999a; Menegon *et al.* 2000; Niederwieser *et al.* 2000; Schneider *et al.* 2004). Some gametocyte-specific protein genes are polymorphic, allowing the technique to be extended to distinguish gametocytes of different genotypes within a single infection (Abdel-Wahab *et al.* 2002). RT-PCR has shown that *P. falciparum* is capable of producing gametocytes, even when it exists as an asymptomatic, sub-patent infection, demonstrating the potential of such infections to sustain the cyclical malaria epidemics in areas of seasonal transmission (Babiker *et al.* 1999a). A shortcoming of the existing RT-PCR protocol is that it cannot quantify gametocytes (Babiker *et al.* 1999a; Babiker *et al.* 2000; Menegon *et al.* 2000; Abdel-Wahab *et al.* 2002). Such quantification is important in order to gain a better understanding of the transmissibility of individual parasite clones that exist in mixed clone infections and the ecological factors that influence them.

Recently, real-time quantitative PCR (qPCR) has been developed and successfully utilized to study the dynamics of different malaria species and genetically distinct clones in mixed infections (Cheesman *et al.* 2003; De Monbrison *et al.* 2003; De Roode *et al.* 2004b). This method enables accurate quantification of genomic DNA of

specific malaria parasite clones within multiple infections, but cannot selectively quantify different life–stages. However, quantitative reverse transcription PCR techniques (qRT-PCR) have also been developed and utilized for the quantification of RNA coding for various genes, including those of malaria parasites (Bruna-Romero *et al.* 2001; Nirmalan *et al.* 2002). The technique has been utilized for the quantification of liver stages of human malaria species and a related but different method (QT-NASBA) has been used to examine dynamics of *Plasmodium falciparum* gametocytes (Witney *et al.* 2001; Perandin *et al.* 2004; Schneider *et al.* 2004).

I report here on the utilization of PCR and RT-PCR protocols to detect parasites and monitor the longevity of *P. chabaudi* infections and their gametocyte production over time. Additionally, I developed RT-PCR and qPCR methods into a protocol for qRT-PCR that can specifically quantify gametocytes of the rodent malaria parasite *P. chabaudi*.

2.3. Materials and Methods

2.3.1. *P. chabaudi* infections

Two independent experiments were carried out to monitor the longevity of gametocyte production of the *P. chabaudi* rodent malaria parasite clone CR, that is known to have moderate virulence in mice (Taylor *et al.* 1998). Infections were established by inoculating 5 (experiment I) and 10 (experiment II) C57Bl/6J 5- to 8-week-old inbred female mice (Harlan Scientific, UK) with 10^6 *P. chabaudi* parasites, into the peritoneal cavity, as described elsewhere (Mackinnon and Read 1999a). Mice were maintained at 21°C (+/- 1°C) and fed *ad libitum* SDS 41B (Harlan Scientific, UK) rat and maintenance diet and 0.05% PABA supplemented drinking water to enhance parasite growth.

Blood samples (10 µl for DNA extraction and 20 µl for RNA extraction), thin blood smears, and red blood cell counts (on 2 µl of blood) from flow cytometry (Coulter Electronics), were taken five days a week, for 3 weeks, starting on days 3 post infection

(PI). After day 22 PI, samples were taken 2-3 times a week until day 87 (experiment I) or day 70 (experiment II). Larger blood volumes were required for RNA extraction to increase sensitivity for gametocytes which are in low abundance compared to asexual stages. Thin blood smears were stained with a 20% Giemsa solution and examined under a microscope at 1000x magnification to determine parasitemia and gametocytemia. Parasite and gametocyte densities were calculated by multiplying parasitemia and gametocytemia respectively by red blood cell density, measured with flow-cytometry.

For qRT-PCR, separate infections of the *P. chabaudi* clone CR were established in three NIH mice using methods described above. The density of gametocytes (gametocytemia) was determined using Giemsa stained thin blood smears, and blood samples were taken for RNA extraction when gametocytes were visible microscopically. These mice were not used for the monitoring of chronic infections via RT-PCR.

2.3.2. DNA extraction

For general PCR, DNA was extracted from 10 µl of blood taken from the tail of *P. chabaudi* infected mice and added to 200 µl of citrate saline (0.85% NaCl, 1.5% tri-sodium citrate), on ice. The samples were centrifuged at 11,000 rcf for 2-3 minutes to pellet red blood cells and the supernatant was removed. The blood pellet was stored at -80°C until DNA extraction. DNA was extracted using InstaGene™ Matrix (BioRad) according to the manufacturer's protocol.

2.3.3. RNA extraction

Parasite RNA was extracted using two methods:

(i) For RT-PCR, RNA was extracted from 20 µl of whole blood, using TRIzol Reagent for total RNA isolation according to the manufacturer's protocol (Invitrogen Life Technologies). On each day of sampling 20 µl of blood was taken from the tail of each mouse and added to 200 µl of citrate saline on ice. The samples were centrifuged at 11,000 rcf for 3 minutes at room temperature to pellet the blood and the supernatant was removed. A 10x volume (200 µl) of TRIzol was added to each pellet and the samples were vortexed immediately and stored at -80°C until RNA extraction. RNA

samples were subjected to DNase treatment to remove co-extracted DNA in a 10 µl reaction containing 5 µl RNA extract, 5mM Tris pH 7.6, 33mM $MgCl_2$, 0.27 U RNasin Ribonuclease Inhibitor and 4.2 U DNase I (Roche, UK), with incubation at 37°C for 15 minutes and 75°C for 10 minutes.

(ii) For qRT-PCR, RNA was extracted using the Applied Biosystems 6100 equipment and protocols. This method allows simultaneous processing of 96 samples and is more time efficient for use with the 96 well qRT-PCR format. To extract gametocyte RNA, 20 µl of blood was taken from the mouse tail and added to 200 µl of citrate saline pre-warmed to 37°C to avoid alterations in expression of RNA believed to occur at temperature changes (Fang and McCutchan 2002). Samples were centrifuged at 11,000 rcf for 3 minutes at room temperature, the supernatant was removed and 20 µl Ca^{2+}/Mg^{2+} free RNase/DNase free PBS (Gibco) and 40 µl of 2X Nucleic Acid Purification Lysis Solution© (Applied Biosystems) were added to the blood pellet. The samples were then stored at -80°C. For completion of the extraction protocol, the samples were thawed and incubated at room temperature for 1 hour to digest blood. RNA was extracted using the Applied Biosystems Isolation of Total RNA from Whole Blood Chemistry protocol on the ABI Prism® 6100 Nucleic Acid PrepStation with the RNA Blood-DNA method (Applied Biosystems). RNA was eluted in 100 µl of Nucleic Acid Purification Elution Solution and stored at -80°C until required for quantification.

2.3.4. PCR and RT-PCR

Primers for PCR and RT-PCR used for longevity experiments were developed to amplify the gametocyte specific *P. chabaudi* gene *pcs230* (GenBank Accession No. EAA15629.1 and EAA22479.1). The *pcs230* sequence was determined by the *P. chabaudi* sequencing project and can be obtained from ftp://ftp.sanger.ac.uk/projects/P.chabaudi. This gene belongs to the *p_s48/45* super-family that encode gametocyte-specific surface proteins in malaria parasites and is only expressed in mature late stage gametocytes (Williamson *et al.* 1993; Lobo and Kumar 1998; Niederwieser *et al.* 2000; Thompson *et al.* 2001; Bozdech *et al.* 2003; Le Roch *et al.* 2003; Hall *et al.* 2005).

To increase the sensitivity of detection, two rounds of PCR were performed, using outer primers, Pc230-(O1), 5'- AAA GAT TCA GGG CAT GGC G - 3' and Pc230(O2), 5'-TTG CCC CAC TTT TTG AGC TAC - 3'and inner primers, Pc230(N1), 5'- ATC TGT TAT GCC TTA TGG GAG - 3'and Pc230(N2), 5'- CTT GAT TAA TAC TTA GAT ACA CCA TAT GAG - 3'. The optimal annealing temperature for each set of primers was determined on a ThermoHybaid PCR machine using a temperature gradient PCR on samples known to be positive for *P. chabaudi* DNA.

To determine longevity of *P. chabaudi* infection, PCR was performed using Roche 2X PCR Master mix in 20 µl reactions containing 2 µl of genomic DNA, 0.5 U Taq DNA polymerase, 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2mM dNTP's, and 0.5 µM of primers Pc230(O1) and Pc230(O2). Samples underwent PCR on an MJ Research DNA Engine, PTC-200; using the following conditions: denaturation at 94°C/180 seconds, 35 cycles of amplification [94°C/30 seconds, 51°C/30 seconds, 70°C/ 60 seconds], and a terminal extension at 70°C/600 seconds. Positive DNA controls and negative controls (all reagents + 2 µl water) were run for each reaction. The outer PCR products were then diluted 10x and 2 µl of the diluted product underwent nested PCR using primers Pc230 (N1) and Pc230 (N2) in a 20 µl PCR reaction using PCR Master mix (Roche) and the PCR conditions described above.

For the detection of longevity of gametocyte production in *P. chabaudi* infection, a nested RT-PCR reaction was carried out to detect *pcs230* mRNA. RNA samples underwent a one-step RT-PCR reaction using the AccessQuick™ RT-PCR system (Promega). This protocol generates cDNA from RNA and then amplifies the cDNA through PCR, in a single reaction. The RT-PCR reaction was carried out in a 20 µl volume on the MJ Research, DNA Engine, PTC-200, with a reaction mixture using the AccessQuick™ Master Mix reagents consisting of 2 µl of RNA, 2 U of AccessQuick™ reverse transcriptase (Promega), 2 U *tfl* DNA polymerase, 0.2 mM dNTP's, 1.5 mM $MgCl_2$, 10 µl AccessQuick™ Master Mix (supplied by Promega as part of kit), 0.5 µM of primers Pc230 (O1) and Pc230 (O2), and 6 µl AccessQuick™ H_2O (Promega).

The RT-PCR reaction and subsequent amplification of cDNA took place under the following conditions, reverse transcription at 50°C/60 minutes, denaturation at 94°C/3 minutes, 39 cycles of amplification [94°C/30 seconds, 61°C/30 seconds, 70°C/6 seconds], and a terminal extension at 70°C/600 seconds. Simultaneously an aliquot of 2 µl RNA from each sample were subjected to the same PCR protocol using the primers and the above conditions minus reverse transcriptase, to detect any carry over genomic DNA that might have been co-extracted with the RNA samples. Positive RNA controls, positive DNA controls and negative controls (all reagents + 2 µl water) were also included in each run. All outer RT-PCR products were diluted 1/10 then underwent a second PCR reaction under the same conditions as outlined for the PCR of DNA.

2.3.5. Detection limits of PCR and RT-PCR

To determine the sensitivity of nested PCR and RT-PCR, samples of known parasite and gametocyte density determined by microscopy (parasites or gametocytes/µl of blood) of *P. chabaudi* were serially diluted. Eight independent samples of extracted DNA were subjected to 10-fold serial dilutions creating a dilution series ranging over seven orders of magnitude from undiluted (neat) to diluted $1/10^7$ (actual parasite density range tested: 1.72×10^5 - 4.3×10^{-3} parasites/µl). Since nested PCR of DNA amplifies genomic DNA from all stages of the parasite, counts of both ring stage parasites and gametocytes were used to determine parasite densities from thin blood smears. To establish the sensitivity of RT-PCR, eleven samples of RNA were subjected to 10 fold serial dilutions, creating a dilution series ranging from neat to diluted $1/10^7$ for 7 samples. None of samples 1-7 were positive below a dilution of 10^{-5}, so four more samples were diluted only to $1/10^6$. The overall dilution range tested for RT-PCR was 1.3×10^4 - 1.0×10^{-4} parasites/µl.

2.3.6. qRT-PCR

A qRT-PCR technique was performed to amplify and quantify RNA coding for the gametocyte specific protein Pcs230, using the Applied Biosystems TaqMan® One-Step RT-PCR Master Mix Reagents Kit, on the ABI Prism® 7000 sequence detection

system. Two primers (Pc230RT-1, 5'- TCT AGT ACA TGC TTT GAA GAA GTA ATA GAA TAT AAT CCT AAT A - 3', and Pc230RT-2, 5'- TGC AAC GAC TTT CTA TAG CTA GTA GGT - 3') and a fluorescent labelled probe (Pc230RT-probe, 5'- AAA AAT GGG ATC GAA ATA AAA - 3') were designed by Applied Biosystems Assays-by-Design^{SM} Service from the *pcs230* gene sequence. As part of this service, Applied Biosystems optimized all primers and probe amplification and annealing conditions and combined them in a master mix.

A one-step qRT-PCR assay was used to generate cDNA from RNA and the cDNA quantified, in a single reaction (Applied Biosystems). This method assumes that reverse transcription takes place at a constant rate between all samples (Nirmalan *et al.* 2002). The reaction mix consisted of 8.5 µl of extracted RNA, 1 µl 20X Assay-by-Design Mix containing primers and probes at a reaction concentration of 900 nM and 250 nM respectively (supplied by Applied Biosystems), and the following components from the Applied Biosystems TaqMan® One-Step RT-PCR Master Mix Reagents Kit: 10 µl 2X AmpliTaq Gold® DNA Polymerase mix (contains AmpliTaq Gold® DNA polymerase, dNTP's with dUTP and buffer components), 5 µl 40X RT enzyme mix (contains MultiScribe™ Reverse Transcriptase and RNase Inhibitor).

Samples were loaded onto 96 well optical reaction plates and placed in the ABI Prism® 7000 Sequence Detection System (Applied Biosystems), then underwent the following reaction conditions: reverse transcription at 48°C for 30 minutes, denaturation at 95°C for 10 minutes, and 50 cycles of amplification [95°C for 15 seconds and 60°C for 60 seconds]. Data was visualized using the ABI Prism 7000 SDS Software (Applied Biosystems). Simultaneously, 8 RNA samples underwent conventional nested PCR reaction using primers Pc230 (O1), Pc230 (O2), Pc230 (N1) and Pc230 (N2) to test for DNA contamination.

2.3.7. Validation of qRT-PCR

The qRT-PCR technique was validated by comparing threshold cycle values (number of cycles after which a sample crosses the threshold of positivity) generated from serial dilutions of samples of known gametocytemia. I expected the threshold

cycle to be negatively correlated with the number of gametocytes present in a sample, showing the same value when the numbers of gametocytes in two different samples are equal. Therefore, a strong correlation in best fit lines for threshold cycle versus gametocyte number generated from serial dilutions with known numbers of gametocytes, will demonstrate that gametocytes are accurately quantified. To further test the robustness of the method, I compared the accuracy of threshold cycle values calculated for two different types of serial dilutions, performing replicates of each set.

The first set of dilution series was prepared blindly with extracted RNA from samples of known gametocyte counts (determined after qRT-PCR, via microscopy). Eleven, ten-fold serial dilutions (neat to $1/10^7$) of extracted RNA (from 3 different mice) diluted in water were prepared. A second type of dilution curves were setup blindly to dilute actual parasites by using uninfected blood from NIH mice to dilute samples of *P. chabaudi* infected whole blood with known gametocyte counts (numbers calculated after qRT-PCR, via microscopy). Two, independent, ten-fold serial dilutions ranging from neat to $1/10^7$ were generated in this manner and the dilution series samples were extracted on the ABI Prism® 6100 Nucleic Acid PrepStation with the RNA Blood-DNA method (Applied Biosystems). The dilution of samples with the uninfected whole blood method more accurately simulates quantification of gametocytes from mice harbouring different levels of gametocyte density, and therefore accurately demonstrates the sensitivity of extraction as well as qRT-PCR.

All 13 sets of RNA dilutions were quantified for *pcs230* mRNA using the TaqMan® One-Step RT-PCR Master Mix Reagents Kit on the ABI Prism® 7000 sequence detection system (Applied Biosystems). The data from all curves was pooled and analysed using Minitab statistical software (version 13.1). Threshold cycle values were regressed against the $\log_{10}$ of the number of gametocytes within samples using ANCOVA. All sets of serial dilutions were compared using a regression to estimate the validity of the qRT-PCR method.

2.4. Results

2.4.1. Detection of *P. chabaudi* infection and gametocytes

Conventional PCR of genomic DNA showed a mean minimum detection limit of 3.68 ± 2.11 (± 1 S.E.) parasites/µl blood. With regard to gametocytes, RT-PCR was found to detect a mean minimum of 27.1 ± 11.7 (± 1 S.E.) gametocytes/µl blood. However, in 5 out of 11 sets of serial dilutions of gametocytes infected blood, qualitative RT-PCR detected down to 1 gametocyte/µl of blood. These detection limits are similar to those found for the detection of *P. falciparum* asexual forms and gametocytes (Roper *et al.* 1996; Babiker *et al.* 1999a). In general the sets of dilution series that started with initial higher gametocytemia resulted in RT-PCR product at lower dilutions (Figure 2.1). In one case a neat sample did not give an RT-PCR product, likely due to pipetting error.

Overall, significantly more blood samples were found to harbour *P. chabaudi* infection revealed by PCR ($\chi^2 = 16.9$, $P < 0.0001$, d.f. = 1), and gametocytes detectable by RT-PCR ($\chi^2 = 24.4$, $P < 0.0001$, d.f. = 1), than by microscopy. Across the 424 blood samples examined, 161 (38%) were gametocyte-positive by nested RT-PCR, whereas only 97 (23%) were gametocyte-positive by microscopy. There were 14 instances (3.30%) where gametocytes were detected by microscopy but not with nested RT-PCR, likely to be due to failed RNA extraction. PCR product from DNA was seen in 110 (27%) out of 405 blood samples when RT-PCR for gametocyte RNA was negative. Because both gametocytes and asexual parasites contain DNA but only gametocytes contain Pc230 RNA, we believe these samples represent cases where asexual parasites were present but gametocytes were absent. Additionally, it has been shown that PCR of parasite DNA is not possible after the parasites have been cleared by the host (Jarra and Snounou 1998).

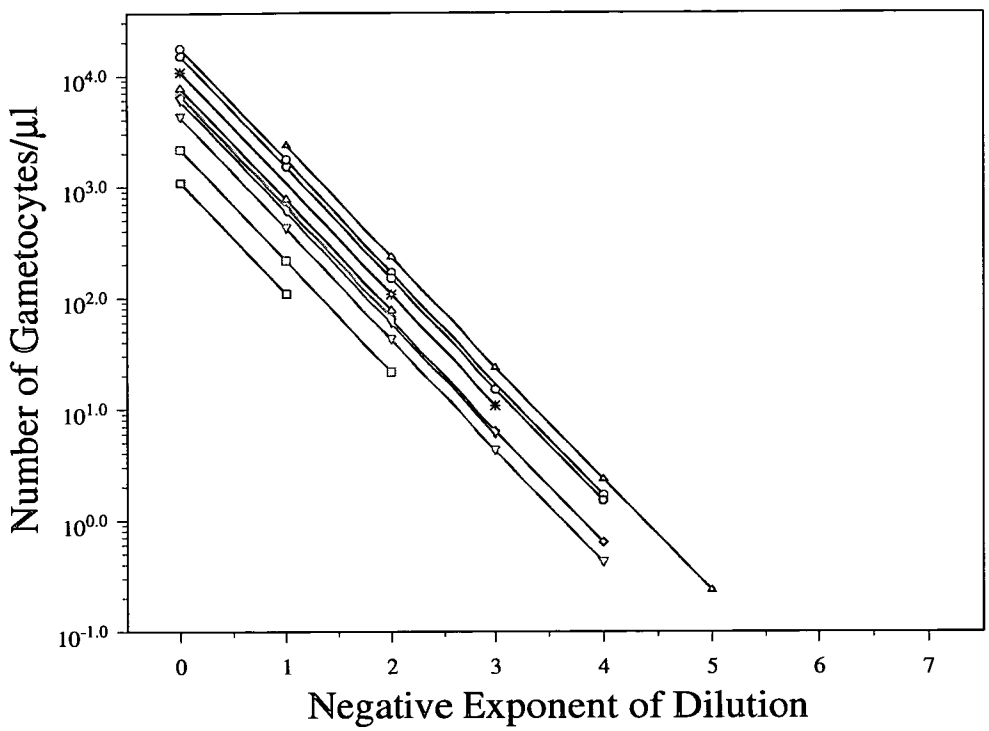


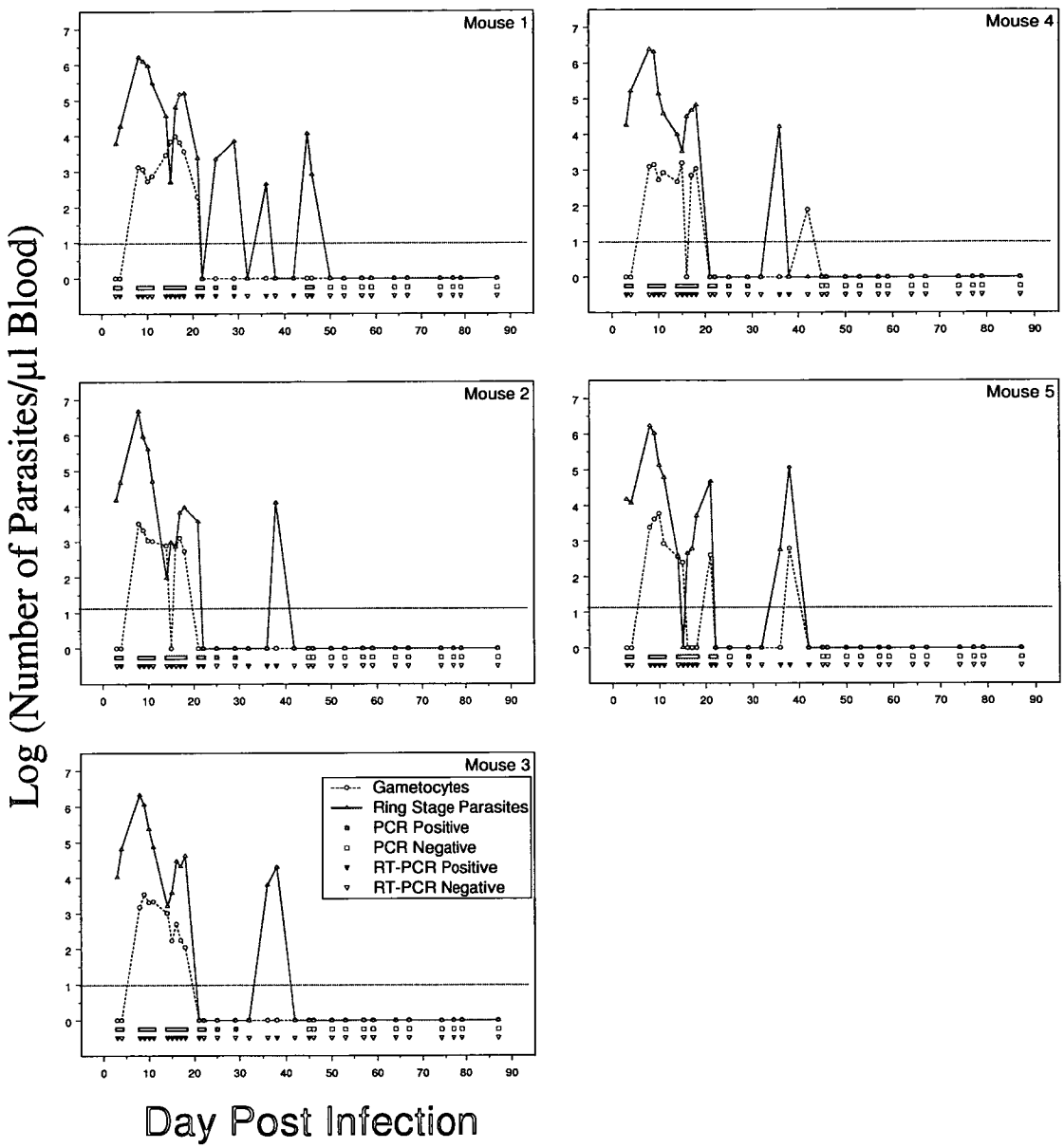
Figure 2.1. Detection level of gametocytes with RT-PCR of RNA coding for the *Pcs230* gene. Each line represents a separate dilution series of independent samples. Y-axis shows number of gametocytes in the sample estimated by microscopy. X-axis shows the position of the sample in the dilution series with 0 being the neat (undiluted) sample and 7 being the seventh dilution in the series (i.e. the neat sample diluted 10^{-7}). A dilution series up to 10^{-7} was performed on seven samples and up to 10^{-6} on four samples. Where symbols are present positive RT-PCR was obtained. Where symbols are absent, RT-PCR was negative. In one case RT-PCR on the undiluted sample was negative, likely due to pipetting error.

2.4.2. Persistence of *P. chabaudi* asexuals and gametocytes from longevity experiments

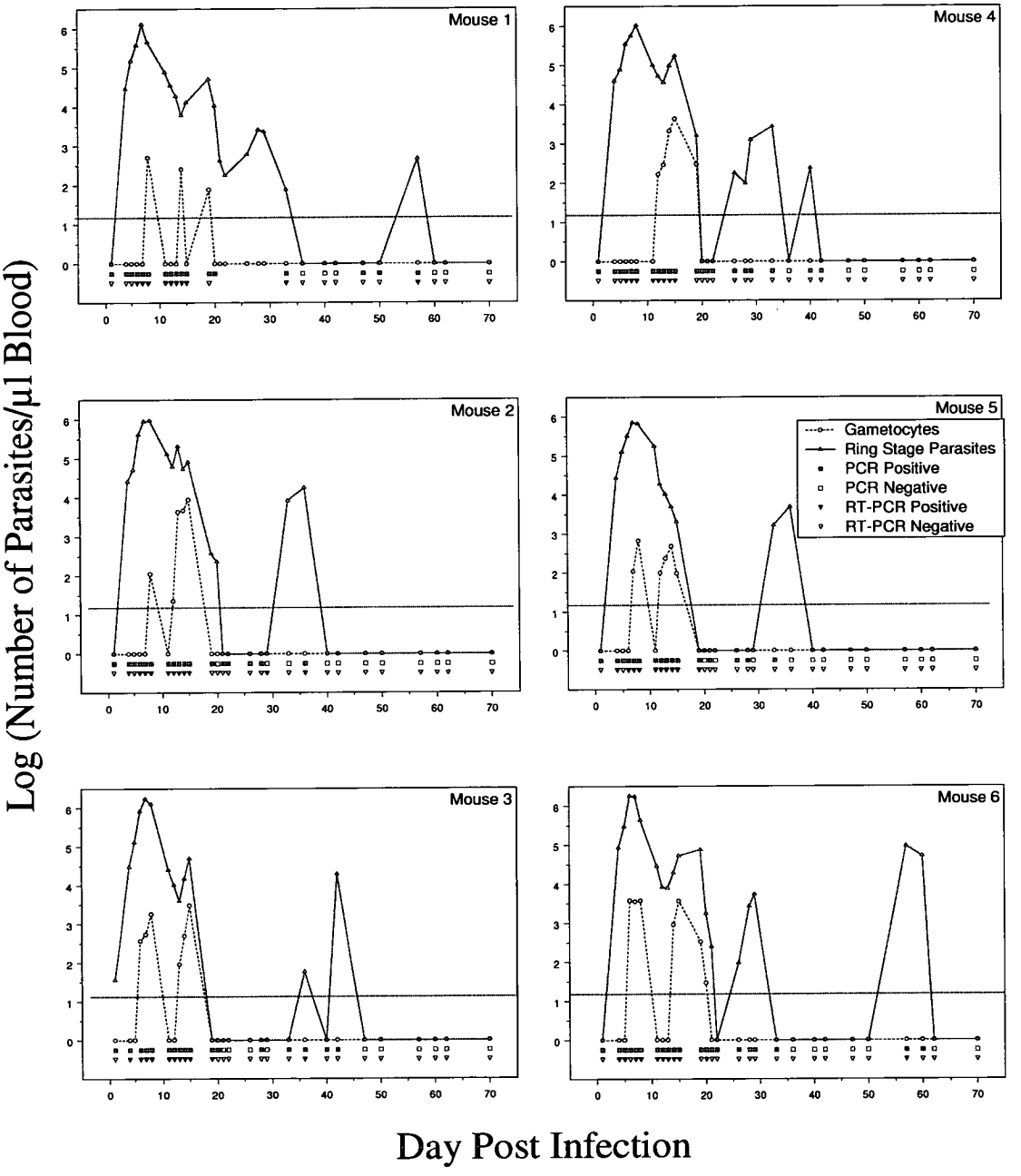
Figure 2.2A and 2.2B show *P. chabaudi* parasite density in chronic infections revealed by microscopy, nested PCR, and RT-PCR, for experiment I and experiment II respectively. In both experiments gametocytes were often detected using RT-PCR several days after they were no longer detectable by microscopy. Mice controlled initial parasitemia after about three weeks. Subsequently, the infections showed one or a few transient peaks of patent parasitemia that recrudesced between days 30-60, during which time gametocytes were frequently only detectable by RT-PCR. In most mice parasites were not detected after day 50 PI and none were detectable either by microscopy or molecular methods after day 60. The median time (± 1 SE) to clearance of gametocytes (i.e. time for 50% of mice to clear gametocytes) was 36 ± 3 days derived by RT-PCR compared to 18 ± 2 days by microscopy (Kaplan-Meier test).

Figure 2.2 (see over-page). *P. chabaudi* asexual forms and gametocytes detected by microscopy, PCR and RT-PCR for mice from experiment I (A) and II (B). Solid lines and dotted lines represent asexual stages and gametocytes density respectively, as determined by microscopy. Squares show qualitative PCR for respective days, filled squares are positive samples, empty squares are negative samples, absence of a square means no sample taken. Triangles represent qualitative RT-PCR samples for *Pcs230* gene on respective days, illustrated in the same manner. PCR and qualitative RT-PCR were more sensitive for detecting asexuals and gametocytes than microscopy as indicated by filled squares and triangles respectively. No RNA samples showed an RT-PCR product when reverse transcriptase was omitted, thus excluding DNA contamination during RNA extraction. (Only mouse 4 - experiment I, and 1 - experiment II, shown in published paper).

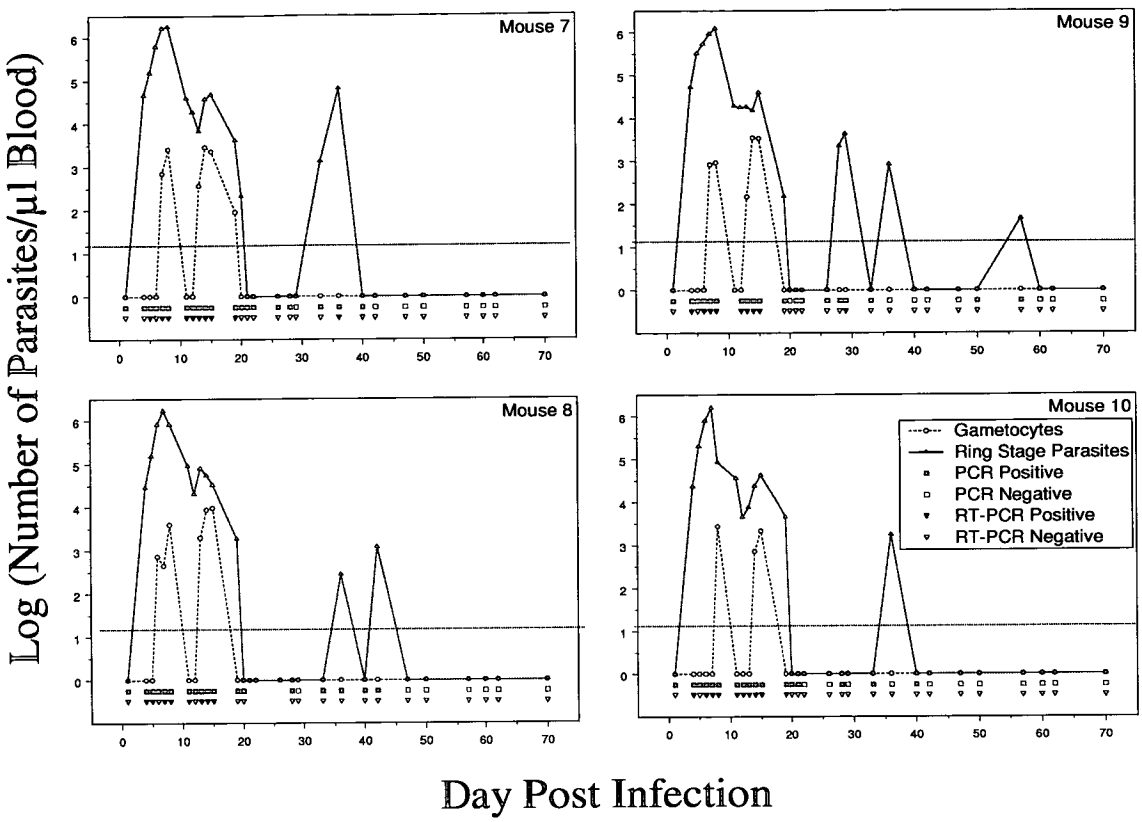
A. Experiment I



B. Experiment II



B. Experiment II - Continued



2.4.3. qRT-PCR

The qRT-PCR technique for gametocyte specific *pcs230* RNA in 13 separate serial dilutions of gametocytes, detected approximately 1 gametocyte per reaction, corresponding to ≤ 1 gametocytes/µl blood (Figure 2.3). As expected, the number of gametocytes was highly related to the threshold cycle ($R^2 = 0.86$, $F_{1,32} = 174$, $P < 0.0001$), with no significant differences in the slope of the relationship between the 13 dilution series. Specifically there was no difference in slope between the 2 dilution series made from samples diluted in whole blood and 11 dilution series made from extracted RNA diluted in water ($P > 0.05$ in all cases). The common slope (± 1 S.E.) of the combined 13 dilution series was CT = - 3.20 (± 0.24). My results are very close to the optimum obtainable slope of -3.3 for PCR amplification during qRT-PCR.

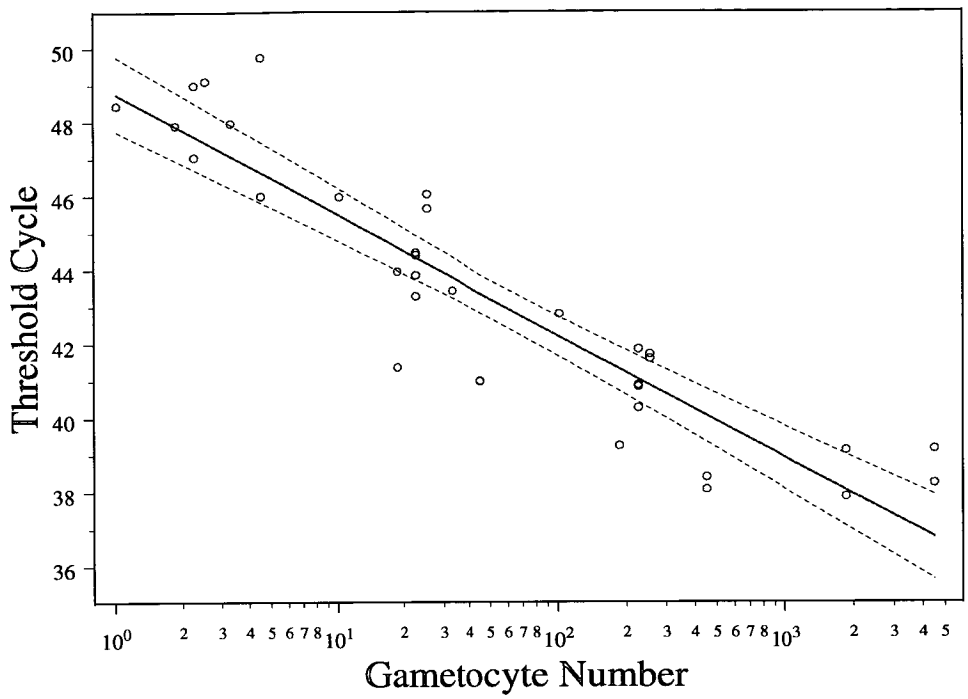


Figure 2.3. Threshold cycle (determined by qRT-PCR) and gametocyte number (determined by microscopy) for 13 dilution series (11 from diluted extracted RNA, 2 from dilutions of actual parasites with whole blood). The lines are the least squares regression line (solid) with 95% confidence intervals (dotted). There is a strong correlation between threshold cycle and gametocyte number ($R^2 = 0.86$, $F_{1,32} = 174$, $P < 0.0001$; slope ± 1 S.E. = - 3.20 ± 0.24). See text for further details.

2.5. Discussion

RT-PCR techniques to detect RNA encoding proteins that are expressed exclusively in *P. falciparum* gametocytes have previously been developed (Babiker *et al.*, 1999, Menegon *et al.*, 2000). Here I have extended this protocol and established robust and sensitive RT-PCR and qRT-PCR methods to monitor the persistence of *P. chabaudi* gametocytes throughout infection. The techniques are based on the amplification of *pcs230* mRNA, which is expressed exclusively in gametocytes (Niederwieser *et al.* 2000; Thompson *et al.* 2001; Hall *et al.* 2005).

I found that the qRT-PCR detection limit ranged from between 1 to 10 gametocytes/μl of blood making it 100 - 1000 times more sensitive than microscopy, with similar sensitivity levels to those found using QT-NASBA for *P. falciparum* gametocytes (Schneider *et al.* 2004). This very low limit of detection was reproducible in eleven independent sets of serial dilutions of gametocytes. Such very low gametocytemia would require an experienced microscopist to examine more than 1000 fields of a well-stained thick blood smear, which could take at least 50 minutes (Babiker *et al.* 1999a). Additionally, quantification by microscopy is often inaccurate when gametocytes persist at the low levels (< 0.01 gametocytes/ red blood cell) common in natural malaria infections (L. Crooks, *Transmission Investment in Malaria*, Ph.D. Thesis, University of Edinburgh, 2004)(Taylor and Read 1997). Thus, comparatively, the qRT-PCR technique for gametocytes presented here provides a specific and highly sensitive estimate to gametocyte density, especially at low levels of gametocytemia. It is theoretically possible that different stages of gametocytes may express varying levels of *Pcs230* RNA. However, even when using blood samples from mice on different days post inoculation, our results showed a strong correlation between expected and observed gametocyte values, showing that expression level is not a source of a large degree of error.

Overall, my protocol is time efficient and capable of processing 96 samples in 8 hours. It therefore has great epidemiological potential, and can easily be adapted to study human malaria parasites. This method differs from current quantification

protocols in that it is to my knowledge the first to quantify *P. chabaudi* gametocytes. Since this technology only requires the design of probes and primers and does not require the use of gene constructs as internal standards, assays can rapidly and cheaply be developed. In addition, by utilizing polymorphic gametocyte specific protein genes, the presented method can easily be adapted for tracking an individual gametocyte genotype when the infection is composed of multiple genotypes. Through the application of newly described sex specific gametocyte protein genes (Khan *et al.* 2005), the method can also be developed for the individual quantification of male or female gametocytes.

I used the developed RT-PCR technique to monitor longevity of gametocyte production of two *P. chabaudi* clones. My results demonstrated that *P. chabaudi* persisted at sub-patent levels, and continued to produce gametocytes for several weeks as chronic infections. This result agrees with that seen in natural *P. chabaudi* infections in the natural host *Thomnomys rutilans*, where chronic infections lasting up to 2 years are often observed (Landau *et al.* 1999). In the present study *P. chabaudi* infection persisted for up to 60 days post-infection, however, the duration of our study was limited to 87 days, and therefore it is possible that additional parasite recrudescence phases may have been missed. In addition all infections were composed of a single *P. chabaudi* clone, whereas, in a recent study of *P. chabaudi,* infections consisting of 3 clones were found to persist longer than did single-clone infections (De Roode *et al.* 2003).

The techniques presented here allow a range of hypotheses to be tested. For instance, chronic persistence of malaria infections in the face of sustained immune attack is probably due to antigenic variation by parasites (Phillips *et al.* 1997). Antigenic variation is often viewed as an adaptation to enhance transmission (Antia *et al.* 1996). Techniques such as that presented here make it possible to determine when transmission stages are present in chronic infections and hence the extent to which antigenic variation can actually contribute to parasite fitness. Of greater practical significance to malaria epidemiology are the findings that chemotherapy led to increased rates of gametocytogenesis in acute infections of *P. chabaudi* and increased gametocyte density

in infections of *P. falciparum* (Buckling *et al.* 1997; Buckling *et al.* 1999b; Buckling *et al.* 1999c); if the same occurs in chronic asymptomatic infections, inappropriate drug use could enhance malarial transmission. Moreover, multi-clone infections are the rule rather than the exception in malaria (Babiker and Walliker 1997; Awadalla *et al.* 2001).

The dynamics of individual clones in mixed infections are complex (Bruce *et al.* 2000; Read and Taylor 2001; De Roode *et al.* 2005b) and the transmission consequences of these dynamics very poorly understood. I am further developing allele-specific gametocyte qRT-PCR, which will make it possible to track gametocyte densities of individual clones in multiple-clone infections (Chapter 4). Such studies will shed more light on the dynamics of malaria parasites and assess biological and environmental factors that influence their gametocytogenesis in nature (Price *et al.* 1999; Bousema *et al.* 2004). Gametocyte-specific quantification thus makes it possible to conduct detailed studies of malaria transmission biology and should help to improve predictions of malaria parasite responses to control measures.

3. TRANSMISSION IN CHRONIC MALARIA INFECTIONS

3.1. Abstract

In many malaria endemic regions individuals develop long lasting chronic infections characterized by extremely low parasite densities. These chronic infections are believed to be the source of seasonal malaria epidemics. However, the degree in which low density infections transmit to vectors remains unclear. In this study I concurrently examined the infectivity of acute and chronic stage infections of the rodent malaria *Plasmodium chabaudi*, through mosquito blood feeds. I found that acute stage infections transmitted to a higher proportion of mosquitoes than chronic stage infections. Despite extremely low parasite densities, chronic infections were still able to transmit to mosquito vectors. Additionally, although acute infections had higher gametocyte and parasite densities, transmission investment and gametocyte infectivity was greater in the chronic stage. My results confirm findings from the field that suggest low level chronic infections are likely an important reservoir for malaria transmission, especially when considering their long duration and potentially high lifetime exposure to vectors.

3.2. Introduction

Unravelling the epidemiology of malaria transmission is critical for successful control strategies. In particular, understanding which cohorts of the population are contributing the most to transmission will help to focus currently overstretched disease prevention efforts. It is largely believed that the bulk of malaria transmission results from high parasitemia acute infections commonly occurring in young children (Githeko *et al.* 1992; Kolk *et al.* 2003). However, in many malaria endemic regions, individuals commonly develop long lasting chronic infections, which are shaped by parasite antigenic variation, host immunity, and seasonal variation in vector prevalence (Su *et al.* 1995; Antia *et al.* 1996; Bottius *et al.* 1996; Druilhe and Perignon 1997; Babiker 1998; Babiker *et al.* 1998; Roper *et al.* 1998; Snounou *et al.* 2000; Abdel-Wahab *et al.* 2002; Hamad *et al.* 2002; Nassir *et al.* 2005). Since chronic infections can last up to several years, they may contribute heavily to disease persistence (Drakeley *et al.* 2000).

Recent data shows that chronic malaria infections are often characterized by extremely low parasite densities which can only be detected through molecular methods (Bottius *et al.* 1996; Roper *et al.* 1996; Babiker *et al.* 1999a; Schneider *et al.* 2006). It is theoretically feasible that these parasite densities are so low they are non-infectious to vectors, particularly when further suppressed by transmission blocking immunity, and indeed some studies support this (Jeffery and Eyles 1955; Mulder *et al.* 1994; Buckling and Read 2001). Alternatively, there is evidence from field data that low level chronic infections may be responsible for triggering seasonal epidemics (Abdel-Wahab *et al.* 2002; Paul *et al.* 2004a; Nassir *et al.* 2005). Additionally, a recent field study of random mosquito feeds after drug treatment, found that a higher proportion of individuals were infectious than those with detectable parasites, even by sensitive molecular methods (Bousema *et al.* 2006). If chronic infections are contributing significantly to the transmission reservoir, then their clearance is essential for control of the parasite. However, if low level parasitemia contributes insignificantly to transmission, treatment comes at an unnecessary financial and resistance evolution cost, particularly when considering that most chronic infections are asymptomatic (Hamad *et al.* 2000).

To directly test the importance of chronic infections for malaria transmission, it is ideal to carry out simultaneous mosquito feeds on individuals maintaining low density chronic and high density acute stage infections, to control for temporal variation in mosquito susceptibility. This type of controlled experiment proves very difficult in a field setting due to chronic and acute infections typically appearing at different times of the year. Therefore, I experimentally investigated the level of transmission from each infection stage, utilizing the laboratory rodent malaria system *Plasmodium chabaudi*.

Like many malaria parasites, including those which infect humans, *P. chabaudi* infections are comprised of both acute and chronic phases (Garnham 1954). The acute phase occurs over the first fortnight of infection and is characterized by a high parasitemia peak which is rapidly controlled by the host. Following the acute phase, infections enter the chronic phase, composed of small recrudescent waves in parasitemia, in which transmission stage gametocytes are intermittently produced (Landau *et al.* 1999; Wargo *et al.* 2006). Similar to human *P. falciparum* infections, during this chronic phase individuals are frequently misdiagnosed as uninfected because parasites persist at very low levels or are sequestered as latent forms in organs, sometimes rendering them undetectable even by highly sensitive molecular methods (Bousema *et al.* 2006; Wargo *et al.* 2006).

I performed concurrent mosquito blood feeds on mice harbouring either acute or chronic stage infections, and then assessed the proportion of mosquitoes infected and mean oocyst burden per infected mosquito, as an indicator of infectivity. I also determined the number of total parasites and gametocytes on the day of the vector feed through qPCR and qRT-PCR, respectively. This enabled me to investigate the transmission investment of parasites in acute and chronic stage infections. I found that acute stage infections had higher asexual densities, gametocyte densities, and transmission to mosquitoes than chronic infections. Despite infecting very few mosquitoes, transmission investment and per gametocyte infectivity was greater in the chronic stage. My results confirm findings from the field that chronic infections are potentially an important malaria reservoir and may have high lifetime transmission due to their long persistence.

3.3. Methods

3.3.1. Parasite, host and vector

In this study I used the rodent malaria parasite *Plasmodium chabaudi* clone CR originally isolated from thicket rats, *Thamnomys rutilans* (Beale *et al.* 1978). This *P. chabaudi* clone was chosen due to its maintenance of long term chronic infections in laboratory mice and generally high infectivity to mosquitoes (Taylor *et al.* 1997a; Ferguson *et al.* 2003; Wargo *et al.* 2006). Hosts were 6-8 week old female C57bl/6J mice which were fed on 41B maintenance diet (Harlan, U.K.) and 0.05% para-amino benzoic acid supplemented drinking water to enhance parasite growth (Jacobs 1964). Vectors were the mosquito species *Anopheles stephensi*, maintained at 25-28°C, 70-80% humidity and fed on 10% glucose in water, supplemented with 0.05% PABA as described elsewhere (Ferguson *et al.* 2003).

3.3.2. Assessment of in-host and transmission dynamics

To compare in-host and transmission dynamics of acute and chronic phase infections, I performed mosquito feeds on 21 mice harbouring infections between 5-8 days post inoculation, and 21 mice between 27-44 days post inoculation, which had previously been infected with 10^6 clone CR parasites, as described elsewhere (Mackinnon and Read 1999a). These infection periods correspond to the acute (0 – 14 days P.I.) and chronic stage (21+ days P.I.) of *P. chabaudi* infections, in which parasites are regularly detected (Wargo *et al.* 2006). Mosquito feeds were performed on individual mice on 4-8 anesthetised mice per day, with roughly equal numbers of chronic and acute phase mice used each day (Figure 3.1). Between 30-65 adult female mosquitoes, 2-8 days past emergence, were fed per mouse for 30 minutes and I subsequently removed all mosquitoes that did not take a blood meal.

On the day of mosquito feeds, thin blood smears and qRT-PCR for the gametocyte specific gene *pcs230* (Wargo *et al.* 2006), were used to ensure the presence of gametocytes before the feed occurred. To determine parasite and gametocyte density at the time of transmission, 20 µl and 5 µl of mouse blood were taken immediately after

the blood feed. I do not believe that mosquito feeding altered asexual parasite or gametocyte numbers (Shutler *et al.* 2005). For quantification of gametocytes, parasite RNA was extracted from 20 µl blood and subject to qRT-PCR of the gametocyte specific gene PC302249.00.0 (forward primer: 5'- GCC CGA ATC ATT ATG TTT TAC TAT AAT GG; reverse primer: 5'- AGA TAA AAT TGC ATA TAT TCC TAA AGT ACT ATC TAC; TaqMan probe: 5'- FAM - CAA AGT AGA CAA TGC ACC AAT - MGB) as described previously (Chapter 2). For quantification of total parasites, DNA was extracted from 5 µl of blood and subjected to qPCR (forward primer: 5'- GAA GAT GCA CGT GTG GAA AAT CA; reverse primer: 5'- GAA TAG TTA TAC CTT TTC CCA TAA CT, TaqMan probe: 5'- FAM - CAT TTA CCT GAA GGT ATT CTA - MGB) as described elsewhere (Bell *et al.* In Press).

To assess transmission success to vectors, mosquito midguts were dissected 9 or 10 days after the blood feed, once oocysts had formed but before their rupture and the release of sporozoites (Killick-Kendrick *et al.* 1978). The number of oocysts were counted at 100x magnification to determine the proportion of mosquitoes infected and the average number of oocysts per infected mosquito. Dissection day had no effect on oocyst detection.

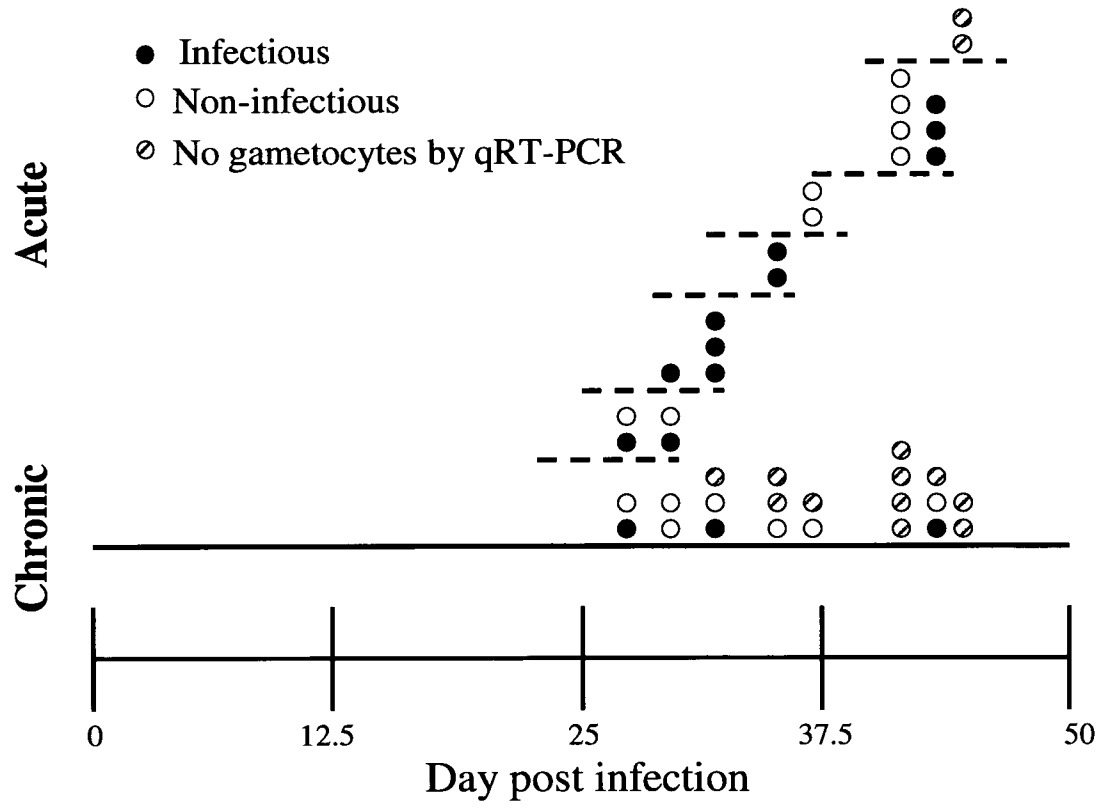


Figure 3.1. Schematic timeline of day post infection in which mosquito feeds occurred. All mice harbouring chronic infections (solid line) were inoculated on the same day, whereas, inoculation of mice harbouring acute infections (dotted lines) was staggered. The day inoculation occurred is represented by the left edge of each line. Mosquito feeds were performed on batches of mice from each infection stage on different days, as shown by circles above respective sampling group. Both infectious (filled circle) and non-infectious (open circle) mice are shown. Thirteen mice (chronic = 11, acute = 2) were later found by qRT-PCR to have no gametocytes (circles with grey stripes) and were dropped from all analysis, as none of these were infectious to mosquitoes.

3.3.3. Statistical analysis

To determine infectivity, the proportion of mosquitoes infected and the number of mice which were infectious in acute versus chronic infections were compared using Mann-Whitney U and chi-square tests, respectively. When comparing gametocyte production in relation to asexual parasites, general linear model analysis was conducted, with infection type as the explanatory variable (acute verses chronic). Parasite densities were log transformed and proportions arcsin-square-root transformed, to meet the assumptions of normality and homogeneity of variance. For the assessment of transmission as a function of gametocyte density, I only utilized positive infections to avoid confounding the analysis with zeros and false negatives, this did not change the observed trends. Since variances were unequal between the two infection stages in this analysis, non-parametric Mann-Whitney U-tests and Spearman's rank correlations were implemented. All statistical tests were carried out in Minitab version 13.

3.4. Results

Thirteen mice (chronic = 11, acute = 2) were found to have zero gametocytes at the time of the blood feed by qRT-PCR (Figure 3.1). This was likely due to host clearance. None of these mice were infectious to mosquitoes and therefore they were dropped from all analyses. Below I refer to the 29 mice remaining with detectable gametocytes. In two mice, parasites were not detectable by qPCR, but gametocytes were detectable by qRT-PCR. This is not surprising considering qRT-PCR is one to two orders of magnitude more sensitive than qPCR. These parasite density values were estimated to be just below the detection level of qPCR (1000/µl blood). Since typically less than 1% of qPCR counts reflect gametocyte densities, for clarity below I refer to counts derived from qPCR as asexual densities and counts from qRT-PCR as gametocytes.

3.4.1. Dynamics from all mice harbouring acute or chronic infections

In general, transmission success was very low, resulting in only 13% (152/1167) of mosquitoes becoming infected. The mean proportion of infected mosquitoes was much greater from blood feeds on acute versus chronic stage infections (Figure 3.2, Man-Whitney U-test: $W = 95$, $P = 0.033$, N = 19, 9). However, there was no significant difference in the number of mice that were infectious to mosquitoes between the two infection stages (Figure 3.2, chronic: 3/10, acute: 11/19; $\chi^2 = 2.04$, $P = 0.15$, d.f. = 1).

On the day of mosquito blood feeds, higher asexual and gametocyte densities were observed in acute compared to chronic infections (Figure 3.3A; asexuals: $F_{1,27} = 68$, $P < 0.001$; gametocytes: $F_{1,27} = 11$, $P = 0.003$). There was a strong linear positive relationship between asexual and gametocyte density (Figure 3.3B, $F_{1,26} = 35$, $P < 0.001$, $R^2 = 0.7$) which had a larger intercept in chronic compared to acute infections (infection type main effect: $F_{1,26} = 5.5$, $P = 0.03$; interaction with covariate: $F_{1,25} = 0.38$, $P = 0.543$). Thus, for a given asexual parasite density, gametocyte densities were greater in the chronic compared to acute stage. The percentage of parasites that were gametocytes was also higher in the chronic stage (Figure 3.3C, $F_{1,27} = 10$, $P = 0.004$), suggesting that parasites increase their transmission investment from 0.2% to 0.7%.

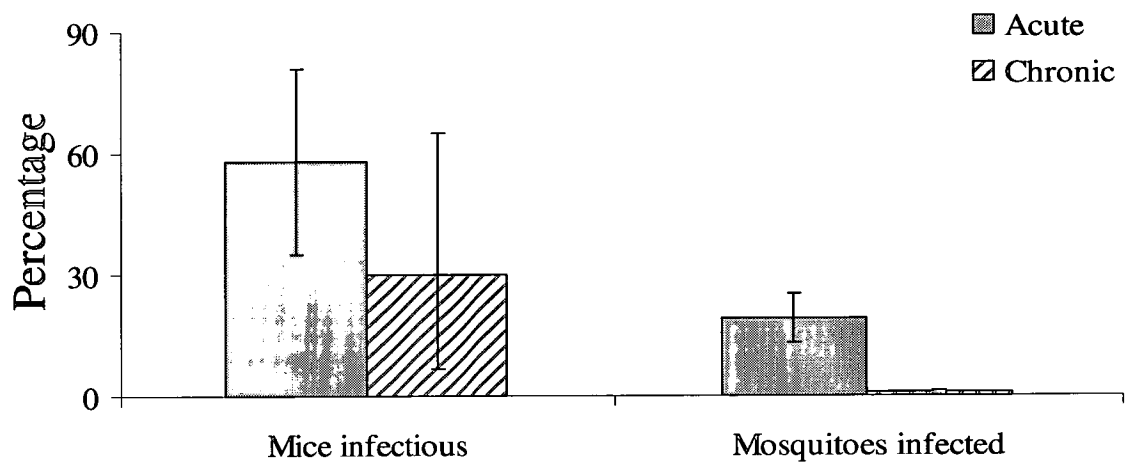


Figure 3.2. Infectivity of acute (grey bars) versus chronic (patterned bars) infection stages. The percentage of mice which transmitted (with 95% binomial confidence interval) to at least one mosquito (left), and the mean percentage of mosquitoes infected (± 1 S.E.M) of those which took a blood meal (right), are given. Infectivity was significantly higher in acute infections.

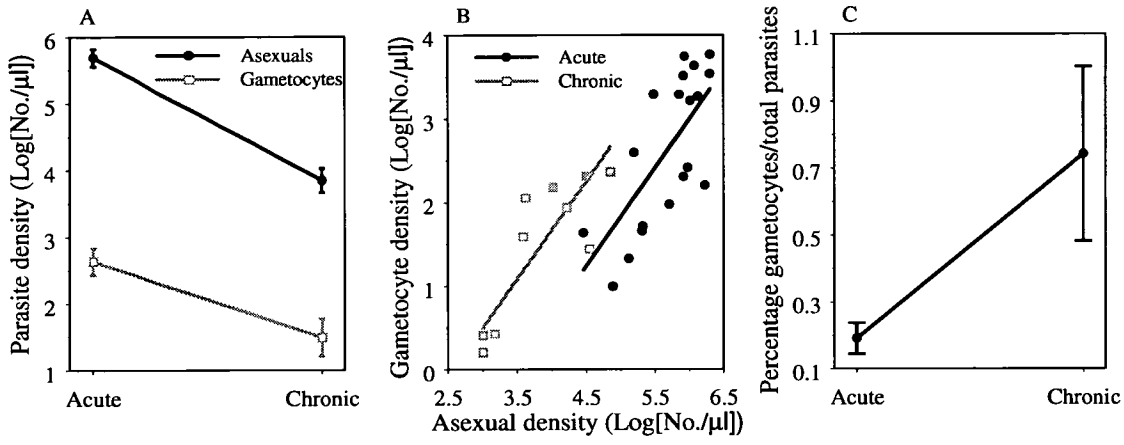


Figure 3.3. The relationship between asexual and gametocyte density in chronic and acute stage infections. (A) Log transformed mean (± 1 S.E.M) asexual (black line) and gametocyte (grey lines) densities were lower in chronic compared to acute infections. (B) There was a strong linear correlation between gametocyte and asexual density, the intercept of which was higher in chronic (grey line) compared to acute (black line) infections. Regression data is shown on log scale and best fit lines are given. (C) The mean percentage of total parasites that were gametocytes (± 1 S.E.M) also revealed higher gametocyte investment in chronic infections.

3.4.2. Dynamics from mice infectious to vectors

When examining just those mice which transmitted to mosquitoes, there was a slightly higher number of oocysts per infected mosquito (Figure 3.4A), and proportion of mosquitoes infected (Figure 3.5A), from blood feeds on acute versus chronic infections, although this difference was only significant for the proportion of mosquitoes infected (Man-Whitney U-test; oocysts/mosquito: $W = 90$, $P = 0.14$, N = 11, 3; proportion infected: $W = 97$, $P = 0.015$, N = 11, 3). There was a weak trend of greater numbers of oocysts (Figure 3.4B) and infected mosquitoes (Figure 3.5B) with increasing gametocyte density (Spearman's rank; oocysts, $Rs = 0.42$, $P = 0.13$; infected mosquitoes, $Rs = 0.76$, $P = 0.002$; N: acute =11, chronic = 3). Closer investigation of this relationship as a ratio, revealed that more oocysts (Figure 3.4C) and infected mosquitoes (Figure 3.5C) were observed for a given gametocyte density in chronic versus acute infections (oocysts: $W = 66$, $P = 0.006$, N = 11, 3; mosquitoes infected: $W = 71$, $P = 0.04$, N = 11, 3). Therefore, although acute infections were generally more infectious than chronic infections, there was some evidence that per gametocyte infectivity was slightly higher during the chronic stage.

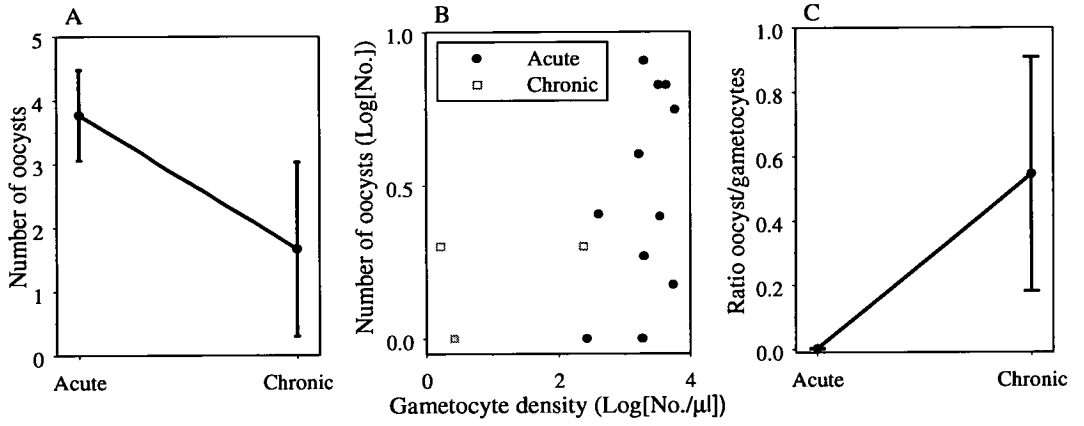


Figure 3.4. Number of oocysts in those mosquitoes which became infected. (A) The mean number of oocysts per infected mosquito (± 1 S.E.M) was not significantly greater in acute compared to chronic infections. (B) There was a weak trend of increasing numbers of oocysts per mosquito with higher gametocyte densities (Spearman's rank) on the day of the blood feed (both log transformed). (C) The ratio of mean oocyst number to mean gametocyte density (± 1 S.E.M) revealed that a given gametocyte density resulted in greater numbers of oocysts per mosquito, in chronic compared to acute infections (Mann-Whitney U-test). Only the 14 mice which were infectious to mosquitoes were included in these analyses (acute =11, chronic = 3).

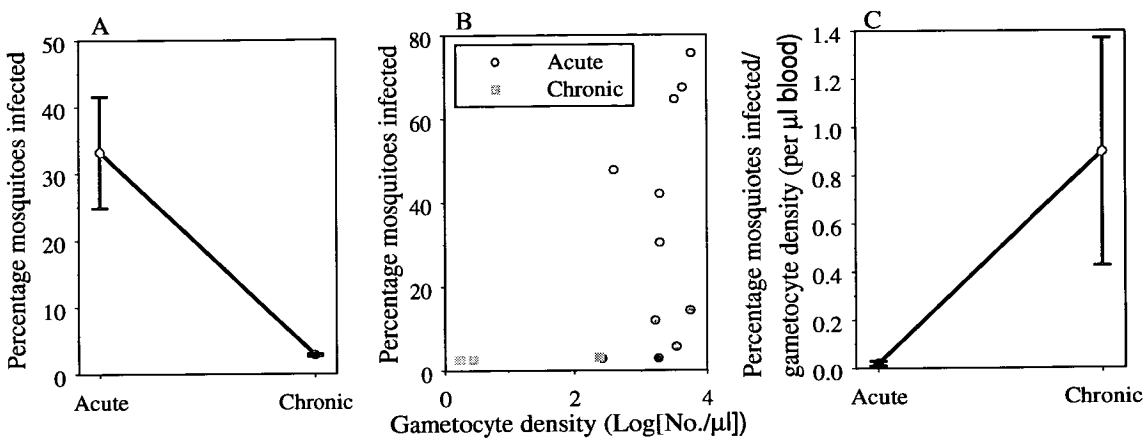


Figure 3.5. Percentage of infected mosquitoes from those mice which were infectious. (A) The mean percentage of infected mosquitoes (± 1 S.E.M) was greater in acute compared to chronic infections. (B) There was a general trend of increasing number of infected mosquitoes with higher gametocyte densities (Spearman's rank). (C) The ratio of mean percentage of infected mosquitoes to mean gametocyte density (± 1 S.E.M) revealed that a given gametocyte density infected greater numbers of mosquitoes in chronic compared to acute infections (Mann-Whitney U-test). Only the 14 mice which were infectious to mosquitoes were included in these analyses (acute =11, chronic = 3).

3.5. Discussion

Long duration, low parasitemia chronic infections which are common in many malaria endemic regions, could potentially be an important transmission reservoir (Babiker *et al.* 1998; Hamad *et al.* 2000; Abdel-Wahab *et al.* 2002; Nassir *et al.* 2005; Schneider *et al.* 2006). However, there is little direct information on whether transmission from such low parasitemia infections occurs and if so, to what degree this transmission will influence the epidemiology of the parasite (Jeffery and Eyles 1955; Paul *et al.* 2004a; Bousema *et al.* 2006). It is theoretically possible that mosquito vectors rarely become infected when taking a blood meal from individuals with parasite densities below a certain threshold.

Here, I conducted concurrent mosquito blood feeds on laboratory mice with acute or chronic stage infections of the rodent malaria *P. chabaudi* and examined direct transmission. I found that parasites in the acute stage infected more mosquitoes than those in the chronic stage. A very limited amount of transmission from chronic stage infections did occur, despite extremely low parasite and gametocyte densities. Additionally, although both gametocyte and parasite densities were higher in acute infections, chronic infections appeared to have greater transmission investment, in that a larger proportion of the circulating parasites were gametocytes. Gametocyte infectivity was also higher in chronic infections, demonstrated by a larger number of oocysts and infected mosquitoes for a given gametocyte density. The combined increased transmission investment and gametocyte infectivity could explain why despite lower gametocyte numbers in chronic infections, there was no significant difference in the proportion of mice which were infectious or the average oocyst number per infected mosquito, when comparing the two infection stages. In this case I cannot, however, rule out the possibility that no differences were observed due to low statistical power.

The mechanisms behind the observed increased gametocyte production and infectivity in chronic infections in this study are unknown. Both could feasibly be controlled by phenotypic plasticity in the parasite. It is well documented that malaria parasites can increase gametocytogenesis in response to environmental cues such as anemia and drug treatment (Gautret *et al.* 1996b; Buckling *et al.* 1999c; Nacher *et al.*

2002; Reece *et al.* 2005; Ali *et al.* 2006). Additionally, alterations in male to female gametocyte sex ratio to optimize fertility, and therefore vector transmission, have also been observed (Paul *et al.* 2000; West *et al.* 2002; Gardner *et al.* 2003; Reece *et al.* 2005). Similar parasite responses to cues such as infection duration, parasite density, immunity, or anemia, could conceivably be in operation in chronic infections.

It is also possible that gametocyte infectivity is influenced indirectly by host condition. Since parasite densities are higher in acute infections I would expect greater levels of red blood cell death and therefore anemia and immunity. Both of these factors have generally been linked to poor quality blood meals and low transmission to vectors (Carter *et al.* 1979; Gautret *et al.* 1996b; Paul *et al.* 2000; Paul and Brey 2003). To counteract these effects, increased gametocytogenesis, sex ratio alteration, and gametocyte infectivity, through the stimulation of erythropoietin (Epo), is believed to occur. However, I assessed acute infections very soon after parasite inoculation (day 5-8), just when anemia begins to take place and before Epo typically elicits a response (Gautret *et al.* 1996a; Gautret *et al.* 1996b; Reece *et al.* 2005). Even if increased transmission investment was occurring in acute infections, the response was clearly smaller than that elicited in chronic infections.

My findings strongly suggest that *P. chabaudi* has evolved strategies for adjusting resource allocation in chronic infections to optimize transmission in the face of extremely low parasite densities. The epidemiological impact of this appears to be very small due to the low proportion of mosquitoes becoming infected from mice in the chronic stage. However, it is important to note that transmission in this study was generally very poor. The reasons for this are unclear but may be partly influenced by *A. stephensi* not being the natural vector of *P. chabaudi*. Unfortunately, the natural vector of *P. chabaudi* is unknown. However, previous studies of *A. stephensi* as a vector for *P. chabaudi* have generally observed much higher transmission success (Mackinnon and Read 1999a). Why transmission was particularly low in my study is unclear, however, I assume that the confounding effects of reduced transmission influenced both infection stages equally, or if anything, underestimated the transmission potential of chronic infections.

A plausible explanation for why infectivity in chronic infections was so low in this study is the possibility of an upper limit to gametocyte investment and transmission which *P. chabaudi* can maintain. If parasite densities are constricted in chronic infections by the host immune response, and a certain threshold of asexual parasites must be maintained to continue persistence in the host, gametocyte density would be restricted to very low levels (Druilhe and Perignon 1997; Taylor and Read 1997). This might explain why even when at their highest per parasite densities in chronic infections, gametocytes still only made up 1% of the total parasites circulating in the host. A strategy around this could be for the parasite to elevate gametocyte production or aggregate when mosquito vectors are present and transmission is guaranteed (Gaillard *et al.* 2003; Paul *et al.* 2004a). One study with *P. chabaudi* did reveal increased gametocytogenesis in response to mosquito probing (Billingsley *et al.* 2005), but a more recent study observed no such response (Shutler *et al.* 2005), in neither case were chronic infections assessed.

It is also important to consider the role of transmission blocking immunity in shaping the epidemiological relevance of chronic versus acute infections. Studies vary on whether transmission blocking immunity waxes or wanes as the duration of the infection increases (Ranawaka *et al.* 1988; Fleck S. L. *et al.* 1994; Mulder *et al.* 1994; Taylor and Read 1997). Transmission blocking immunity is hypothesized to be stronger when gametocyte densities are higher (Naotunne *et al.* 1991; Boudin *et al.* 2004; Drakeley *et al.* 2006), but the proportion of parasites which are gametocytes in the blood stream may play an equally important role. I observed gametocyte infectivity was lowest during the acute phase where gametocyte densities were highest, and increased in chronic infections where gametocyte densities were lowest. Therefore, my results suggest that transmission blocking immunity is greater at the start of infection, and the effect wanes when densities drop below a certain threshold. This agrees with the results of Drakeley *et al.* (2006), who found higher transmission blocking immunity and gametocytemia in children, compared to adults.

In conclusion, these results suggest that, although acute infections are likely to contribute to the bulk of transmission, chronic infections may play an important role in the spread of disease, particularly due to their long duration and therefore increased potential for exposure to vectors. This could be particularly critical when mosquito densities are very low or fluctuate dramatically between widely spaced seasons. These findings support those from the field which suggest transmission from chronic infections maintains seasonal malaria epidemics (Abdel-Wahab et al. 2002; Paul et al. 2004a; Nassir et al. 2005). Therefore, the consideration and treatment of low level chronic infections may be critical for designing optimal control strategies and resource investment.

4. TRANSMISSION INVESTMENT OF MALARIA PARASITES IN RESPONSE TO IN-HOST COMPETITION

4.1. Abstract

Conspecific competition occurs in a multitude of organisms, particularly in parasites, where several genotypes are commonly sharing limited resources inside their host. This competition dynamic creates an evolutionary dilemma where genotypes face a trade-off between in-host replication and transmission. Theory is contradictory on whether increased or decreased transmission investment is the best strategy to maximize genotype fitness in the face of competition, and experimental evidence is limited. I developed and utilized a genotype-specific method of qRT-PCR to quantify *Plasmodium chabaudi* in-host replication and transmission stages in mixed-genotype infections, in two mouse strains. I found that in infections composed of an avirulent and virulent parasite genotype, competitive suppression of both genotypes occurred, with the virulent genotype obtaining overall competitive superiority. In response to competitive suppression, there was little evidence of an adaptive alteration in transmission stage investment, apart from a small shift of resources away from transmission towards in host replication by one clone, in one mouse genotype. The plasticity in commitment to transmission did not result in a competitive advantage, although it might have reduced the disadvantage. Generally, competitive suppression of blood-stage malaria parasites resulted in equally reduced transmission stage production. Thus, this study supports current literature, which predicts that conspecific within-host competition will result in a competitive advantage and positive selection for virulent genotypes and the evolution of virulence.

4.2. Introduction

Parasites exhibit a wide array of complex life cycles, which commonly involve different life stages for in-host replication and between-host transmission. Protozoan parasites for example, often undergo clonal expansion within their host and produce morphologically distinct propagules for transmission to vectors. Likewise, parasitic worms, such as cestodes, typically grow and mature within their host and then release eggs into the environment. Ultimately, resources available to a parasite must be divided between the production of transmission stages and in-host replication, thereby creating a replication-transmission trade-off, analogous to the more general growth versus reproduction trade-off found in many organisms (Perrin and Sibly 1993; Amarasekare and Nisbet 2001).

Several factors, such as environmental conditions, are likely to shape the life history strategies that parasites choose to maximize fitness while balancing the trade-off between replication and transmission (Gautret *et al.* 1996b; Buckling *et al.* 1997; Buckling *et al.* 1999b; Eisen and DeNardo 2000; Dezfuli *et al.* 2001; Mackinnon *et al.* 2002a; Osgood *et al.* 2003; De Roode *et al.* 2004a; Paul *et al.* 2004b; De Roode *et al.* 2005b; Vizoso and Ebert 2005; Michaud *et al.* 2006; Bell *et al.* In Press). How pathogens solve this fundamental life history dilemma will likely impact on medically and epidemiologically relevant traits (Day 2003), since disease severity is frequently a consequence of densities of in-host replication stages, and infectiousness a consequence of transmission stage densities.

Like many protozoan parasites, malaria is subject to a replication-transmission trade-off (Taylor and Read 1997). For much of its life cycle a malaria parasite undergoes asexual stage replication in the mammalian host. During asexual replication a small proportion of asexual forms become committed to develop into sexual stage gametocytes. Gametocytes are the only life stage infectious to vectors and they cannot replicate within the vertebrate host, therefore, their only contribution to parasite fitness is through transmission. Phenotypic plasticity in gametocyte production, in response to environmental changes such as drug pressure and anaemia, is commonly reported and

generally demonstrates higher gametocyte numbers with deteriorating in-host conditions (Buckling *et al.* 1999b; Buckling *et al.* 1999c; Nacher *et al.* 2002; Paul *et al.* 2004b; Ali *et al.* 2006).

An important environmental factor that malaria parasites face is the presence of genetically unrelated genotypes in the mammalian host, typically resulting in conspecific interactions (Babiker and Walliker 1997; Druilhe *et al.* 1998; Anderson *et al.* 2000; Read and Taylor 2001). There is now strong experimental and indirect epidemiological evidence that these interactions are often competitive, such that malaria genotypes suffer reduced replication in the presence of competing genotypes (Daubersies *et al.* 1996; Mercereau-Puijalon 1996; Arnot 1998; Paul *et al.* 2004b; De Roode *et al.* 2005b; Bell *et al.* In Press).

In-host competition is believed to play a critical role in the evolution of traits such as virulence, which is predicted to be positively linked with competitive ability (Mosquera and Adler 1998; Brown *et al.* 2002). However, transmission has rarely been examined as a component of competitive performance. In malaria there appears to be a broad correlation between the total proportional composition of parasite genotypes in the mammalian host and insect vector; however, the transmission investment of individual genotypes was not assessed (Taylor and Read 1998; De Roode *et al.* 2005b). This lack of transmission investment data can be partly attributed to that fact that, until very recently, it has been impossible to quantify clone frequencies in the malaria transmission stage population.

In response to competitive suppression it is plausible that genotypes will divert more resources into transmission to maximize fitness. An alternative strategy might be for parasite genotypes to shift resources away from transmission towards replication, so as to maximize in-host numbers and competitive success. Mechanisms to drive either of these responses in malaria are certainly feasible, as parasites could detect crowding in their environment, anaemia, or even the presence of other clones through genotype specific antigens (Yap and Stevenson 1994; Gautret *et al.* 1996b; Nacher *et al.* 2002; Paul and Brey 2003). Many theoretical studies have invoked such phenotypic plastic responses (Koella and Antia 1995; van Baalen and Sabelis 1995; Antia *et al.* 1996; West

et al. 2001) and they have been observed in experimental studies in some systems outside of malaria, typically suggesting increased transmission investment (Davies *et al.* 2002; Stockley and Parker 2002; Michaud *et al.* 2006).

Currently, there is not enough evidence to distinguish between increased or decreased transmission investment of malaria genotypes in response to conspecific competition. Unravelling these two theories requires a method for genotype-distinguishing stage-specific quantifications. Here I report on the development and utilization of a quantitative reverse transcriptase PCR assay (qRT-PCR) for clone-specific quantification of gametocytes, of a competing avirulent and virulent clone in mixed infections of the rodent malaria *Plasmodium chabaudi*. I estimated the transmission investment of each clone during conspecific competition in two mouse strains, using the qRT-PCR technique, as well as asexual growth and replication using a quantitative PCR (qPCR) assay (Bell *et al.* In Press). I found that each clone experienced competitive suppression of both asexual and transmission stage production in mixed infections. There was evidence that the less virulent clone shifted resources away from transmission investment towards in-host replication, in response to competition. This response only occurred in one mouse genotype and did not affect the overall performance of the clone, suggesting that competitive suppression of blood-stage malaria parasites results in reduced transmission to the insect vector.

4.3. Methods

4.3.1. Parasites and hosts

I used two genetically distinct *P. chabaudi* clones AJ and AS, originally isolated from thicket rats, *Thamnomys rutilans* (Beale *et al.* 1978). Clone AS reaches lower within-host densities, has a lower competitive ability and causes lower virulence than clone AJ (De Roode *et al.* 2005b; Bell *et al.* In Press). Hosts were 6-8 week female C57bl/6J and CBA/ca inbred mice (herein referred to as C57 and CBA) fed on 41B

maintenance diet (Harlan, U.K.) and water supplemented with 0.05% PABA to enhance parasite growth (Jacobs 1964). Mice were kept on a 12:12 h light-dark cycle.

I inoculated 14 C57 and 20 CBA mice per treatment with 10^6 AJ, 10^6 AS, or 10^6 AS + 10^6 AJ parasites (2 x 10^6 total parasites) as described elsewhere (Mackinnon and Read 1999a). I utilized equal numbers of each parasite clone for both single and mixed infections in order to compare the dynamics of each clone when it is alone versus in the presence of a competitor.

4.3.2. Monitoring infection dynamics

Total parasitemia (percentage of red blood cells infected) and gametocytemia were monitored from day 3-17 post infection using thin blood smears of tail blood fixed in methanol and stained in 10% Giemsa. To determine parasite and gametocyte density, flow-cytometry (Coulter Electronics) counts for red blood cell density were taken. Clone-specific parasite density of both AS and AJ in single and mixed infections was monitored using qPCR on 5 µl of whole blood taken each day of sampling, as described elsewhere (Bell *et al.* In Press). Since qPCR relies on the quantification of parasite DNA, it cannot selectively differentiate between parasite life stages and therefore provides an estimate of the total number of parasites circulating in the blood. To accurately quantify production of transmission stages, gametocyte density was monitored for clones AS and AJ individually on each sampling day, except days 3 and 9, using qRT-PCR as previously described (Chapter 2), but with the following minor modifications.

Briefly, for gametocyte qRT-PCR, 20 µl of mouse tail blood was sampled and placed in a 90 µl 1:2 volume mix of Ca^{2+}/Mg^{2+} free PBS (Gibco) and 2X Nucleic Acid Purification Lysis Solution© (Applied Biosystems). To prevent possible alterations in gene expression and degradation of RNA (Fang and McCutchan 2002), all blood samples were immediately placed in lysis buffer, vortexed gently and stored at -80°C. Total RNA was then extracted on the ABI Prism® 6100 machine as outlined elsewhere (Chapter 2). To generate cDNA, the extracted RNA underwent reverse transcription using the High-Capacity cDNA Archive Kit (Applied Biosystems) in a 50 µl reaction

containing 25 µl of RNA and 25 µl of kit reagents with the following components: 5 µl 10X RT buffer, 2 µl 25X dNTP mixture, 5 µl 10X random primers, 2.5 µl (5U/µl) MultiScribe reverse transcriptase, and 10.5 µl RNase-free H_2O). The reaction was then incubated on an MJ Research DNA Engine, PTC-200 at 25°C for 10 minutes followed by 37°C for 2 hours and 4°C hold, then stored at -80°C.

To complete gametocyte quantification, cDNA converted from RNA underwent qPCR on an ABI Prism® 7000 using genotype-differentiating forward primers for *P. chabaudi* clones AS (5'- AAG TTT ACC TGA GAG TAC AAA TAT AAT AGG TGT A - 3') and AJ (5'- TGA CAG TAC AAA TAT AAT AAG CGC AGT T - 3') in conjunction with a conserved reverse primer (5'- GCT GCT ATA CGT GTT ATA AAT CCT ATT ACT - 3') and TaqMan® MBG probe (5'- 6FAM – TGT TAT AAT TGT GTT CAC CCT ATC - 3'). The qPCR reactions were set up for each clone at a final volume of 25 µl using 7 µl of cDNA, 900 nM forward and reverse primers, 250 nM probe, and 1X concentration (supplied at 2X) TaqMan® Universal PCR Master Mix (contains AmpliTaq Gold DNA Polymerase, dNTP's with dUTP, Passive Reference, Optimized Buffer) (Applied Biosystems). All reactions were run on the ABI Prism® 7000 sequence detection system under the following conditions: 50°C for 2 minutes, 95°C for 10 minutes, then 45 cycles of: 95°C for 15 seconds and 60°C for 1 min. All primer and probe concentrations were determined through optimizations of minimum C_T values.

The primers and probes for gametocyte qRT-PCR quantification were designed to amplify the gametocyte-specific *P. chabaudi* gene PC108476.00.0 (herein referred to as *pcs16*), the homolog of the *Plasmodium falciparum* gametocyte gene *pfs16,* known to be exclusively expressed in gametocytes. Expression of *pfs16* begins in gametocyte-committed ring stages, as one of the first events in sexual differentiation, and continues into late stage gametocytes and gametes (Baker *et al.* 1994; Bruce *et al.* 1994; Dechering *et al.* 1997; Dechering *et al.* 1999; Niederwieser *et al.* 2000; Schneider *et al.* 2004). Although less is known about *pcs16* in *P. chabaudi*, many genes, including those expressed in gametocytes, are highly conserved between *P. falciparum* and rodent malaria species such as *P. chabaudi* (Janse *et al.* 1994; Carlton *et al.* 1998; Janssen *et al.* 2001; Thompson *et al.* 2001; Carlton *et al.* 2002; Waters 2002; Khan and Waters 2004; Hall *et al.* 2005).

To verify the gametocyte specificity of *pcs16*, I quantified an array of samples for both clones AS and AJ with varying levels of gametocytes using the *pcs16* assay and a separate qRT-PCR assay developed for the *P. chabaudi* gene PC302249.00.0, which is the highly conserved homologue of the *Plasmodium berghei* gene PB000198.00.0 (*P. chabaudi* primers: forward primer 5'- CAC AAT ATA GTA TAA AAG TAG GAC TTG AAA ATA ATA GTA G - 3', reverse primer 5'- GGA ATA TGG GAT ATT GTC AAA GGA TAT AC - 3', probe 5'- FAM - TTT TCC ACT TAC AAC TCC A - 3'). In *P. berghei* this gene has been demonstrated to have specific expression in late stage gametocytes using GFP and promoter analysis (Khan *et al.* 2005).

I found a strong correlation between the qRT-PCR counts from the *pcs16* and PC302249.00.0 assays for each clone across gametocyte densities spanning more than four orders of magnitude (AJ: $R^2 = 0.82$, $F_{1,43} = 131$, $P < 0.001$, slope = 1.08 ± 0.095, intercept = -0.23 ± 0.28; AS: $R^2 = 0.6$, $F_{1,26} = 41$, $P < 0.001$, slope = 1.14 ± 0.18, intercept = -0.35 ± 0.6) as well as with microscope blood smear counts across gametocyte densities spanning more than one order of magnitude ($R^2 = 0.33$, $F_{1,8} = 5.4$, $P = 0.049$, slope = 1.56 ± 0.66, intercept = -1.8 ± 2.1).

These results, combined with continued accurate quantification of both AJ and AS clones in artificial mixtures using the *pcs16* assay (correlation observed vs. expected: AJ: $R^2 = 0.95$, $F_{1,51} = 896$, $P < 0.001$, slope = 0.954 ± 0.032, intercept = 0.12 ± 0.08; AS: $R^2 = 0.88$, $F_{1,8} = 234$, $P < 0.0001$, slope = 0.99 ± 0.064, intercept = -0.035 ± 0.15), further verified gametocyte specificity and accuracy of qRT-PCR.

Standards for qRT-PCR were developed using single infections of genotypes AS and AJ with high levels of gametocytes and few asexual parasites. RNA was extracted and converted to cDNA as outlined above and a six step ten-fold dilution series of cDNA was set up for each quantification run. The same clone AS and AJ standards were utilized throughout the experiment, creating an internal control for variation in levels of *pcs16* RNA.

4.3.3. Statistical analysis

All statistical analyses were carried out using general linear models in Minitab version 14. Competitive performance was examined by comparing the performance of a clone on its own in single infections versus in the presence of a competitor in mixed infections, with explanatory variables mouse 'strain' (C57 or CBA), parasite 'clone' (AS or AJ) and infection type 'competition' (clone alone or in mixed infection). For all GLM analyses, maximal models were tested (variation in factor = 'clone' + 'strain' + 'competition' + 'clone'*'strain' + 'clone'*'competition' + 'strain'*'competition' + 'clone'*'strain'*'competition') and terms were dropped from the model if insignificant ($P > 0.05$) until the minimal significant model was obtained. When testing for correlations, terms were added as covariates in the analysis. Due to the same mice hosting both genotypes AS and AJ in mixed infections, I compared the performance of the two genotypes to each other in single infections but not in mixed infections, to avoid violating assumptions of independence. Where necessary, data was log (counts) or arcsin-square-root transformed (proportions) to meet assumption of normality and homogeneity of variance; likewise, gametocyte proportion data was first arcsin-square-root and then Box-Cox transformed.

When examining total parasite or gametocyte production I summed densities up to day nine which was the latest day in which enough mice survived to compare the treatment groups. To analyse the proportion of AS parasites in mixed infections I sampled asexual density and then gametocyte density from the same mouse one day later.

4.4. Results

4.4.1. Disease severity

Higher than anticipated levels of host morbidity in all infection types resulted in the euthanasia or death of several mice during the experiment. In infections composed of a single clone, 3 out of 34 (8.8%) AS-infected and 14 out of 34 (41%) AJ-infected mice died, whereas in mixed infections 11 of 34 (32%) mice died. Thus, as in previous experiments, AJ was the more virulent clone ($\chi^2 = 15.5$, $P = 0.008$, d.f. = 5).

Approximately equal levels of host mortality were observed in CBA (18/60 or 30%) and C57 mice (10/42 or 24%). In most cases, mouse death occurred around peak parasitemia, when disease symptoms were most severe. Mouse death prevented some treatment groups from being tracked for the duration of the experiment; however, all groups were followed until at least day 9 post-infection (Figure 4.1). In spite of host mortality, I was still able to utilize over 2000 qPCR and qRT-PCR samples for analysis.

4.4.2. In-host asexual and sexual densities

Total parasite density and total gametocyte density showed similar kinetics during the infections, peaking around day 8 and then trailing off (Figure 4.1). Transmission stage production closely mirrored in-host replication for each clone, with the number of gametocytes an order of magnitude or two lower than total parasites on any given day (Figure 4.1). Since less than 10% and usually less than 1% of DNA total parasite qPCR counts reflect gametocyte densities, below I refer for clarity to counts derived from qPCR as asexual densities and counts from qRT-PCR as gametocytes.

In infections containing only a single clone, genotype AS produced fewer asexuals and gametocytes than genotype AJ (Figure 4.2, asexuals: $F_{1,29} = 20$, $P < 0.001$, gametocytes: $F_{1,30} = 132$, $P < 0.001$), with the difference in asexual density between the two parasite clones being significantly greater in C57 compared to CBA mice (parasite clone*host strain interaction: asexuals, $F_{1,29} = 8$, $P = 0.009$; gametocytes, $F_{1,29} = 1.86$, $P = 0.183$). In general more asexuals and gametocytes were produced in CBA compared to C57 mice (asexuals, $F_{1,29} = 46$, $P < 0.001$, gametocytes, $F_{1,30} = 78$, $P < 0.001$).

For both parasite clones, the presence of the other clone reduced asexual and gametocyte densities (Figure 4.2). Thus, each clone experienced competitive suppression (asexuals: AS, $F_{1,32} = 62$, $P < 0.001$; AJ, $F_{1,28} = 13$, $P < 0.001$; gametocytes: AS, $F_{1,31} = 29$, $P < 0.001$; AJ, $F_{1,26} = 7$, $P = 0.012$). The level of suppression of asexuals was similar in both host strains (competition*host strain interaction: AS, $F_{1,31} = 0.23$, $P = 0.635$; AJ, $F_{1,26} = 0.83$, $P = 0.370$). However, competitive suppression of gametocytes was greater in CBA compared to C57 mice for clone AS (competition* host strain interaction: $F_{1,31} = 6$, $P = 0.019$) but not clone AJ ($F_{1,26} = 3$, $P = 0.088$).

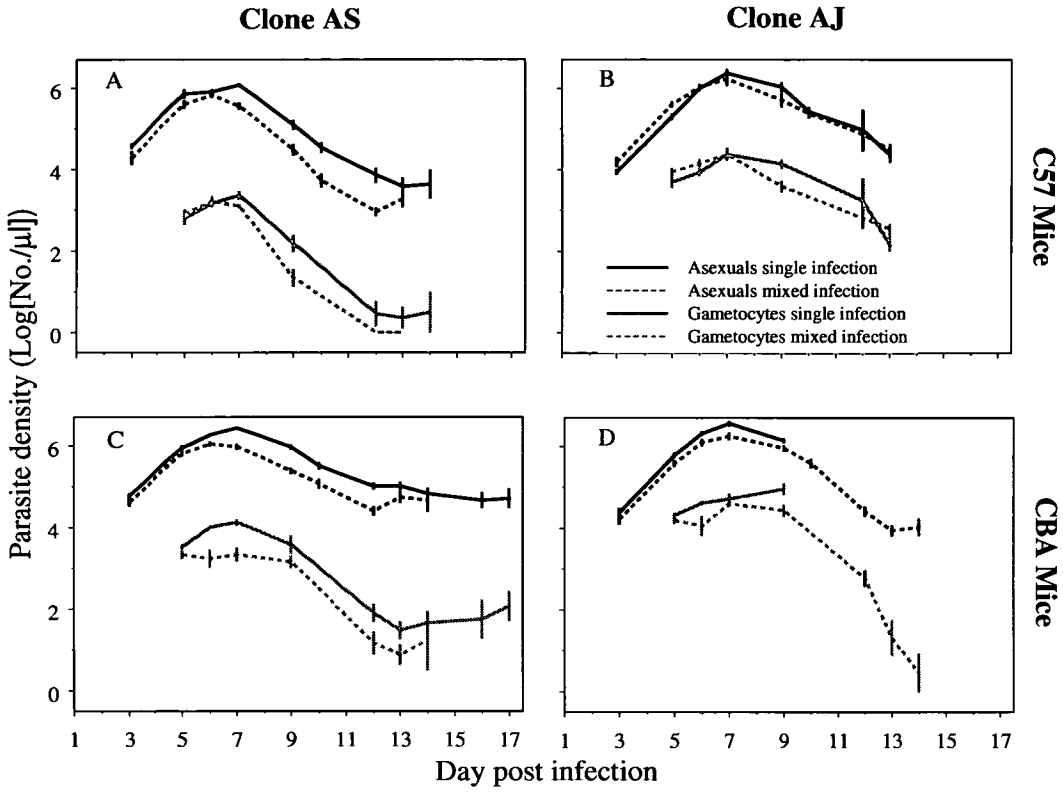


Figure 4.1. Asexual and gametocyte densities during the course of infection for parasite clones AS (A, C) and AJ (C, D) in mouse strains C57 (A, B) and CBA (C, D). Clone performance is shown in single-clone infections (solid lines) or in the presence of the other clone (dotted lines). Gametocyte densities were determined by clone-specific qRT-PCR (grey lines) and asexual densities by clone-specific qPCR (black lines). Plotted points are mean (± 1 S.E.M) of up to 12 mice; lines end when no mice were alive in respective treatment groups.

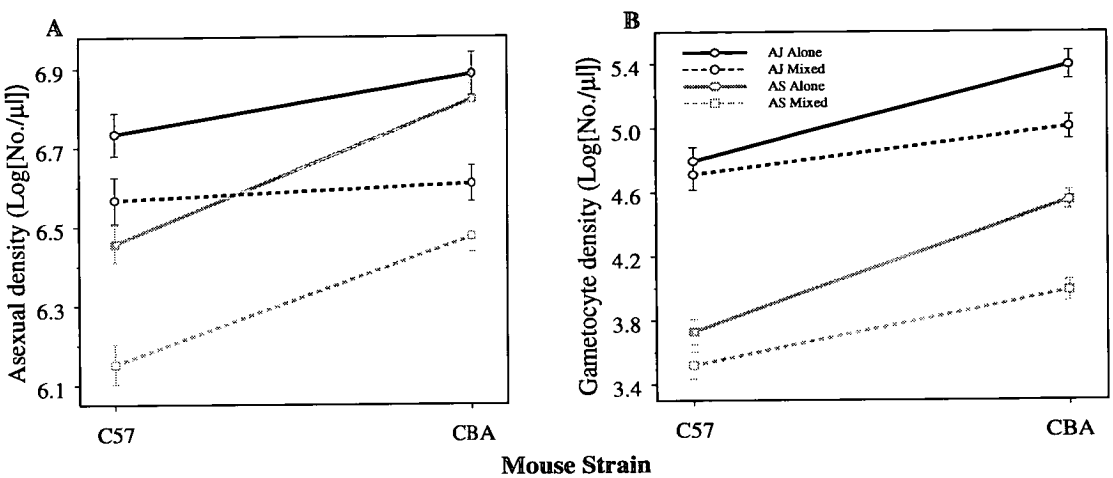


Figure 4.2. Total number of asexual parasites (A) and gametocytes (B) up to day 9 post infection. Plotted points are least squares means (± 1 S.E.M) for clone AJ (black lines) or AS (grey lines) in mixed (dotted lines) or single infections (solid lines), for each mouse strain. Only the 65 mice surviving up to day 9 post-infection were used to determine the mean for each treatment group (CBA mice: AS alone = 12, AS mixed = 10, AJ alone = 7, AJ mixed =10; C57 mice: AS alone = 7, AS mixed = 6, AJ alone = 7, AJ mixed = 6). In all cases there was competitive suppression of parasites.

4.4.3. Transmission investment in relation to total parasite production

For both clones, there was a significant positive relationship between the total number of asexual parasites present during an infection and the total number of gametocytes (Figure 4.3A and B; AS: $R^2 = 0.86$, $F_{1,30} = 17$, $P < 0.001$; AJ: $R^2 = 0.74$, $F_{1,26} = 42$, $P < 0.001$; all higher order interactions with the covariate were non-significant). Overall, more gametocytes were produced per asexual parasite in CBA compared to C57 mice (Figure 4.3; AS, $F_{1,30} = 8$, $P = 0.007$; AJ, $F_{1,26} = 21$, $P < 0.001$). I found no effect of competition on the relationship between total gametocyte and asexual parasite density for clone AJ (Figure 4.3B, competition: $F_{1,21} = 0.34$, $P = 0.57$; competition*asexual density: $F_{1,21} = 0.01$, $P = 0.92$), or for clone AS in C57 mice (Figure 4.3A). However, in CBA mice, clone AS (Figure 4.3A) produced fewer gametocytes for a given asexual parasite density when in competition as compared to on its own (mouse strain*competition interaction: $F_{1,30} = 7$, $P < 0.013$).

An alternative way of looking at transmission investment is to examine the proportion of parasites in an infection that were gametocytes (Figure 4.3C, D). An average of 3% of parasites produced by clone AJ were gametocytes and 0.3% produced by clone AS (Figure 4.3C, D), with the proportional gametocyte production being higher in CBA than C57 mice (mouse strain main effects: AS, $F_{1,31} = 26$, $P < 0.001$; AJ, $F_{1,26} = 24$, $P < 0.001$). There was no effect of competition on proportional gametocyte composition of clone AJ (Figure 4.3D; $F_{1,26} = 0.6$, $P = 0.44$) or of clone AS in C57 mice (Figure 4.3C). In CBA mice, however, clone AS (Figure 4.3C) had reduced proportional gametocyte investment in mixed compared to single infections (mouse strain*competition interaction: $F_{1,31} = 7$, $P = 0.011$).

Thus, both covariate and proportional analysis (Figure 4.3A, B and 4.3C, D) revealed the same picture. Competition reduced the number of asexual parasites (Figure 4.2) and proportionately, the number of gametocytes of clone AJ (Figure 4.3). This was also true for clone AS in C57 mice, but not in CBA mice, where there were disproportionately fewer gametocytes (Figure 4.2 and 4.3).

To evaluate the kinetics of transmission investment, I examined the frequency of AS clones in the asexual and gametocyte populations in mixed infections through time [number of AS clones/(number of AS clones + number of AJ clones)]. The frequency of AS gametocytes closely tracked the frequency of AS asexuals one day earlier (Figure 4.4; $R^2 = 0.51$, $F_{1,58} = 62$, $P < 0.001$), as expected from 1 day maturation time of *P. chabaudi* gametocytes (Gautret *et al.* 1996a). This correlation was stronger than the correlations with asexuals on the same day, or two or three days earlier.

Summed over the infection, the proportion of parasites in mixed infections which were clone AS was less than 50%, demonstrating higher parasite production of clone AJ. The frequency of clone AS in the asexual population in mixed infections was greater than the frequency in the gametocyte population ($F_{1,29} = 50$, $P < 0.001$). This is likely because clone AJ produced more gametocytes per asexual parasite than clone AS did. Additionally, the frequency of clone AS in the asexual and gametocyte populations were higher in CBA compared to C57 mice ($F_{1,29} = 50$, $P < 0.001$). This suggests that although both clones produced more parasites in strain CBA compared to strain C57, parasite production of clone AS increased by slightly more. Despite this, clone AS remained the minority clone in both mouse strains. There were no apparent changes in the relationship between proportion of AS gametocytes and total parasites as clone AS became increasingly rare in the infections (days 7 -12, Figure 4.4).

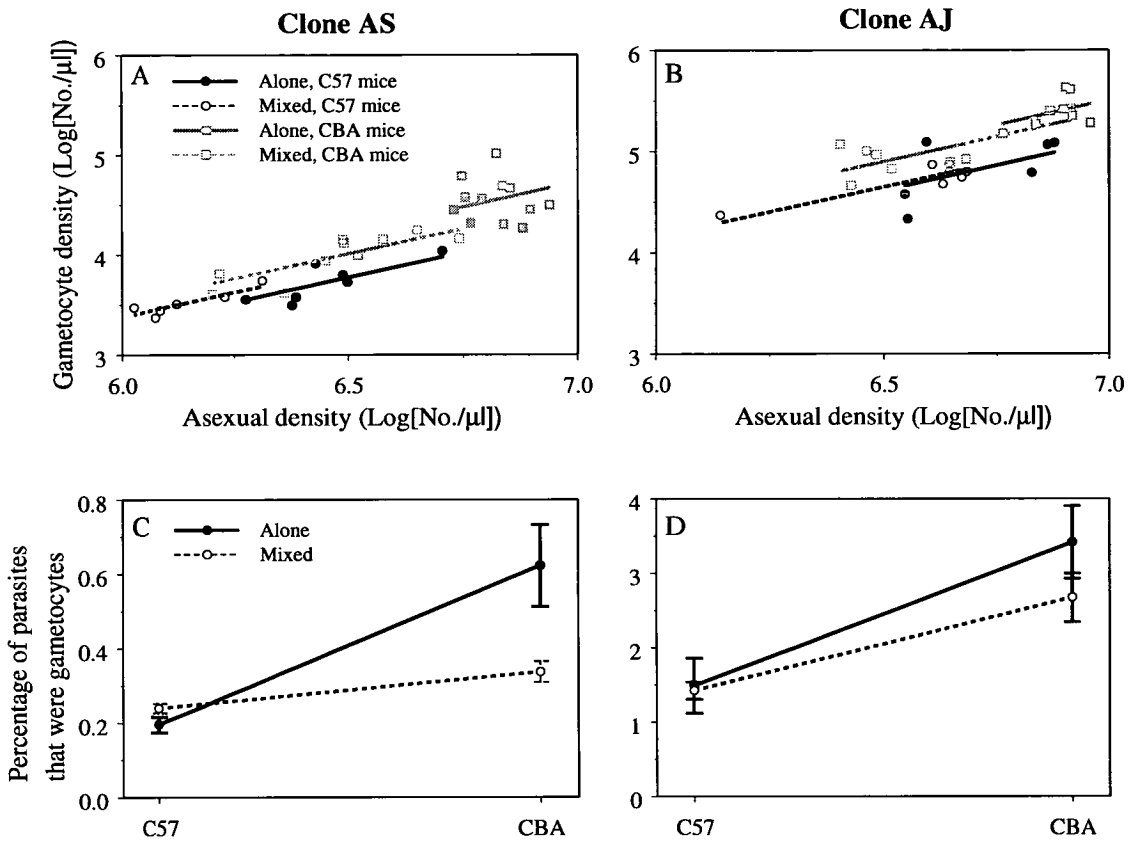


Figure 4.3. The relationship between total gametocyte and asexual parasite production. Total gametocyte production was compared to total parasite production for each mouse alive on day 9 post-infection (A and B). Least squares regression fit lines are given for clones AS (A) and AJ (B) in single (solid lines) and mixed infections (dotted lines) in C57 (black circles) and CBA mice (grey squares). I also compared percentage of parasites that were gametocytes (C and D). Plotted points are mean percentage (± 1 S.E.M) for clones AS (C) and AJ (D) in single (solid line) and mixed infections (dotted line). Overall I found that significantly more gametocytes were produced for a given parasite density in CBA compared to C57 mice. Additionally, clone AS had higher proportional gametocyte production in single versus mixed infections of CBA mice.

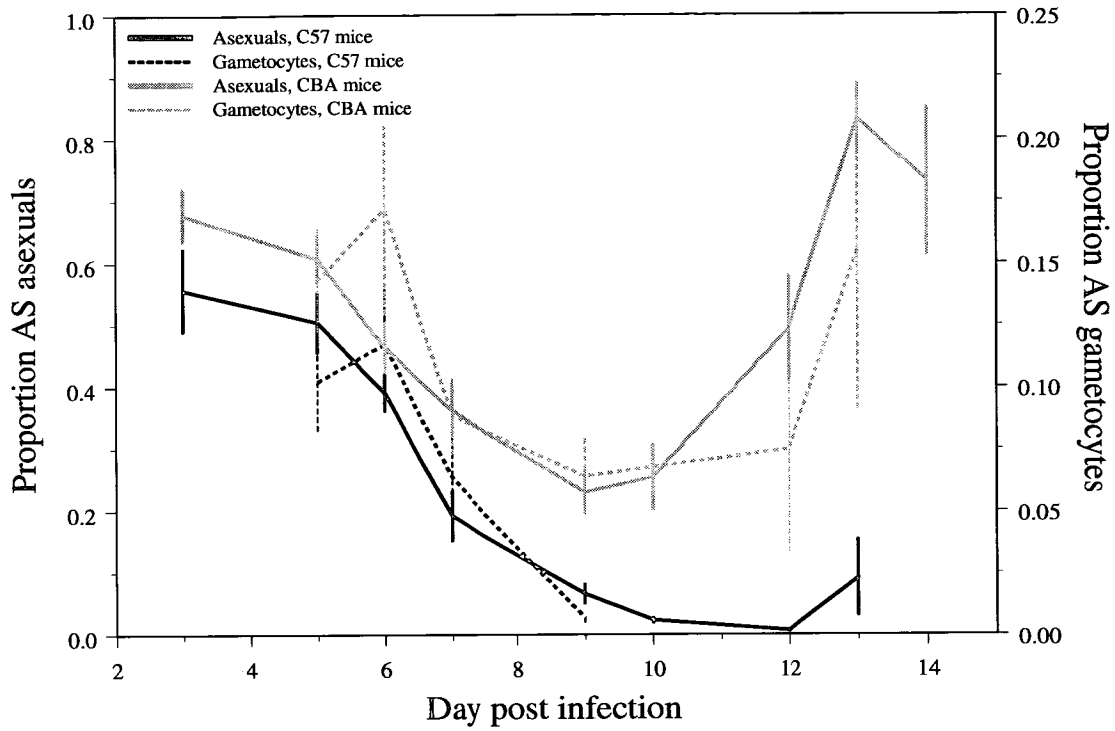


Figure 4.4. Proportion of parasites in mixed infections that were clone AS. The proportion of AS asexuals (left y-axis, solid lines) and gametocytes (right y-axis, dotted lines) were tightly correlated through time in both C57 (black lines) and CBA mice (grey lines). Standard error bars (± 1 S.E.M) are given, with values based on days which both clones were detected via qPCR or qRT-PCR, and two or more mice were alive, in respective treatment groups.

4.5. Discussion

Life history strategies of parasite genotypes during intra-specific competition have to my knowledge rarely been examined. In this study I uniquely measured life history traits of the rodent malaria *Plasmodium chabaudi* during conspecific competition, to assess the role of within-host replication and transmission investment in shaping the overall fitness of genotypes. I observed competitive suppression of total parasite and gametocyte production for both the virulent (AJ) and less virulent clone (AS) in mixed genotype infections. Despite competitive suppression, the more virulent clone produced more total parasites and gametocytes than the less virulent clone, and maintained a higher frequency in the parasite population during competition. The mechanism behind the observed competitive suppression remains unclear, but could be due to direct competition for resources such as red blood cells and glucose, or indirect host-mediated competition through strain-transcending immune responses (Hellriegel 1992; Yap and Stevenson 1994; Gravenor *et al.* 1995; Hetzel and Anderson 1996; Buckling and Read 2001; Almogy *et al.* 2002; Råberg *et al.* 2006).

The in-host competitive suppression I observed is directly translatable to the transmission success of individual clones, since the proportion of gametocytes within the host closely tracked total parasite population throughout the infection. Overall, both clones maintained the same level of transmission stage production throughout the infection, with the competitively superior and more virulent genotype having a 10-fold higher transmission investment. Therefore, my results do not support the theoretical possibility that competitively suppressed genotypes may maintain lower total parasite densities in the host and put more resources into transmission (Koella and Antia 1995; van Baalen and Sabelis 1995; Antia *et al.* 1996). On the contrary, in one of the mouse genotypes studied, I observed that the competitively inferior clone AS actually produced relatively fewer transmission stages when under competitive suppression.

There are multiple factors which could have contributed to the observed reduced transmission investment for clone AS in CBA mice, as a result of competition. One possibility is that competitive suppression was simply higher for gametocytes compared to asexuals in CBA mice, possibly due to a stronger gametocyte-specific immune

response in this mouse strain. This hypothesis is supported by results which typically demonstrate lower transmission success from CBA compared to C57 mice (unpublished data). Why I did not observe a similar relative reduction in gametocyte investment for clone AJ may be due to strain-specific immunity, or simply the greater ability of clone AJ to persist in a hostile in-host environment, supported by its general competitive superiority (De Roode *et al.* 2004b; Råberg *et al.* 2006; Bell *et al.* In Press).

Regardless of a transmission investment response by clone AS, it still remained the minority and ultimately competitively inferior clone. Phenotypic plasticity in transmission investment therefore did not alleviate competitive suppression, as envisaged by several theoretical studies, and since this response only occurred in one mouse strain, it is unlikely to have a very strong impact on the population genetics of the parasite. I cannot rule out the possibility that clones may respond to competition in other situations such as the case of super-infection, where phenotypic plasticity for transmission investment would be particularly important if the first clone to infect the host has a strong advantage (Nowak and May 1994; Hood 2003; De Roode *et al.* 2005a). Genotypic adjustments in commitment towards transmission may also be epidemiologically relevant when mixed infections are composed of drug resistant and sensitive clones. For example, if resistant genotypes can increase transmission investment in competition with sensitive genotypes, in response to environmental cues, and maintain higher fitness, it could enhance the spread and evolution of drug resistance (Buckling *et al.* 1999c; Hastings and Donnelly 2005).

My results suggest that competition in malaria infections selects for fixed strategies that increase parasite growth rates, virulence, and competitive abilities; rather than plasticity in growth or transmission stage production. Thus, this study supports current literature which predicts that conspecific within-host competition will result in a competitive advantage and positive selection for virulent genotypes and the evolution of virulence (Nowak and May 1994; van Baalen and Sabelis 1995; Ebert 1998; Mosquera and Adler 1998; Mackinnon and Read 1999b; Read and Taylor 2001; Brown *et al.* 2002; Davies *et al.* 2002; Hood 2003; Schjorring and Koella 2003; Bell *et al.* In Press).

5. COMPETITIVE FACILITATION OF DRUG RESISTANCE IN MIXED CLONE MALARIA INFECTIONS

5.1. Abstract

The infection kinetics of resistant and sensitive genotypes co-circulating in the same host plays a critical role in the evolution of malaria drug resistance. In particular, the timing of drug treatment and level of clearance of the sensitive clone will impact the subsequent competition dynamics and therefore the performance of the resistant clone. In this study I competed a drug resistant and sensitive clone of the rodent malaria *Plasmodium chabaudi* and applied, at the onset of clinical symptoms, three drug treatment regimes: full, half, and quarter-duration of the recommended dosage. I observed that under full and half treatment the drug resistant clone experienced competitive facilitation, in that it produced more total parasites and transmission stages in mixed infections than when initiated as a single infection. Reducing drug treatment to a quarter-duration resulted in the competitive release but not facilitation of the resistant clone, likely due to the preservation of the sensitive clone and maintenance of within host competition. My results suggest that optimal drug treatment strategies for slowing the evolution of resistance may be to reduce drug treatment to levels which alleviate clinical symptoms in the host, but do not completely clear sensitive parasites. This contradicts current thinking on the subject, which asserts that incomplete drug treatments will accelerate the rate of resistance evolution.

5.2. Introduction

Resistance in human malaria parasites against almost all anti-malarial drugs is now well documented and in most cases rapidly evolved within a few years of first deployment of treatment in the field (Hyde 2005). Evidence is building that the speed at which drug resistance spreads is shaped by the fitness of resistant mutants in relation to wild type drug sensitive strains (Hastings and Donnelly 2005). Intra-host dynamics are likely to play a particularly critical role in shaping this fitness relationship, due to the high genetic diversity of malaria infections and frequent co-circulation of resistant and sensitive genotypes in the same host (Babiker *et al.* 1999b; Anderson *et al.* 2000; Awadalla *et al.* 2001; Jafari *et al.* 2004). Additionally, there is now strong experimental and indirect epidemiological support that in-host competition between genotypes shapes malaria parasite population structure (Daubersies *et al.* 1996; Mercereau-Puijalon 1996; Arnot 1998; Paul *et al.* 2004b; De Roode *et al.* 2005b; Hastings 2006; Bell *et al.* In Press).

Recent studies have shown that resistant malaria clones have lower fitness and are competitively inferior in the absence of drug treatment compared to sensitive clones, likely due to a resource cost of the resistance phenotype (De Roode *et al.* 2004a; Hastings and Donnelly 2005; Walliker *et al.* 2005). This cost is believed to be outweighed by the fitness advantage resistant clones experience under drug treatment. However, the level of this fitness advantage is not explicitly clear in that resistant clones could perform in different ways after the drug clearance of sensitive clones, greatly influencing the rate in which drug resistance evolves (Hastings and D'Alessandro 2000).

It is often assumed that in mixed genotype infections, resistant clones will fill the space provided by the drug clearance of sensitive clones in a mechanism of competitive release (Hastings and D'Alessandro 2000), and indeed this has been observed in studies with prophylactic drug treatment (De Roode *et al.* 2004a). However when individuals seek medical attention at the onset of clinical symptoms, parasites will be at higher densities in the host and immune function as well as host condition may play an important role in shaping infection dynamics (Gupta and Anderson 1999).

The presence of parasites for several days before drug clearance of the sensitive clone, may allow the immune system to become primed and suppress the competitive release of the resistant clone (Cravo *et al.* 2001). Alternatively, if there is a lag between drug clearance of the sensitive genotype and an immune response directed towards the resistant genotype, it could result in a period of unchecked enhanced growth for the resistant clone (Almogy *et al.* 2002).

Ultimately, the performance of the resistant clone will depend heavily on the level of clearance of the sensitive clone and the rate of re-infection. This is particularly important when considering that both failure to complete drug treatment regimes and exposure to infectious bites soon after treatment ends are frequent in the field, likely resulting in the persistence of sensitive clones in the infection. If the persistence of mixed infections maintained in-host competition, this competition could potentially inhibit the release and fitness advantage of resistance clones, observed with prophylactic drug treatment (De Roode *et al.* 2004a). This is supported by the observation that the percentage of resistance alleles has stabilized well below the predicted 100% level in many endemic malaria regions (Hastings 2006). In theory, drug treatment should result in a higher proportional composition and higher fitness of resistant clones compared to drug cleared sensitive clones. However, the response resistant clones elicit could lead to different outcomes in that suppression, release, or enhanced growth, will vary dramatically in the overall numbers of resistant genotypes produced, and their epidemiological impact on the evolution of resistance (Hastings and D'Alessandro 2000).

Here I investigated the dynamics of a drug resistant clone in competition with a drug sensitive clone of the rodent malaria *Plasmodium chabaudi*, after four, two, one or zero days of pyrimethamine treatment. Infected hosts were treated at the onset of clinical symptoms to mimic a natural field situation of individuals seeking medical attention after illness occurs. The dynamics of each clone was monitored individually using qPCR for total parasite production and qRT-PCR for transmission stage production.

I found that drug clearance of the sensitive clone, after four and two days of anti-malarial treatment, resulted in enhanced growth of the resistant clone, which experienced elevated total parasite and transmission stage production compared to its performance alone. One day of drug treatment only partially cleared the sensitive clone and subsequently, the resistant clone performed as well but not better than it did in single infections, therefore demonstrating competitive release but not competitive facilitation.

My findings suggest that reducing drug treatment to levels which just alleviate clinical illness and reduce, but do not attempt to eliminate the infection, may be the best strategy for slowing the spread of drug resistance, by maintaining in-host competition. This contradicts current theory that ascertains incomplete drug treatment will accelerate drug resistance evolution which, to my knowledge, has not been previously experimentally investigated.

5.3. Methods

5.3.1. Parasites and hosts

A pyrimethamine resistant *Plasmodium chabaudi* clone and a genetically distinct pyrimethamine sensitive clone were used for this study. Both clones were originally isolated from thicket rats *Thamnomys rutilans* (Beale *et al.* 1978). The drug resistant clone was derived from ancestral clone AS, which was subjected to pyrimethamine drug selection during several rounds of concurrent serial passage to evolve drug resistance, becoming clone ASpyr-1B, herein referred to as clone R. The drug sensitive clone, herein referred to as clone S, was derived from ancestral clone AJ and has been subjected to very few serial passages so as to maintain its original phenotype. Both clones have been extensively investigated in the experimental literature and clone R is known to have lower in host total parasite production, competitive ability and virulence compared to clone S (De Roode *et al.* 2004a; De Roode *et al.* 2005b; Walliker *et al.* 2005).

This study consisted of two separate experiments. In both experiments, mice were inoculated with 10^6 S, 10^6 R, or 10^6 S + 10^6 R parasites (2 x 10^6 total parasites), in order to compare the dynamics of each clone when it is alone versus in the presence of a competitor (see Table 5.1 for sample sizes)[1]. Parasites were inoculated into 6 – 8 week old female mice by diluting singly infected donor mouse blood in 0.1 ml of calf serum solution which was injected into the intraperitoneum, as described elsewhere (Mackinnon and Read 1999a). Mice were kept on a 12:12 h light-dark cycle and fed on 41B maintenance diet (Harlan, U.K.) with 0.05% para-amino benzoic acid supplemented drinking water to enhance parasite growth (Jacobs 1964). Different mouse strains were used for experiment I and II due to high disease severity and host mortality observed in CBA mice during experiment I (Table 5.1).

5.3.2. Drug treatment

In an effort to mimic the natural field scenario of sick individuals seeking medical treatment upon the onset of clinical symptoms, anti-malaria drug treatment began on day 7 post-infection, after mice first showed signs of weight loss and anaemia. The malaria antifolate drug pyrimethamine was dissolved in DMSO and 100 µl administered orally by gavage at a concentration of 8 mg/kg of mouse body weight, previously shown to clear all AJ parasites after four days of treatment (Richard Culleton, personal communication). Mice either received zero, one, two or four days of drug treatment as outlined in Table 5.1. All mice, including negative controls, received a gavage of 100 µl DMSO on each treatment day. The drug treatment regime administered allowed me to monitor the performance of the resistant clone upon complete (experiment I) and partial drug clearance (experiment II) of the sensitive clone. Drug treatment in experiment II resulted in some mouse death which was determined through post-mortem to have been gavage-induced. These mice were dropped from all figures and analyses (Table 5.1), and prevented the analysis of malaria induced mortality in experiment II.

[1] These two clones are the same clones used in Chapter 4, there labeled AS (resistant) and AJ (sensitive).

Table 5.1. Mice in each treatment group from experiment I and II. Values given are final number of mice used for analysis with the corresponding numbers in brackets representing the mice which were excluded due to death (left value), or failed inoculation (right value). Some treatment groups were not carried out in both experiments as shown by empty cells. Figures for experiment I of red blood cell (Figure 5.1) and parasite density (Figure 5.3) through time, track some mice which later died and were excluded from the analysis, as death was believed to be parasite induced. All mice that died in experiment II were excluded from all figures and analysis since the majority of death was gavage induced.

		DRUG TREATMENT (DAYS)			
EXPERIMENT	**Clone**	**0**	**1**	**2**	**4**
I (CBA/ca mice)	R	7 [13 + 0]	-	-	5 [14 + 1]
	S	0 [18 + 2]	-	-	9 [9 + 2]
	R+S	3 [16 + 1]	-	-	4 [13 + 3]
II (C57bl/6J mice)	R	2 [9 + 0]	5 [5 + 1]	4 [7 + 5]	-
	S	3 [8 +1]	5 [7 + 0]	6 [8 + 4]	-
	R+S	2 [10 + 0]	5 [6 + 1]	7 [11 + 0]	-

5.3.3. Monitoring infection dynamics

To assess parasite virulence, mouse red blood cell density was measured daily using flow-cytometry (Coulter Electronics), with a baseline taken one day prior to the start of infection. Clone-specific parasite density of clones S and R, in single and mixed infections, was monitored using qPCR on parasite DNA extracted from 5 µl of whole blood taken each day of sampling, as detailed elsewhere (Bell *et al.* In Press). Transmission stage densities were monitored for clones S and R, in single and mixed infections, using clone specific qRT-PCR on gametocyte RNA extracted from 20 µl (experiment I) or 10 µl (experiment II) of blood, as previously described (Chapter 2 and 4). Since typically less than 1% of qPCR counts reflect gametocyte densities, for clarity I refer to counts derived from qPCR as asexual densities and counts from qRT-PCR as gametocytes.

Sampling began on day 3 (experiment I), or day 2 (experiment II), post infection and occurred daily till day 17 for experiment I (day 3 and 9 - only qPCR taken; 4,8,11, and 15 - no sample taken) and day 21 for experiment II. In experiment II, qRT-PCR for gametocytes was only performed from day 12 onwards, after asexual parasites of the sensitive clone reached their lowest densities from drug treatment. Quantification with qPCR made it possible to back calculate the exact number of parasites injected into the host and confirm the inoculation accuracy. Any mice which were found to have received less than 10^6 parasites were dropped from the analysis (Table 5.1).

5.3.4. Statistical analysis and trait definition

The effects of competition and drug treatment on infection virulence, parasite growth, and transmission investment, were examined using general linear models (GLM). For all GLM analysis maximal models were tested and non-significant terms dropped to obtain the significant minimal model. Experiments I and II were analysed separately due to a not fully factored experimental design (Table 5.1). Where necessary, data was log (counts) or arcsin-square-root transformed (proportions) to meet assumption of normality and homogeneity of variance.

To assess virulence through GLM, I analysed mean red blood cell density after drug treatment as a response variable with initial red blood cell density as a covariate. The explanatory variables included 'drug treatment' (zero, one, two, or four days of drugs) and 'infection type' (S alone, R alone, or mixed). Higher virulence was defined as lower red blood cell density. Virulence in experiment I, was further assessed using chi-square tests on the number of mice found dead in cages, between the treatment groups (does not include euthanized mice).

To quantify competition dynamics by GLM analysis, response variables included mean parasite density, mean gametocyte density, and mean proportion of total parasites which were gametocytes; all of which were calculated after the period of drug treatment. The performance of each clone was then analysed with explanatory variables 'drug treatment' (zero, one, two, or four days of drugs), 'parasite clone' (R or S), and 'competition' (clone alone or in mixed infection). Competitive suppression was defined as a reduction in total in-host parasite production by a clone in mixed infections compared to single infections.

5.4. Results

5.4.1. Virulence

Generally, drug treatment reduced virulence, but a longer duration of treatment did not result in greater alleviation of clinical symptoms.

5.4.1.1. Experiment I

I found no significant differences in host mortality between treated or untreated single infections of clone R (10% untreated, 25% treated), or mixed infections (30% treated, 30% untreated). Higher host death was observed for untreated clone S infections (50%), compared to treated (5%) infections ($\chi^2 = 15.5$, $P = 0.002$, d.f. = 3), suggesting that virulence was reduced due to drug clearance of the sensitive clone, but only when the drug resistant clone was not present.

As a further measure of virulence I examined the average red blood cell density after completion of drug treatment (Figure 5.1 and 5.2). Four days of drug treatment in experiment I appeared to reduce the virulence of single infections of the sensitive clone (Figure 5.1), but I could not test this statistically because the untreated mice died. When examining mixed and single resistant clone infections (Figure 5.1 and 5.2A), I found no differences in red blood cell loss in the absence of drug treatment (infection type main effect: $F_{2,16} = 2.1$, $P = 0.165$), or any evidence of alleviated virulence due to four days of drugs, for either infection type (drug treatment main effect: $F_{2,15} = 0.1$, $P = 0.9$).

5.4.1.2. Experiment II

In experimental block II there was greater red blood cell loss from single sensitive clone and mixed infections, compared to single resistant clone infections, in the absence of drug treatment (Figure 5.1 and 5.2B). One day of drug treatment resulted in a significant alleviation of red blood cell loss, in both mixed and single infections of the sensitive clone (Figure 5.2B). Red blood cell loss was further alleviated in single sensitive clone infections as a result of two days of pyrimethamine, but not in mixed infections (Figure 5.2; drug treatment*infection type interaction: $F_{4,29} = 4.5$, $P = 0.006$). Therefore, although drug treatment reduced the virulence of mixed infections, higher drug dosage did not provide any further reduction in host morbidity. Interestingly, red blood cell loss appeared to be highest for clone R after 1 day of drug treatment (Figure 5.1 and 5.2B), but when analyzing clone R individually, this difference was not found to be significant ($P > 0.1$).

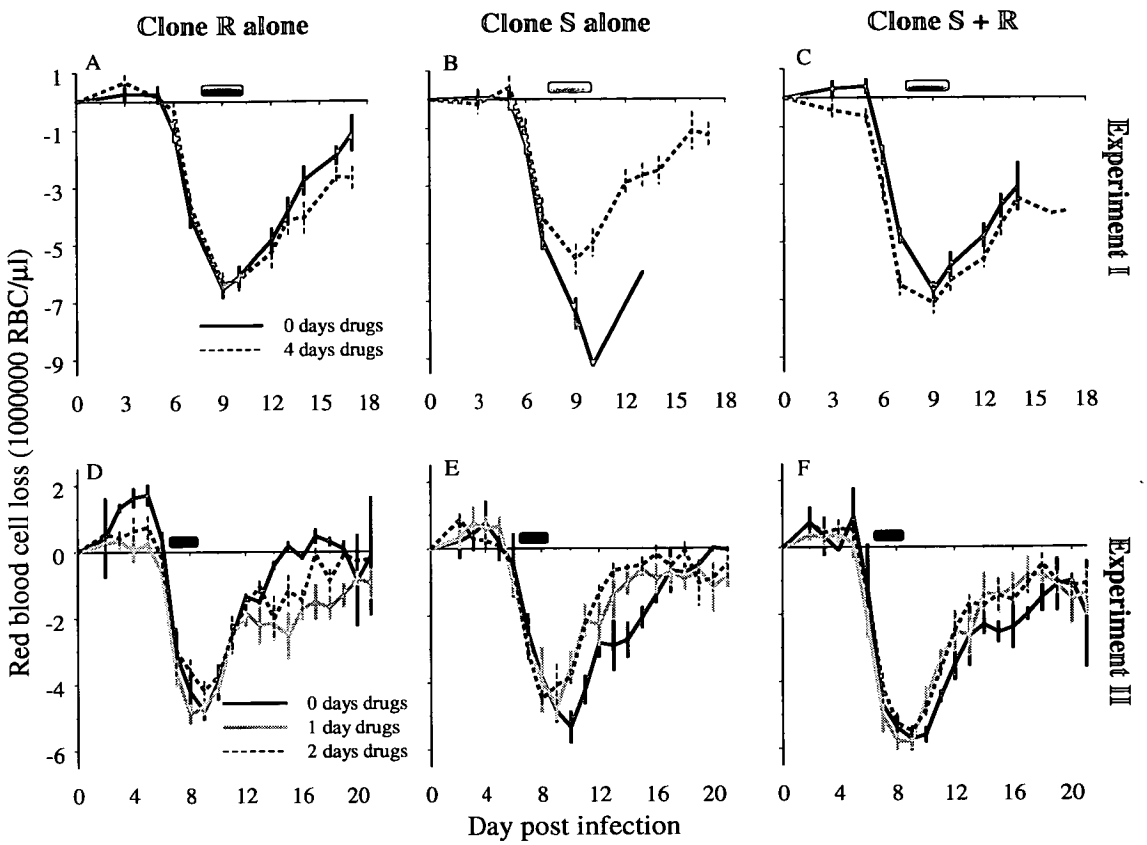


Figure 5.1. Mean red blood cell loss (± 1 S.E.M) throughout the infection (RBC density each day – initial RBC density). Lower values correspond to greater virulence. In experiment I the red blood cell loss of single resistant (A), single sensitive (B) and mixed clone infections (C) are shown after zero (solid black line) or four days (dotted black line) drug treatment. In experiment II (D, E, F) the red blood cell loss of the three infection types are shown after zero (solid black line), one (grey line), or two days (dotted black line) drug treatment. The black bars above the x-axis represent the time period on which drug treatment was given. Standard errors were calculated from all mice which were still alive on that sampling day and lines end when no mice remained living.

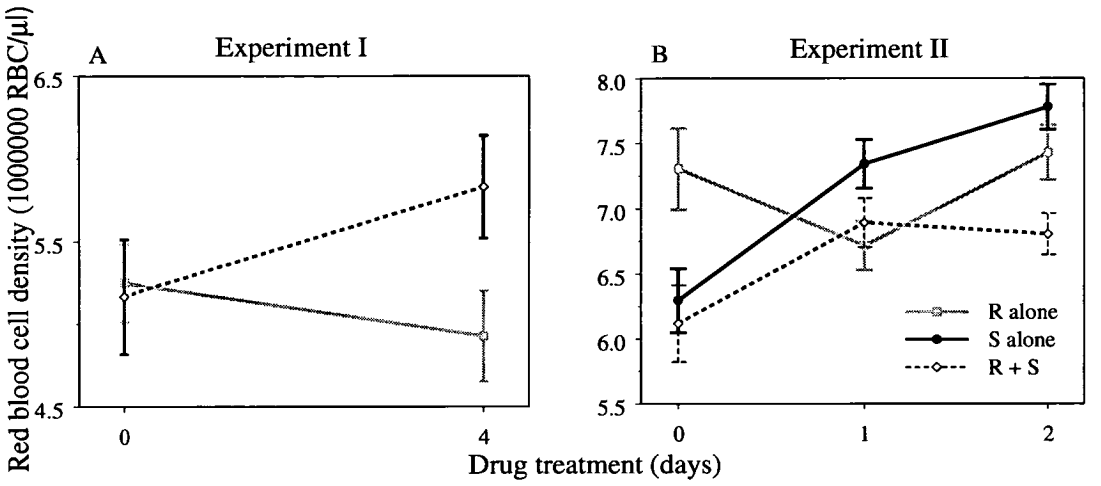


Figure 5.2. Red blood cell density after drug treatment. Lower values indicate greater red blood cell loss and higher virulence. In experiment I (A), drug treatment had no effect on the anemia induced by either single (solid grey line), or mixed infections (dotted line), of the resistant clone. The anemia induced by the sensitive clone could not be assessed due to high mortality in clone S infections, without treatment. In experiment II (B), drug treatment reduced the virulence of single infections of the sensitive clone (black lines), and mixed infections (dotted line), having no effect on single infections of the resistant clone (grey line). Points represent the least-squares-mean of log transformed red blood cell density (± 1 S.E.M) calculated over days 12-14 (experiment I) and days 12-21 (experiment II), from the mice surviving through the sampling period in each treatment group (see Table 5.1).

5.4.2. Parasite growth dynamics

Generally, drug treatment cleared the sensitive clone, releasing the resistant clone from competition. The resulting dynamics of the resistant clone then depended heavily on the drug treatment regime.

5.4.2.1. Experiment I

Four days of pyrimethamine treatment in experiment I completely cleared the sensitive clone in both single and mixed infections (Figure 5.3C, D). I observed a one day lag between asexual and gametocyte drug clearance (Figure 5.3D), as predicted by gametocyte resistance to pyrimethamine and their 8-16 hour half-life (Reece *et al.* 2003). The performance of the sensitive clone in the absence of drug treatment could not be assessed, due to the complete mortality of mice harbouring single infections before the end of the analysis period (Table 1 and Figure 5.3).

The resistant clone experienced competitive suppression in the absence of drug treatment, resulting in the production of fewer asexual parasites in mixed compared to single infections (Figure 5.3A, B and 5.4A). Drug clearance of the sensitive clone released the resistant clone from competition, resulting in a higher number of asexual parasites and gametocytes (Figure 5.3A, B and 5.4) than it produced in treated single clone infections (competition*drug treatment interaction, asexuals: $F_{1,15} = 4.5$, $P = 0.05$; gametocytes: $F_{1,15} = 6$, $P = 0.03$). This observed competitive facilitation was not attributed to a direct physiological effect of pyrimethamine, since drug treatment did not influence the resistant clone when it began the infection in the absence of the sensitive clone, besides a slight reduction in gametocyte density (Figure 5.4).

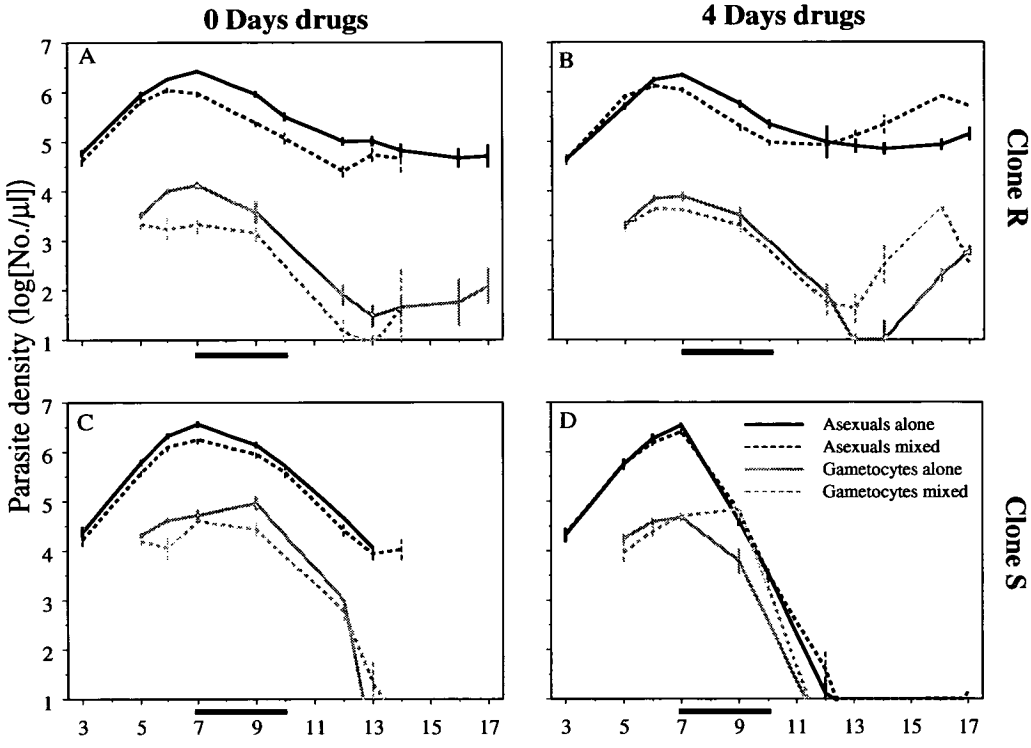


Figure 5.3. Parasite density growth curves of experiment I. Asexual density from qPCR (black lines) and gametocyte density from qRT-PCR (grey lines), are given for the resistant (A and B) and sensitive clone (C and D), in single (solid line) and mixed infections (dotted line). Drug treatment was administered for zero (A and C; sham injection), or four days (B and D) from days 7-10, as shown by the black bars below the x-axis, with day 12 representing the first sampling point after treatment. All graphs are drawn to the same log scale with minimum y-axis value representing the lowest reliable detection threshold of qPCR. Mean densities (± 1 S.E.M) were calculated from of all mice which were alive on the respective sampling day.

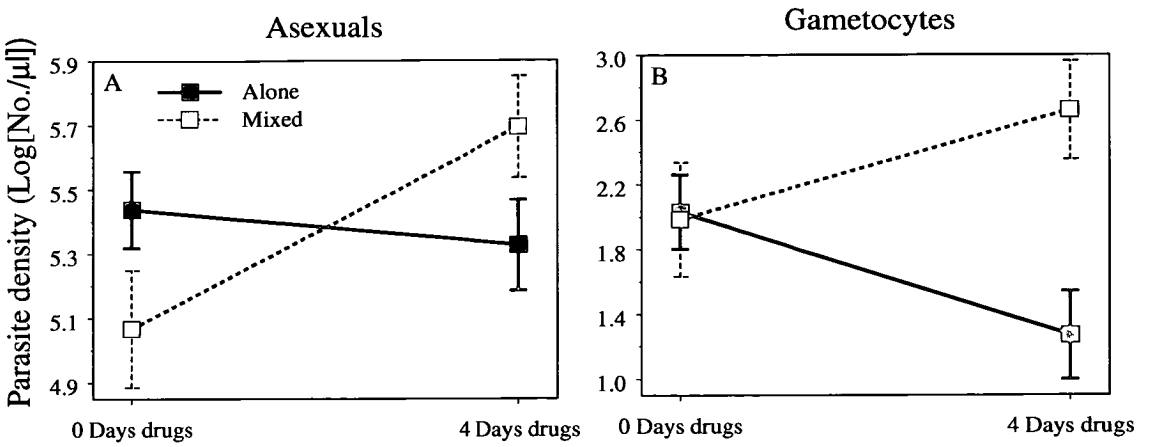


Figure 5.4. Mean parasite density of drug resistant clone in experimental I. After four days of drug treatment the resistant clone produced significantly more asexual parasites (A) and gametocytes (B) when in the presence the sensitive clone (dotted line) compared to alone (solid line), experiencing competitive facilitation. Data points represent least squares mean (± 1 S.E.M) of log transformed total asexual or parasite density over days 12-14 (asexuals) and 13-14 (gametocytes) post infection, from the mice surviving till the end of sampling period (see Table 1). I could not examine parasite or gametocyte density of the drug sensitive clone in experiment I, due to complete parasite clearance after four days of drug treatment.

5.4.2.2. Experiment II

One and two days of drug treatment in experiment II greatly reduced but did not completely clear both gametocytes and asexuals of the sensitive clone (Figure 5.5D, E, F; 5.6D, E, F and 5.7; treatment main effect; asexuals: $F_{2,24} = 31$, $P < 0.001$, gametocytes: $F_{2,24} = 47$, $P < 0.001$). Lower parasite densities were observed in single infections of the sensitive clone after two compared to one day of treatment (5.5D, E, F; 5.6D, E, F and 5.7; $F_{1,9} = 6.6$, $P = 0.03$), and therefore the combined results from experiments I and II suggest higher drug efficacy with increased treatment duration (Figure 5.3 – 5.7).

Competitive suppression also reduced asexual and gametocyte densities of the sensitive clone (5.5D, E, F; 5.6D, E, F and 5.7; competition main effect; asexuals: $F_{1,24} = 19$, $P < 0.001$; gametocytes: $F_{1,24} = 8.6$, $P = 0.007$), with a suggestive trend of increased competitive suppression with drug treatment (Figure 5.7), however, this was not significant (drug treatment*competition interaction; asexuals: $F_{2,22} = 2.2$, $P = 0.14$; gametocytes: $F_{2,22} = 1.21$, $P = 0.32$).

Drug treatment regime had a profound affect on the resistant clone in competition. In the absence of pyrimethamine, clone R was competitively suppressed by the sensitive clone and produced fewer asexual parasites (Figure 5.5A and 5.8), as observed in experiment I. After 2 days of drug treatment, the resistant clone was released from competitive suppression and then experienced enhanced growth by producing more asexual parasites in the presence of the sensitive clone, compared to when inoculated as a single infection (Figure 5.5A, C and 5.8). Thus, competitive facilitation of the resistant clone was observed both as a result of four (experiment I) and two (experiment II) days of drug treatment (Figure 5.4 and 5.8).

Alternatively, one day of drug treatment did not lead to the enhanced growth of the resistant clone (Figure 5.5A, B and 5.8). Instead, clone R produced only as many asexual parasites as it did in the absence of competition, but not significantly more, therefore demonstrating competitive release rather than competitive facilitation (drug treatment*competition interaction; asexuals: $F_{2,19} = 6.8$, $P = 0.006$). This suggests that the incomplete clearance of the sensitive clone as a result of reduced drug treatment maintained in-host competition, preventing the enhanced growth of the resistant clone.

Gametocyte density of the resistant clone in experimental block II was difficult to assess due to values hovering around the accurate detection threshold of qRT-PCR (Figure 5.6 and 5.8). However, in mixed infections it was evident that drug treatment resulted in a higher number of gametocytes (treatment main effect, mixed infections: $F_{2,13} = 8$, $P = 0.008$), and more gametocytes were produced in drug treated mixed infections compared to single infections (Figure 5.6 and 5.8), supporting the enhanced growth demonstrated with asexual densities.

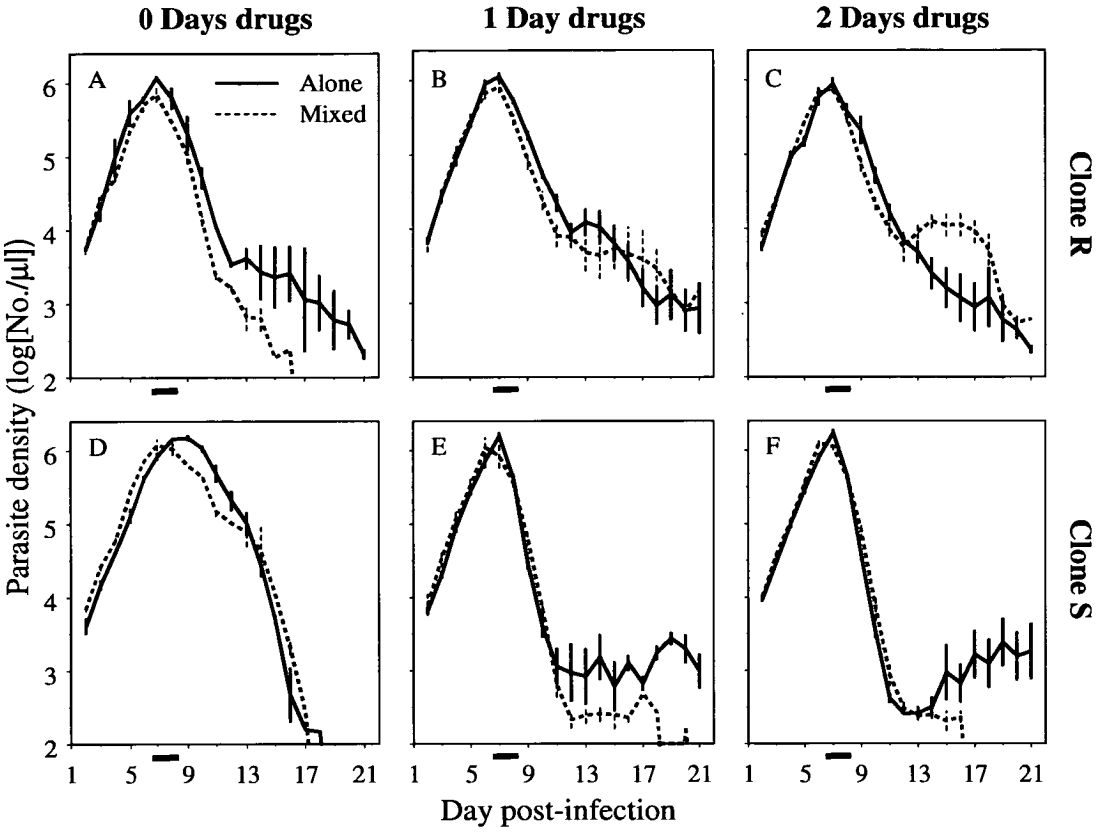


Figure 5.5. Asexual parasite density over the course of infection for experiment II, determined by qPCR. The mean densities (± 1 S.E.M) of the resistant (A, B, C) and sensitive clone (D, E, F), in single (solid line) and mixed (dotted line) infections, are shown for the zero (A, D), one (B, E), and two day drug treatment groups (C, F). Drug treatment began on day 7 post-infection, as indicated by the back bars below the x-axis. The sensitive clone was cleared by the drugs, with higher drug dosage resulting in lower parasite densities (E, F). Two days of drug pressure and high reduction in the density of the sensitive clone, resulted in the enhanced growth of the resistant clone in mixed infections (C). One day of drug treatment resulted in competitive release but not enhanced growth of the resistant clone (B). All figures are drawn to the same log scale.

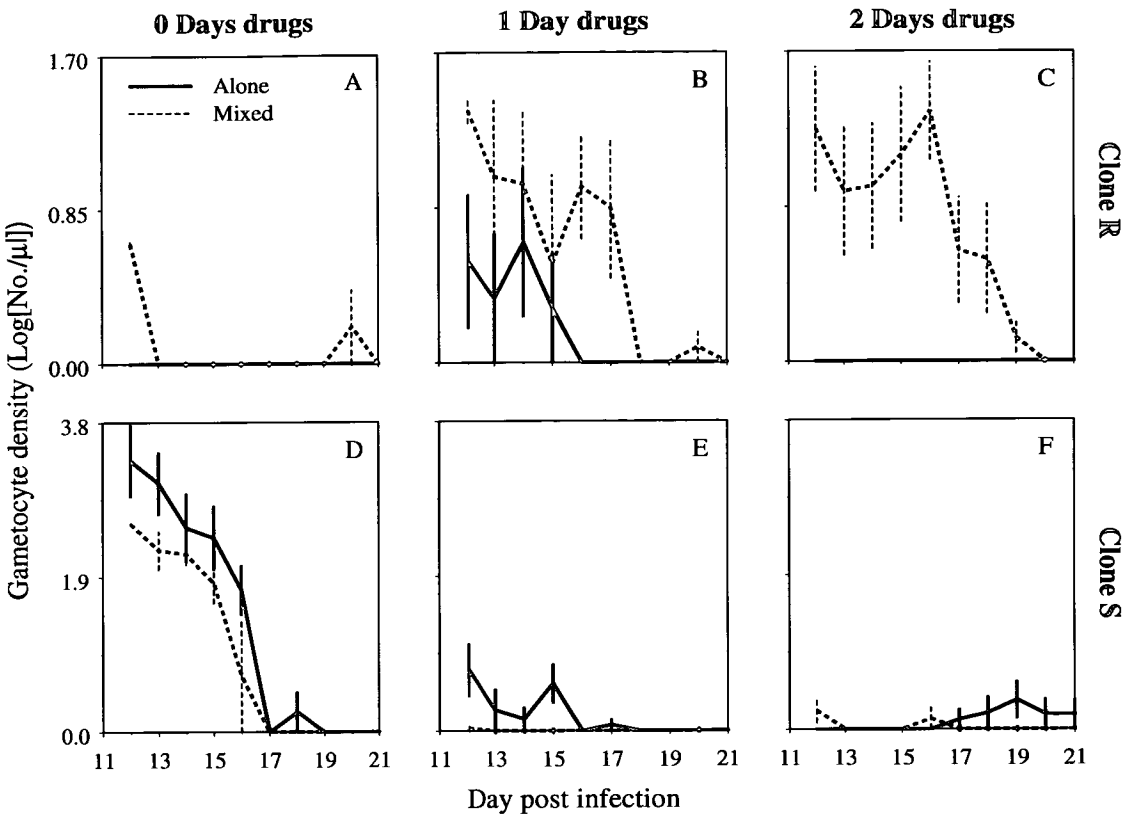


Figure 5.6. Gametocyte density after drug treatment for experiment II, determined by qRT-PCR. The mean densities (± 1 S.E.M) of the resistant (A, B, C) and sensitive clone (D, E, F), in single (solid line) and mixed (dotted line) infections, are shown for the zero (A, D), one (B, E), and two day drug treatment groups (C, F). Drug treatment occurred on day 7-9 post infection, before gametocyte density was assessed. Figures for each clone are drawn to the same y-axis log scale, and all plots have the same x-axis scale. Absence of lines indicates values were below the detection threshold of qRT-PCR.

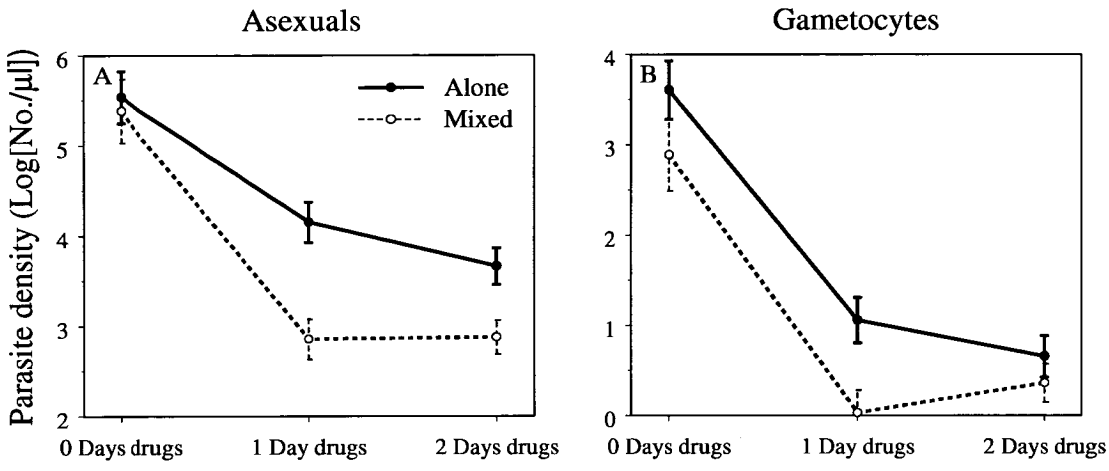


Figure 5.7. Mean parasite density of the drug sensitive clone after drug treatment, in experiment II. Drug treatment resulted in a pronounced reduction of asexuals (A) and gametocytes (B), in single (solid line) and mixed infections (dotted line). Points represent least squares mean (± 1 S.E.M) of log transformed total parasite density from days 12 -21 post infection. Only the mice surviving till the end of the sampling period, were included in the analysis (see Table 5.1).

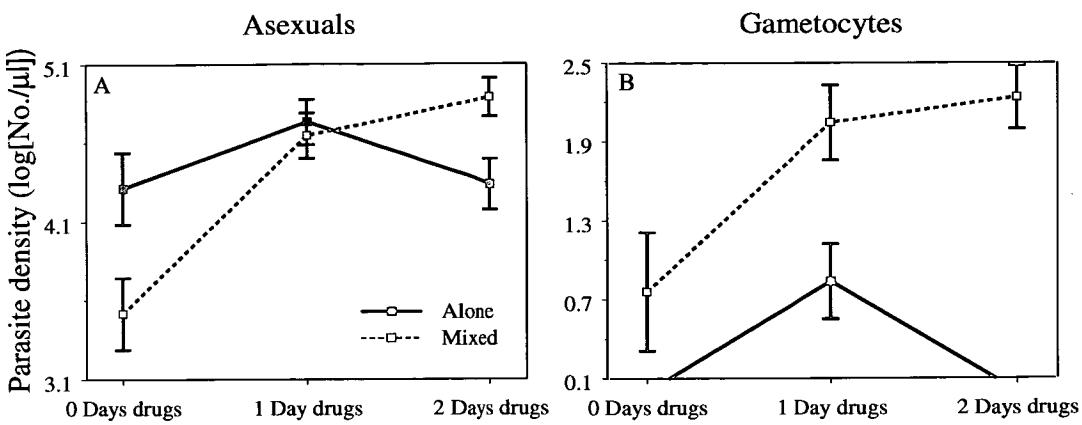


Figure 5.8. Mean parasite density of drug resistant clone in experimental II. After two days of drug treatment, the resistant clone produced significantly more asexual parasites (A) and gametocytes (B) when in the presence of a competitor (dotted line) compared to alone (solid line), experiencing competitive facilitation. After one day of drug treatment, the resistant genotype performed as well as on its own but not better, experiencing competitive release. Points represent least squares mean (± 1 S.E.M) of log transformed total parasite density for days 12 -21 post infection. Only the mice surviving till the end of the sampling period, were included in the analysis (see Table 5.1). Gametocyte density dropped below the accurate level of quantification with qRT-PCR in the single infection, zero and two day drug treatment groups.

5.4.3. Transmission investment

Generally, transmission investment was not influenced by drug treatment, for either clone. The resistant clone however, had higher transmission investment in competition than when alone.

5.4.3.1. Sensitive clone

The average proportion of gametocytes produced per total parasites was examined as an indicator of transmission investment in response to drug treatment (Figure 5.9C). Due to the complete drug clearance of the sensitive clone after 4 days of drug treatment, clone S transmission investment could only be assessed in experiment II. In the absence of drug treatment, the drug sensitive clone produced more gametocytes per total parasites in single versus mixed infections (Figure 5.9C). After two days of drug treatment this relationship switched, in that a higher proportion of gametocytes were produced by the sensitive clone in mixed versus single infections (drug treatment*competition interaction: $F_{2,22}=3.5$, $P = 0.048$). This was primarily due to a decrease in gametocyte production per asexual parasite in single infections as a result of drug treatment (Figure 5.9C).

5.4.3.2. Resistant clone

In both experiment I and II the resistant clone had higher per parasite gametocyte production in competition compared to alone (Figure 5.9A, B); this could only be tested formally in experiment I, due to gametocyte values in single infections below the detection level of qRT-PCR in experiment II (experiment I: $F_{1,17}= 5$, $P = 0.04$). There was no significant affect of drug treatment on proportional gametocyte production (Figure 5.9A, B, $P > 0.1$ experiment I and II). Therefore, the resistant genotype appeared to increase transmission investment in response to competition but not drug treatment. This result is unsurprising considering that clone R is resistant to the pyrimethamine and drug treatment had no direct effects on its parasite density (Figure 5.8).

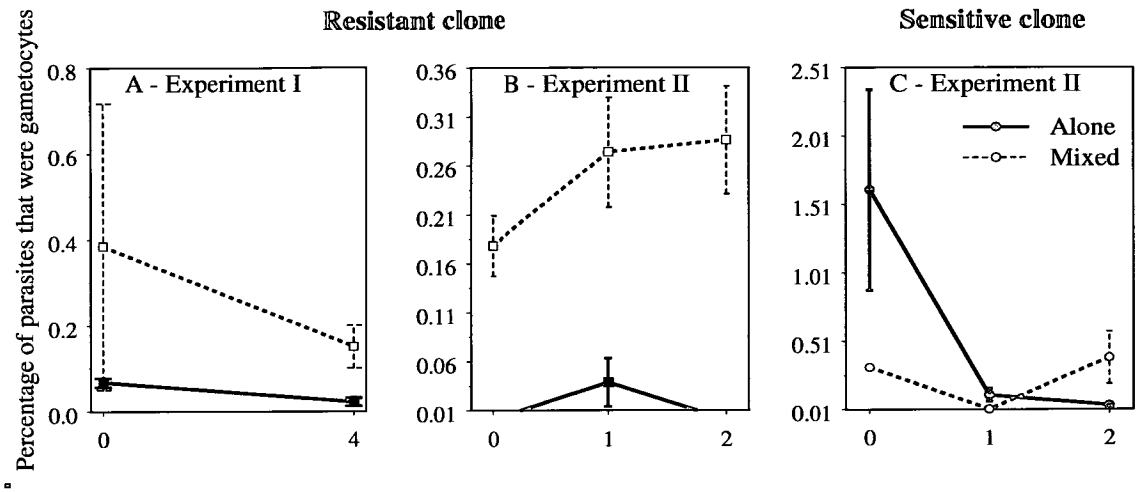


Figure 5.9. Percent of total parasites produced that were gametocytes after drug treatment for the resistant clone in experiment I (A) and II (B), and the sensitive clone in experiment II (C). Four days of drug treatment completely cleared the sensitive clone in experiment I, and therefore the percentage of parasites that were gametocytes could not be assessed. The resistant clone (A and B) tended to produce more gametocytes per parasite in mixed compared to single infections, with drug treatment having no significant effect on gametocyte investment. The sensitive clone (C) appeared to decrease transmission investment in single infections (solid line), but not mixed infections (dotted line) after drug treatment drugs. In experimental II, the single resistant clone infection zero and two day drug treatment groups had proportional gametocyte values, below the detection threshold of qRT-PCR. Data points represent mean (± 1 S.E.M) of total gametocyte density over total parasite density from days 13-14 (block I) and 12-21 (block II) post-infection. Only the mice which survived till the final day of sampling, were included in the analysis (see Table 5.1).

5.5. Discussion

Mixed genotype malaria infections are common in many malaria endemic regions, where drug resistant and sensitive genotypes frequently co-circulate in the same host (Babiker *et al.* 1999b; Anderson *et al.* 2000; Awadalla *et al.* 2001; Jafari *et al.* 2004). Therefore, intra-host dynamics plays a critical role in the evolution of drug resistance. In particular, how drug resistant clones respond to the drug clearance of sensitive clones is likely to greatly impact the rate at which drug resistance spreads, and potentially the population structure of resistance (Hastings and D'Alessandro 2000; Hastings 2006). I found that a drug resistant *P. chabaudi* malaria clone experienced enhanced growth upon drug clearance of a sensitive clone, by producing more total parasites and transmission stages in mixed infections than when inoculated as a single infection. This competitive facilitation of the resistant clone was observed after both four and two days of anti-malarial drug treatment, when the highest levels of sensitive clone clearance were obtained. Reducing drug treatment to one day led to the competitive release of the drug resistant clone, which produced as many asexual parasites and gametocytes as it would have done in the absence of competition. However, one day of drug treatment did not result in the competitive facilitation of the resistant clone, likely due to incomplete clearance of the sensitive clone and the maintenance of within host competition.

The competitive release of the resistant clone observed in this study, in response to sensitive clone drug clearance, is similar to results reported after prophylactic drug treatment (De Roode *et al.* 2004a). Most likely competitive release can be attributed to the removal of in-host competition for limited resources (Yap and Stevenson 1994). Unraveling why competitive facilitation occurred as a result of high drug treatment is less obvious and the exact mechanisms in operation here are unknown. One possibility is a lag period between drug clearance of the sensitive clone and an immune response directed towards the resistant clone. Before drug treatment, it is plausible that the immune system is forced to focus on the sensitive genotype due to its representation as the majority clone in this phase of the infection.

After drug clearance of the sensitive clone there may be a delay in the ability of the immune system to recognize the rapid change in the in-host antigenic composition, which hinders the elicitation of a specific response targeted towards the resistant genotype (Gupta and Anderson 1999). The resistant clone might then experience a period of unchecked growth.

A lag in genotype specific immunity has been observed in HIV infections where minority antigenic types are overlooked due to immune commitment to majority antigens in the population (Almogy *et al.* 2002). Antigenic distinction is highly plausible in our *P. chabaudi* system due to the high degree of genetic variation between the two clones and evidence from nude mice experiments of an immune mediated component to competition (Råberg *et al.* 2006). If the delayed immune response hypothesis is correct, I would expect that drug clearance of a drug resistant and sensitive clone which are antigenically identical, would not result in competitive facilitation, due to strong transcending immunity, and may even lead to the prevention of competitive release. This hypothesis proves difficult to test explicitly since the qPCR technique, which makes it possible to track the growth dynamics of individual clones in competition, requires genetic variation for clonal differentiation.

Regardless of the mechanisms involved, competitive facilitation could be predicted in the field due to the common co-circulation of genotypes which are genetically distinct. Some suggestive evidence of increased parasite production in natural *Plasmodium falciparum* drug resistant infections, after drug treatment, has been observed (Robert *et al.* 2000; Schneider *et al.* 2006). If occurring, competitive facilitation must greatly affect the rate at which drug resistance spreads, likely accelerating it beyond current model predictions (Hastings and D'Alessandro 2000). Ultimately, this is dependent on how in-host dynamics translates to transmission. In general, I found that transmission stage production closely tracked asexual parasite production throughout the experiment. Therefore, my results provide a good estimate of the transmission success of individual clones and the impact the observed dynamics will have on the overall population structure and epidemiology of the parasite (Taylor and Read 1998; Mackinnon and Read 1999a).

Contradictory to previous studies, I found no hint of increased transmission investment in response to drug treatment, so that although competitive facilitation resulted in increased asexual and gametocyte production, it did not lead to an increase in the percentage of parasites that were gametocytes (Buckling *et al.* 1999a). The dichotomy between my findings and those of Buckling *et. al.* (1999) may be due to the different sampling time periods, drug treatment regimes, and *P. chabaudi* clones studied. In particular, here I investigated the response of a clone to drug treatment, which was resistant to the drugs and therefore suffered no direct consequences of anti-malarial treatment.

Interestingly, I did find that the drug sensitive clone appeared to decrease transmission investment as a result of drug treatment in single but not mixed infections, perhaps due to a shift of resources into asexual parasite production, which did not drop as dramatically as total gametocyte production (Figure 5.7). The confounding effects of competition could have prevented this response in mixed infections. This is supported by my observation of a trend of increased transmission investment in response to competition, particularly by the drug resistant clone, which is known to suffer the most from competition (De Roode *et al.* 2004a; De Roode *et al.* 2005b). Although such an increased transmission investment response to competition was not observed in my previous research (Chapter 4), it is important to consider that here it was measured in late stage infections after drug treatment, whereas previously I investigated the response over the entire course of infection.

My results, demonstrating small changes in transmission investment in mixed infections, merely further support the conclusion that four and two days of drug treatment led to much greater transmission potential of the drug resistant clone in mixed compared to single infections. In contrast, after one day of drug treatment transmission potential was no higher in mixed compared to single infections. These findings suggest that the optimal drug treatment strategy to slow the evolution of drug resistance may be to reduce dosages to levels which just alleviate clinical symptoms and prevent host death, but do not completely clear sensitive parasites.

The preservation of sensitive clones will maintain a competition environment within the host which should block the competitive facilitation of the resistant clone and possibly hinder competitive release. Although zero days of drug treatment unsurprisingly results in the most favorable evolutionary outcome of competitive suppression, this must be balanced with the health and welfare of the sick host.

I found that both two and one day of drug treatment reduced host morbidity. However, there was no added benefit to increasing drug treatment and mixed infections after four days of drug treatment were just as virulent as those left untreated, potentially due to the enhanced growth of the drug resistant clone. Therefore, not only did the lowest drug dosage provide the most favorable evolutionary outcome, but the greatest observed benefit to the host. My findings contradict current theory which ascertains that incomplete drug treatment will accelerate the evolution of drug resistance, which to my knowledge, has not been experimentally investigated.

6. GENERAL DISCUSSION

6.1. Should we care about gametocytes?

Predictions about the epidemiology of malaria are often based on in-host dynamics rather than transmission. The assumption is frequently made that the dynamics observed in the host directly relate to parasite transmission. In this doctoral thesis I explicitly sought to test this assumption, asserting that we simply did not have enough information to determine how transmission relates to parasite in-host ecology. I went on to examine transmission investment in three different contexts: infection duration, mixed genotype infections, and drug resistance evolution. I discovered that transmission dynamics depend heavily on the context examined.

In respect to infection duration, I found that gametocyte investment increased in long lasting chronic infections. Additionally, during the chronic stage, per gametocyte infectivity appeared to be higher. Most importantly, extremely low parasite densities that were not detected by microscopy, were infectious to vectors. The typical field study using microscopy as a method of parasite detection would therefore have incorrectly believed these hosts to be non-infectious; see Schneider *et al.*, 2006. This sheds light on why the importance of low level parasite density infections as a transmission reservoir, has been grossly underestimated (Bottius *et al.* 1996; Babiker *et al.* 1999a; Bousema *et al.* 2006). Therefore, in this context, examining transmission is crucial, as in-host asexual parasite densities offer a poor predictor of transmissibility.

In the context of mixed genotype infections, the link between transmission and in-host dynamics was more varied. Conspecific competition was a strong driving force of the in-host ecology of acute stage mixed infections[2]. Investigation of transmission investment during conspecific competition initially revealed only a small degree of phenotypic plasticity in gametocyte production (Chapter 4). Here, one clone was found to reduce transmission investment in response to competition, but only in one host

[2] This is likely to be true for chronic infections but was not tested here, for more information see: De Roode *et. al.* 2005b, and Bell *et. al.* In press.

genotype. However, in two further experiments (Chapter 5), which utilized the same clones as previously examined (Chapter 4), I observed a markedly increased transmission investment in response to competition. The conflicting results observed between these two chapters were attributed to different sampling periods. This suggested that phenotypic plasticity in response to competition can occur and may have added temporal variability. Although phenotypic plasticity did not appear to influence the overall competitive outcome in any of the situations in which it was observed, it would have altered the proportional composition of clones in the vector population beyond what was predicted by in-host dynamics.

It is interesting to note that in all cases where phenotypic plasticity was observed during competition, it was elicited by the same competitively inferior clone. It is unclear whether this was a consequence of competitive inferiority, or merely a genetic trait of clone AS (also referred to as clone R in Chapter 5). Two further experiments to test this would be useful: Firstly, an examination of transmission stage production with a completely different clonal pair during conspecific competition, ensuring that one clone was competitively inferior to the other. Secondly, a competition experiment with the competitively superior clone in our study, AJ (also called S in Chapter 5), as the competitively inferior clone. It would then be very interesting to observe if clone AJ altered its life history and elicited phenotypic transmission investment plasticity.

Surprisingly, in this thesis I observed no clear alterations in transmission investment in response to drug treatment, where they were most expected. Increased transmission stage production as a result of pyrimethamine drug treatment has frequently been observed, and therefore is likely to play an important role on the epidemiology of the parasite (Buckling *et al.* 1999c; Bousema *et al.* 2006). However, I do not believe it has been examined in the context of conspecific competition, as was done here. It may be that competition is simply a more important cue for the parasite. It should be noted that a limitation of my study was the short sampling period available for examining transmission stage production, due to high host mortality. However, since the bulk of parasites were produced in the acute stage I examined, this is where I expected the effects on transmission stage production to be most prevalent.

In general, my thesis provides evidence that transmission stage production can vary substantially from the production of in-host asexual forms. Therefore, broad epidemiological conclusions based on the in-host dynamics of malaria may by incorrect. One could of course take this a step further and ask, do the separate antigenic types of each clone, which are produced throughout the infection, differ in their transmission investment (Biggs *et al.* 1991; Su *et al.* 1995; Antia *et al.* 1996; Druilhe and Perignon 1997; Phillips *et al.* 1997; Snounou *et al.* 2000; Viney and Read 2002)? It proves very difficult to test this hypothesis as it demands a method for distinguishing clonal antigenically distinct forms. My results suggest that such a response in malaria is unlikely, due to small degree of transmission phenotypic plasticity that was observed even at the clonal level.

6.2. What about mosquitoes?

There is one important assumption made throughout this doctoral work which was not explicitly tested: gametocyte density is positively correlated with vector transmission. Generally, studies have found that this is the case (Mackinnon and Read 1999a). But, ultimately, I only directly measured transmission to vectors in chapter three, when examining chronic infections, and there it was observed that per gametocyte infectivity to mosquitoes changed throughout the infection. This result suggests that the relationship between gametocyte density and transmission might not be straight forward. In each subsequent chapter mosquito blood feeds were attempted, but resulted in less than 5% of mosquitoes becoming infected. The limited transmission observed did portray similar patterns to those observed in gametocytes, but no solid reportable conclusions could be drawn.

It is unclear why transmission was so low in my research whereas previous work has observed much higher transmission success of *P. chabaudi* into the vector *A. stephensi* (Mackinnon and Read 1999a; Ferguson and Read 2004). The poor transmission quality of *P. chabaudi* in this thesis was one of the greatest limitations of the rodent malaria system, and it is evident that unknown factors affected transmission, which were not occurring in past research. The identification of the natural vector

species could potentially alleviate this limitation. There is also ongoing research to improve mosquito transmission success of *P. chabaudi*, by examining the effects of factors such as crowding, feeding regime, water cleanliness, age, and temperature. This type of investigation could in itself constitute an entire doctoral thesis and prove interesting not only from a practical perspective, but also for unraveling vector evolutionary ecology and control, which remain poorly understood (Donnelly *et al.* 2002; Scott *et al.* 2002; Killeen *et al.* 2003). This however, was not an aim of my doctoral research, and gametocyte investigation offers a large stepping stone above the transmission knowledge we currently have available. Now we are in a better position to begin considering factors that may further shape parasite transmission and epidemiology, which revolve around mosquito ecology (Ferguson and Read 2002; Ferguson *et al.* 2003).

6.3. Is qRT-PCR a good method?

Assessing the relevance and importance of the findings in this thesis heavily depends on the reliability of the qRT-PCR technique. During development, qRT-PCR was robustly validated against traditional microscope blood smear gametocyte quantification. I found a significant 1:1 correlation between these two methods, but the R^2 value was surprisingly low (Chapter 4). This was likely due to the much greater sensitivity and accuracy of qRT-PCR. Previous studies have found that microscope smear quantification of gametocytes is subject to a high degree of error due to their low densities (Crooks 2004). In essence this makes it very difficult to find a 'gold standard' against which to validate qRT-PCR.

I attempted to reinforce validation with microscope smears by further testing the qRT-PCR method against itself, as done in other studies (Witney *et al.* 2001; Schneider *et al.* 2004). My reasoning was that the technology has been utilized in many contexts, with high levels of success, and is in a way its own gold standard (Witney *et al.* 2001; Blair *et al.* 2002; Cheesman *et al.* 2003; Munir and Kibenge 2004; Vanlandingham *et al.* 2004; Bejon *et al.* 2005; Bjarnadottir and Jonsson 2005; De Roode *et al.* 2005a; De Roode *et al.* 2005b; Purcell *et al.* 2006; Yue *et al.* 2006; Bell *et al.* In Press). I therefore

quantified gametocytes using two different gametocyte specific genes, *pcs16* and *pc302249.00.*0, under the assumption that the genes were largely independent, and a good correlation in the results would provide further support for the validity of the method. Indeed a strong correlation was observed. However, although both of these genes have been well characterized in *P. falciparum* and other rodent malaria species such as *Plasmodium berghei*, very little proteomics work has been done on *P. chabaudi.* I assumed that the genes are playing functionally similar roles in *P. chabaudi* due to the highly conserved genetics between malaria species (Janse *et al.* 1994; Vinkenoog *et al.* 1995; Carlton *et al.* 1998; Thompson *et al.* 2001; Carlton *et al.* 2002; Waters 2002), and based on my qRT-PCR results this appears to be the case.

The ideal test to ensure that both the genes I have used for gametocyte quantification are indeed exclusively expressed in gametocytes, would be to isolate different life stages of *P. chabaudi.* Attempts at such purification were made throughout my research, using a variety of methods such as gradient purification, and selective killing of asexuals with drug treatment. Neither method could ensure 100% pure gametocytes. However, a critical discovery during my thesis was that drug treatment resulted in rapid reductions in the number of asexual parasites detected by qPCR, with a 24 hour lag in the reduction of gametocytes detectable by qRT-PCR (Chapter 5). This was expected due to the resistance of gametocytes to drugs and their 8-16 hour half-life (Reece *et al.* 2003), thus confirming that qRT-PCR was indeed quantifying gametocytes.

Currently, work is ongoing to transfect *P. chabaudi* with fluorescence protein markers (Sarah Reece and Joanne Thompson, personal communication). This technology has been utilized previously to purify *P. berghei* gametocytes, even to the level of splitting males from females (Khan *et al.* 2005). Transfection of *P. chabaudi* has however, proven difficult, but if successful it could provide an alternative method for gametocyte purification and quantification, to reinforce my results. Two limitations of this method do apply. Firstly, it is much less sensitive than qRT-PCR, and secondly, it cannot distinguish between *P. chabaudi* genotypes, particularly those naturally occurring in the host.

Genotype differentiation proves to be one of the most difficult hurdles for clone specific gametocyte quantification, due to the degree of genetic variation required, even using qRT-PCR technologies. In general, gametocyte genes are very highly conserved, likely due to their functional importance (Andy Waters, personal communication). The gene *pcs16* utilized in this thesis for clone specific gametocyte quantification (Chapter 4 and 5), was the only one of several tested that exhibited enough variation between clones. Since *pcs16* is believed to begin expression in early stage gametocytes, it may not have been the obvious first choice; especially considering mature gametocytes are believed to contribute the most to transmission (Gautret *et al.* 1996a; Gautret 2001). The alternate gametocyte specific gene *pc302249.00.0*, used for internal qRT-PCR validation during my research (Chapter 4), is believed to be expressed in late stage gametocytes and may be a more suitable candidate for gametocyte quantification. Currently it is under further investigation for clone differentiation, but the assay appears to have a much higher gametocyte number detection threshold.

6.4. Impact of findings on human malaria epidemiology

This thesis attempted to not only address some fundamental ecological questions, but also relate them to human malaria, so as to provide insights for disease management. The types of controlled experiments desired demanded the use of *P. chabaudi* as a model system. As I outlined in the introduction, there are many reasons that make *P. chabaudi* a useful model for the human malaria species, *P. falciparum*. But it is a model and in that regard, is subject to some limitations. I will attempt to address some of these limitations here as they directly relate to my research.

One very fundamental difference between *P. falciparum* and *P. chabaudi* is the level of parasitemia obtained in the host. *P. falciparum* is characterized by low parasitemia which does not typically rise above 5%, *i.e.* 10^5 parasites per µl of blood (Field and Niven 1937; Collins and Jeffery 1999). In contrast, I observed *P. chabaudi* infections frequently reaching parasitemia levels in excess of 20%, *i.e.* 10^6 parasites per µl. This likewise corresponds to different death rates in the natural hosts, which are estimated at 0.1 – 1% in humans, and between 5 – 20% in laboratory mice (Drakeley *et*

al. 2000; Breman 2001; Mackinnon and Read 2004; Snow *et al.* 2005). I raise this point here because it may have direct implications for the competition dynamics of the parasite. I would expect competition to be greater for *P. chabaudi* than *P. falciparum* infections, because in the former case crowding is greater and resources, such as red blood cells, are more limited. In fact, although there is good epidemiological evidence that competition in *P. falciparum* is occurring, it has not been directly tested, most likely for ethical reasons (Daubersies *et al.* 1996; Mercereau-Puijalon 1996; Arnot 1998; Read and Taylor 2001). If competition in *P. falciparum* does not occur, or even if the strength of competition is much weaker than for *P. chabaudi*, it might exhibit less transmission stage production plasticity, because clones may behave more independently. To my knowledge, this has not been investigated.

Similar to overall parasitemia, differences in gametocyte density and prevalence are also believed to exist between *P. chabaudi* and *P. falciparum.* In my work, I found that during the acute phase, *P. chabaudi* gametocytes were regularly detected in nearly all infected hosts, although at low densities. In *P. falciparum*, gametocyte prevalence appears to be much lower, with the number of individuals harboring microscopically detectable gametocytes typically below 20% (Taylor and Read 1997). This could partly be influenced by the different gametocyte maturation time of 24-36 hours in the rodent system, compared to approximately 7 days for the human parasite (Gautret *et al.* 1996a; Eichner *et al.* 2001; Gautret 2001). Alternatively, this discrepancy could be due to the frequent persistence of gametocytes at such low levels in *P. falciparum*, they are below the detection threshold of microscopy (Babiker *et al.* 1999a; Schneider *et al.* 2006). Despite the underlying cause, gametocytes appear to be a more limited resource for *P. falciparum.* It is difficult to say whether this will make phenotypic plastic responses in gametocyte production more important for one species or the other, but it is likely that optimized transmission life history strategies for *P. falciparum* would be critical.

A further difference between *P. falciparum* and *P. chabaudi*, is the importance of acquired immunity. Humans in malaria endemic regions are regularly exposed to the parasite and therefore have high levels of acquired immunity (Breman 2001), whereas, in my work none of the mice were exposed to infection before the start of the

experiment. Acquired immunity is likely to have a large impact on in-host competition dynamics, especially in the case of clone transcending immune function. Additionally, there is evidence that drug efficacy is higher with a primed immune response, which could impact the results of my drug resistance studies (Buckling and Read 2001; Cravo *et al.* 2001; Mackinnon and Read 2003). An examination of the effect of acquired immunity on the transmission of malaria clones in competition would be very interesting.

On a final note of the differences between *P. chabaudi* and *P. falciparum*, in this thesis, for practical reasons I examined in-host competition using only two genetically distinct clones. Humans are likely exposed to a multitude of genetic lineages (Babiker and Walliker 1997; Arnot 1998; Babiker *et al.* 1999b; Anderson *et al.* 2000). I cannot conclude that the dynamics here would hold true for all clonal combinations, and what would ensue if greater than 2 clones were present has not been experimentally tested (see De Roode *et al.*, 2003, for general asexual dynamics). However competition dynamics have been observed for a multitude of other *P. chabaudi* clonal combinations, and the addition of more clones in the host is likely to simply increase in-host competition (Bell *et al.* In Press). Likewise, similar transmission investment responses might be predicted as those observed here, although they could be more substantial if competition is greater. Ultimately, co-infection of 2-3 *P. falciparum* clones is regularly observed in nature (Babiker *et al.* 1999b) and therefore, my experiments do have relevance to the human malaria in host competition environment.

Despite the limitations of the *P. chabaudi* model, it does provide insights into important phenomena to study in its human counterpart. In particular, many of the same technologies that were used here in this thesis could be directly implemented in the field to study *P. falciparum*. Quantification of *P. falciparum* gametocytes has already begun using a real-time quantitative nucleic acid sequence-based amplification technology (QT-NASBA), and similar to what I observed, it has shown that densities are regularly below the detection threshold of microscopy (Schneider *et al.* 2004; Schneider *et al.* 2006). These studies have also demonstrated that gametocyte densities can persist after drug treatment, although in the case of *P. falciparum* they lasted for several weeks (Bousema *et al.* 2006), whereas, I only observed their persistence for 1-2 days. This

discrepancy is likely due to the greater gametocyte half-life of *P. falciparum* compared to that of rodent malaria (Smalley and Sinden 1977; Eichner *et al.* 2001; Reece *et al.* 2003). Most interestingly, the QT-NASBA technology has revealed that some anti-malarial drugs result in a decrease in gametocyte densities, while others do not (Bousema *et al.* 2006). This is congruent with the findings of Buckling *et al.* (1999) who observed that some types of anti-malarial drugs increase gametocytogenesis in *P. chabaudi* and others do not. These results suggest that different drug types may vary in their influence on the dynamics and fitness of drug resistant clones, and therefore their impact on the rate of resistance evolution (see Chapter 5).

A current limitation to the field QT-NASBA technology is the inability to distinguish between *P. falciparum* clones. Clonal differentiation could easily be implemented with the qRT-PCR technique I have developed (Chapter 2 and 4), to assess the dynamics of individual clones in natural human *P. falciparum* infections (Decuypere *et al.* 2003). This would require the sequencing of *P. falciparum* clones regularly circulating in humans, and could lead to the missed detection of some lineages. Other practical limitations must also be borne to mind. Most notably the high cost of qRT-PCR and the rapid degradation of RNA in a hot environment, both of which can be hindering factors to field work in malaria endemic regions. Despite this, the technology should make huge advances for the field of human malaria epidemiology, especially in the context of drug resistance evolution.

6.5. A few burning questions

Science is the process of discovery, with each answer, come a host of new questions. The results I have obtained in this doctoral thesis, likewise, raise many questions and my aim here is to suggest possible avenues of future research.

1. Do chronic infections contribute significantly to the reservoir of human malaria?

There is growing evidence that low level chronic *P. falciparum* infections are transmissible to vectors (Hamza Babiker, personal communication, Bousema *et al.* 2006). Using the techniques I have outlined, it is now possible to quantify gametocytes

in chronic and acute *P. falciparum* infections and compare their infectivity. This could provide insights into the mechanism behind the transmission of extremely low parasite densities. It would also then be possible to provide an accurate estimate of what percentage of the *P. falciparum* transmission reservoir is maintained in chronic infections.

2. What is the epidemiological relevance of transmission investment plasticity?

Although, the transmission investment plasticity responses I observed were very weak, they were unexpected and warrant further investigation, as some key questions remain unanswered. In particular: How much does this plastic response actually effect the transmission of the parasite? What dynamic is observed when the infection is made up of more than two clones? Is there a genetic component to this variation? Does *P. falciparum* alter gametocyte production? Does plasticity have an impact on the evolution of virulence? Mosquito vector transmission experiments are warranted here.

3. Does competitive facilitation, as a result of drug treatment, impact the rate of resistance evolution?

This is an area of research which is just beginning to blossom. An array of hypotheses remain to be tested, for which the qPCR and qRT-PCR methods prove to be a powerful tool. First and foremost, what is the mechanism behind this response? Is the response genotype dependent? Will different types of drugs elicit a different response due to their varied mechanisms of operation? Will we observe the same response in *P. falciparum*? As far as I know, this type of dynamic has never been fitted into model simulations, therefore, how does it impact the theoretical predictions of resistance evolution?

4. Can we harness in-host ecology to slow resistance evolution?

The experiments in chapter 5 suggest that reducing drug treatment maintained in-host competition, thereby inhibiting the performance of the drug resistant clone. However, competitive release was ultimately still observed, and therefore, resistance

evolution would be expected. It would be very interesting to investigate if further reductions in drug treatment could maintain in-host competition to such a degree, that the resistant clone remained competitively suppressed, inhibiting its transmission. Balancing this drug treatment regime with the welfare of the host must also be considered. This type of investigation has perhaps the most critical relevance to malaria control.

6.6. Concluding remarks

In this doctoral thesis, I took a step back, and attempted to tap into fundamental natural selection principles for the study of parasite evolution. Transmission is the most critical driving force for parasite fitness and demands further investigation from an evolutionary ecology perspective, if successful malaria control strategies are to be achieved. My research provided clues into the dynamic nature of transmission, and brought to question the validity of assumptions between observed in-host ecology and transmission dynamics. Ultimately, both in-host ecology and transmission shape each-other: higher levels of transmission increase mixed infection rates. The goal here was not to provide all the answers, but rather to raise some important questions that may have never been asked. Malaria is a challenging beast, which has proven difficult to tame. Each summit we reach only reveals how many more there are left to conquer, but it's that final Mt. Everest on the distant horizon that makes the journey worth taking.

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8. THESIS APPENDIX

Scientific publications

Wargo, A. R., Randle, N., Chan, B. H. K., Thompson, J., Read, A. F. & Babiker, H.A. 2006 *Plasmodium chabaudi*: Reverse transcription PCR for the detection and quantification of transmission stage malaria parasites. *Experimental Parasitology* 112, 13-20. *(Based on Chapter 2)*

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Plasmodium chabaudi: Reverse transcription PCR for the detection and quantification of transmission stage malaria parasites

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Abstract

We have developed two reverse transcription polymerase chain reaction (RT-PCR) techniques to detect and quantify the transmission stages (gametocytes) of *Plasmodium chabaudi* malaria parasites. Both the qualitative and quantitative techniques are based on the amplification of mRNA coding for the *P. chabaudi* protein Pcs230, which is expressed exclusively in gametocytes. The quantitative RT-PCR (qRT-PCR) technique was developed and validated by examining serial dilutions of known gametocyte densities. The method generated a high correlation between calibration curves of blind samples ($R^2 = 0.86$). The technique was found to be specific, reproducible, and time efficient for quantification of both patent and sub-patent gametocytemia with a sensitivity level 100–1000 times greater than microscopy. The qualitative RT-PCR (RT-PCR) technique was used to monitor the persistence and dynamics of *P. chabaudi* gametocytes following acute infection. Mice in two independent experiments were sampled for up to 87 days post-infection. RT-PCR showed that gametocytes can persist for up to 8 weeks, post-infection, whereas microscopy could only detect gametocytes up to 6 weeks. Potential applications of the above techniques for studying the ecology, evolution, and epidemiology of malaria transmission are discussed.

Index Descriptors and Abbreviations: Plasmodium chabaudi; Gametocyte; Malaria transmission; pcs230; Chronic infection; DNA, deoxyribonucleic acid; RNA, ribonucleic acid; dNTPs, deoxyribonucleotide triphosphate mix; RT-PCR, reverse transcription of the RNA followed by PCR amplification of the cDNA; cDNA (RNA to complementary DNA); qPCR, quantitative real time PCR; qRT-PCR, quantitative real time reverse transcription PCR

1. Introduction

Molecular methods are now becoming widely utilized to study malaria parasites both in field and laboratory settings. The power of these techniques is increasingly clear, particularly for studying the ecology and evolution of parasites. In nature, malaria infections often occur at sub-patent levels where parasites cannot be detected by microscopy. This is very common in areas such as eastern Sudan, where the transmission of *Plasmodium falciparum* is highly seasonal and chronic sub-patent infections are prevalent in the dry season (Abdel-Wahab et al., 2002; Babiker and Walliker, 1997; Hamad et al., 2002).

Numerous studies have been conducted to examine the diversity and dynamics of asexual forms of malaria parasites, but the ecology of gametocytes is still poorly understood. Recently, reverse transcription PCR (RT-PCR) techniques have been used to detect RNA of proteins that are expressed exclusively in gametocyte stages, distinguishing gametocytes from asexual stages of *P. falciparum*, even at sub-patent levels (Babiker et al., 1999; Menegon et al., 2000; Niederwieser et al., 2000; Schneider et al., 2004). Some gametocyte-specific protein genes are polymorphic, allowing the technique to be extended to distinguish game-

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tocytes of different genotypes within a single infection (Abdel-Wahab et al., 2002). RT-PCR has shown that *P. falciparum* is capable of producing gametocytes, even when it exists as asymptomatic, sub-patent infection, demonstrating the potential of such infections to sustain the cyclical malaria epidemics in areas of seasonal transmission (Babiker et al., 1999). A shortcoming of the existing RT-PCR protocol is that it cannot quantify gametocytes (Abdel-Wahab et al., 2002; Babiker et al., 1999, 2000; Menegon et al., 2000). Such quantification is important to gain a better understanding of the transmissibility of individual parasite clones that exist in mixed clone infections and the ecological factors that influence them.

Recently, real-time quantitative PCR (qPCR) has been developed and successfully utilized to study the dynamics of different malaria species and genetically distinct clones in mixed infections (Cheesman et al., 2003; De Monbrison et al., 2003; De Roode et al., 2004; Perandin et al., 2004). This method enables accurate quantification of genomic DNA of specific malaria parasite clones within multiple infections, but cannot selectively quantify different life-stages. However, quantitative reverse transcription PCR techniques (qRT-PCR) have also been developed and utilized for the quantification of RNA coding for various genes, including those of malaria parasites (Bruna-Romero et al., 2001; Nirmalan et al., 2002). The technique has been utilized for the quantification of malaria parasite liver stages and a related but different method (QT-NASBA) has been used to examine dynamics of *P. falciparum* gametocytes (Schneider et al., 2004; Witney et al., 2001).

We report here on the utilization of PCR and RT-PCR protocols to detect parasites and monitor the longevity of *Plasmodium chabaudi* infections and their gametocyte production over time. Additionally, we developed RT-PCR and qPCR methods into a protocol for qRT-PCR that can specifically quantify gametocytes of the rodent malaria parasite *P. chabaudi*.

2. Materials and methods

2.1. Plasmodium chabaudi infections

Two independent experiments were carried out to monitor the longevity of gametocyte production of the *P. chabaudi* rodent malaria parasite clone CR that is known to have moderate virulence in mice (Mackinnon and Read, 1999). Infections were established by inoculating 5 (experiment I) and 10 (experiment II) C57Bl/6J 5 to 8-week-old inbred female mice (Harlan Scientific, UK) with 10^6 *P. chabaudi* parasites, into the peritoneal cavity, as described elsewhere (Mackinnon and Read, 1999). Mice were maintained at 21 °C (±1 °C) and fed ad libitum SDS 41B (Harlan Scientific, UK) rat and maintenance diet and 0.05% PABA supplemented drinking water to enhance parasite growth. Blood samples (10 μl for DNA extraction and 20 μl for RNA extraction), thin blood smears, and red blood cell counts (on 2 μl of blood) from flow-cytometry (Coulter Electronics) were taken 5 days a week, for 3 weeks, starting on day 3 post-infection (PI). After day 22 PI, samples were taken 2–3 times a week until day 87 (experiment I) or day 70 (experiment II). Larger blood volumes were required for RNA extraction to increase sensitivity for gametocytes which are in low abundance compared to asexual stages. Thin blood smears were stained with a 20% Giemsa solution and examined under a microscope at 1000× magnification to determine parasitemia and gametocytemia. Parasite and gametocyte densities were calculated by multiplying parasitemia and gametocytemia, respectively, by red blood cell density, measured with flow-cytometry.

For qRT-PCR, separate infections of the *P. chabaudi* clone CR were established in three NIH mice using methods described above. The density of gametocytes (gametocytemia) was determined using Giemsa stained thin blood smears, and blood samples were taken for RNA extraction when gametocytes were visible microscopically. These mice were not used for the monitoring of chronic infections via RT-PCR.

2.2. Molecular methods for detecting parasites

2.2.1. DNA extraction

For general PCR, DNA was extracted from 10 μl of blood taken from the tail of *P. chabaudi* infected mice and added to 200 μl of citrate saline (0.85% NaCl, 1.5% trisodium citrate), on ice. The samples were centrifuged at 11,000 rcf for 2–3 min to pellet red blood cells and the supernatant was removed. The blood pellet was stored at −80 °C until DNA extraction. DNA was extracted using InstaGene Matrix (Bio-Rad) according to the manufacturer's protocol.

2.2.2. RNA extraction

Parasite RNA was extracted using two methods:

(i) For RT-PCR, RNA was extracted from 20 μl of whole blood, using TRIzol Reagent for total RNA isolation, according to the manufacturer's protocol (Invitrogen Life Technologies). On each day of sampling, 20 μl of blood was taken from the tail of each mouse and added to 200 μl of citrate saline on ice. The samples were centrifuged at 11,000 rcf for 3 min at room temperature to pellet the blood and the supernatant was removed. A 10× volume (200 μl) of TRIzol was added to each pellet and the samples were vortexed immediately and stored at −80 °C until RNA extraction. RNA samples were subjected to DNase treatment to remove co-extracted DNA in a 10 μl reaction containing 5 μl RNA extract, 5 mM Tris, pH 7.6, 33 mM $MgCl_2$, 0.27 U RNasin Ribonuclease Inhibitor and 4.2 U DNase I (Roche, UK), with incubation at 37 °C for 15 min and 75 °C for 10 min.

(ii) For qRT-PCR, RNA was extracted using the Applied Biosystems 6100 equipment and protocols. This method allows simultaneous processing of 96 samples and is more time efficient for use with the 96-well qRT-PCR format. To extract gametocyte RNA, 20 μl of blood was taken from the

mouse tail and added to 200 μl of citrate saline pre-warmed to 37 °C to avoid alterations in expression of RNA believed to occur at temperature changes (Fang and McCutchan, 2002). Samples were centrifuged at 11,000 rcf for 3 min at room temperature, the supernatant was removed, and 20 μl Ca^{2+}/Mg^{2+} free RNase/DNase free PBS (Gibco) and 40 μl of 2× Nucleic Acid Purification Lysis Solution (Applied Biosystems) were added to the blood pellet. The samples were then stored at −80 °C. For completion of the extraction protocol, the samples were thawed and incubated at room temperature for 1 h to digest blood. RNA was extracted using the Applied Biosystems Isolation of total RNA from Whole Blood Chemistry protocol on the ABI Prism 6100 Nucleic Acid PrepStation with the RNA Blood-DNA method (Applied Biosystems). RNA was eluted in 100 μl of Nucleic Acid Purification Elution Solution and stored at −80 °C until required for quantification.

2.2.3. PCR and RT-PCR

Primers for PCR and RT-PCR used for longevity experiments were developed to amplify the gametocyte-specific *P. chabaudi* gene *pcs230* (GenBank Accession No. EAA15629.1 and EAA22479.1). The *pcs230* sequence was determined by the *P. chabaudi* sequencing project and can be obtained from ftp://ftp.sanger.ac.uk/projects/P.chabaudi. This gene belongs to the *p_s48/45* super-family that encode gametocyte-specific surface proteins in malaria parasites and is only expressed in mature late stage gametocytes (Bozdech et al., 2003; Hall et al., 2005; Le Roch et al., 2003; Lobo and Kumar, 1998; Niederwieser et al., 2000; Thompson et al., 2001; Williamson et al., 1993). To increase the sensitivity of detection, two rounds of PCR were performed, using outer primers, Pc230(O1), 5′-AAAGATTC AGGGCATGGCG-3′ and Pc230(O2), 5′-TTGCCCCA CTTTTTGAGCTAC-3′and inner primers, Pc230(N1), 5′-AT CTGTTATGCCTTATGGGAG-3′and Pc230(N2), 5′-CTT GATTAATACTTAGATACACCATATGAG-3′. The optimal annealing temperature for each set of primers was determined on a ThermoHybaid PCR machine using a temperature gradient PCR on samples known to be positive for *P. chabaudi* DNA.

To determine longevity of *P. chabaudi* infection, PCR was performed using Roche 2× PCR Master mix in 20 μl reactions containing 2 μl of genomic DNA, 0.5 U *Taq* DNA polymerase, 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, and 0.5 μM of primers Pc230(O1) and Pc230(O2). Samples underwent PCR on an MJ Research DNA Engine, PTC-200; using the following conditions: denaturation at 94 °C/180 s, 35 cycles of amplification [94 °C/30 s, 51 °C/30 s, and 70 °C/60 s], and a terminal extension at 70 °C/600 s. Positive DNA controls and negative controls (all reagents + 2 μl water) were run for each reaction. The outer PCR products were then diluted 10× and 2 μl of the diluted product underwent nested PCR using primers Pc230(N1) and Pc230(N2) in a 20 μl PCR using PCR Master mix (Roche) and the PCR conditions described above.

For the detection of longevity of gametocyte production in *P. chabaudi* infection, a nested RT-PCR was carried out to detect *pcs230* mRNA. RNA samples underwent a one-step RT-PCR using the AccessQuick RT-PCR system (Promega). This protocol generates cDNA from RNA and then amplifies the cDNA through PCR, in a single reaction. The RT-PCR was carried out in a 20 μl volume on the MJ Research DNA Engine PTC-200, with a reaction mixture using the AccessQuick Master Mix reagents consisting of 2 μl of RNA, 2 U of AccessQuick reverse transcriptase (Promega), 2 U *tfl* DNA polymerase, 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 10 μl AccessQuick Master Mix (supplied by Promega as part of kit), 0.5 μM of primers Pc230(O1) and Pc230(O2), and 6 μl AccessQuick H_2O (Promega). The RT-PCR and subsequent amplification of cDNA took place under the following conditions, reverse transcription at 50 °C/60 min, denaturation at 94 °C/3 min, 39 cycles of amplification [94 °C/30 s, 61 °C/30 s, and 70 °C/6 s], and a terminal extension at 70 °C/600 s. Simultaneously, an aliquot of 2 μl RNA from each sample was subjected to the same PCR protocol using the primers and the above conditions minus reverse transcriptase, to detect any carry over genomic DNA that might have been co-extracted with the RNA samples. Positive RNA controls, positive DNA controls, and negative controls (all reagents + 2 μl water) were also included in each run. All outer RT-PCR products were diluted 1/10 then underwent a second PCR under the same conditions as outlined for the PCR of DNA.

2.2.4. Detection limits of PCR and RT-PCR

To determine the sensitivity of nested PCR and RT-PCR, samples of known parasite and gametocyte density determined by microscopy (parasites or gametocytes/μl of blood) of *P. chabaudi* were serially diluted. Eight independent samples of extracted DNA were subjected to 10-fold serial dilutions, creating a dilution series ranging over seven orders of magnitude from undiluted (neat) to diluted $1/10^7$ (actual parasite density range tested: 1.72×10^5–4.3×10^{-3} parasites/μl). Since nested PCR of DNA amplifies genomic DNA from all stages of the parasite, counts of both ring stage parasites and gametocytes were used to determine parasite densities from thin blood smears. To establish the sensitivity of RT-PCR, 11 samples of RNA were subjected to 10-fold serial dilutions, creating a dilution series ranging from neat to diluted $1/10^7$ for seven samples. None of the samples 1–7 were positive below a dilution of 10^{-5}, so four more samples were diluted only to $1/10^6$. The overall dilution range tested for RT-PCR was 1.3×10^4–1.0×10^{-4} parasites/μl.

2.2.5. qRT-PCR

A qRT-PCR technique was performed to amplify and quantify RNA coding for the gametocyte-specific protein Pcs230, using the Applied Biosystems TaqMan One-Step RT-PCR Master Mix Reagents Kit, on the ABI Prism 7000 sequence detection system. Two primers (Pc230RT-1, 5′-T CTAGTACATGCTTTGAAGAAGTAATAGAATATA ATCCTAATA-3′, and Pc230RT-2, 5′-TGCAACGACTT

TCTATAGCTAGTAGGT-3′) and a fluorescent labeled probe (Pc230RT-probe, 5′-AAAAATGGGATCGAAAT AAAA-3′) were designed by Applied Biosystems Assays-by-Design Service from the *pcs230* gene sequence. As part of this service, Applied Biosystems optimized all primers and probe amplification and annealing conditions and combined them in a master mix.

A one-step qRT-PCR assay was used to generate cDNA from RNA and the cDNA quantified, in a single reaction (Applied Biosystems). This method assumes that reverse transcription takes place at a constant rate between all samples (Nirmalan et al., 2002). The reaction mix consisted of 8.5 μl of extracted RNA, 1 μl of 20× Assay-by-Design Mix containing primers and probes at a reaction concentration of 900 and 250 nM, respectively (supplied by Applied Biosystems), and the following components from the Applied Biosystems TaqMan One-Step RT-PCR Master Mix Reagents Kit: 10 μl 2× AmpliTaq Gold DNA Polymerase mix (contains AmpliTaq Gold DNA polymerase, dNTP's with dUTP and buffer components), 5 μl of 40× RT enzyme mix (contains MultiScribe Reverse Transcriptase and RNase Inhibitor). Samples were loaded onto 96-well optical reaction plates and placed in the ABI Prism 7000 Sequence Detection System (Applied Biosystems), then underwent the following reaction conditions: reverse transcription at 48 °C for 30 min, denaturation at 95 °C for 10 min, and 50 cycles of amplification [95 °C for 15 s and 60 °C for 60 s]. Data were visualized using the ABI Prism 7000 SDS Software (Applied Biosystems). Simultaneously, eight RNA samples underwent conventional nested PCR using primers Pc230(O1), Pc230(O2), Pc230(N1), and Pc230(N2) to test for DNA contamination.

2.2.6. Validation of qRT-PCR

The qRT-PCR technique was validated by comparing threshold cycle values (number of cycles after which a sample crosses the threshold of positivity) generated from serial dilutions of samples of known gametocytemia. We expected the threshold cycle to be negatively correlated with the number of gametocytes present in a sample, showing the same value when the numbers of gametocytes in two different samples are equal. Therefore, a strong correlation in best fit lines for threshold cycle versus gametocyte number generated from serial dilutions with known numbers of gametocytes will demonstrate that gametocytes are accurately quantified. To further test the robustness of the method, we compared the accuracy of threshold cycle values calculated for two different types of serial dilutions, performing replicates of each set. The first set of dilution series was prepared blindly with extracted RNA from samples of known gametocyte counts (determined after qRT-PCR, via microscopy). Eleven, 10-fold serial dilutions (neat to $1/10^7$) of extracted RNA (from three different mice) diluted in water were prepared. A second type of dilution curves were set up blindly to dilute actual parasites by using uninfected blood from NIH mice to dilute samples of *P. chabaudi* infected whole blood with known gametocyte counts (numbers calculated after qRT-PCR, via microscopy). Two, independent, 10-fold serial dilutions ranging from neat to $1/10^7$ were generated in this manner and the dilution series samples were extracted on the ABI Prism 6100 Nucleic Acid PrepStation with the RNA Blood-DNA method (Applied Biosystems). The dilution of samples with the uninfected whole blood method more accurately simulates quantification of gametocytes from mice harbouring different levels of gametocyte density, and therefore accurately demonstrates the sensitivity of extraction as well as qRT-PCR.

All 13 sets of RNA dilutions were quantified for *pcs230* mRNA using the TaqMan One-Step RT-PCR Master Mix Reagents Kit on the ABI Prism 7000 sequence detection system (Applied Biosystems). The data from all curves were pooled and analysed using Minitab statistical software (version 13.1). Threshold cycle values were regressed against the $\log_{10}$ of the number of gametocytes within samples using ANCOVA. All sets of serial dilutions were compared using a regression to estimate the validity of the qRT-PCR method.

3. Results

3.1. Detection of P. chabaudi infection and gametocytes

Conventional PCR of genomic DNA showed a mean minimum detection limit of 3.68 ± 2.11 (±1 SE) parasites/μl blood. With regard to gametocytes, RT-PCR was found to detect a mean minimum of 27.1 ± 11.7 (±1 SE) gametocytes/μl blood. However, in 5 out of 11 sets of serial dilutions of gametocyte-infected blood, qualitative RT-PCR was detected down to 1 gametocyte/μl of blood. These detection limits are similar to those found for the detection of *P. falciparum* asexual forms and gametocytes (Babiker et al., 1999; Roper et al., 1996). In general, the sets of dilution series that started with initial higher gametocytemia resulted in RT-PCR product at lower dilutions (Fig. 1). In one case, a neat sample did not give an RT-PCR product, likely due to pipetting error.

Overall, significantly more blood samples were found to harbour *P. chabaudi* infection revealed by PCR ($\chi^2 = 16.9$, $P < 0.0001$, df = 1), and gametocytes detectable by RT-PCR ($\chi^2 = 24.4$, $P < 0.0001$, df = 1), than by microscopy. Across the 424 blood samples examined, 161 (38%) were gametocyte-positive by nested RT-PCR, whereas only 97 (23%) were gametocyte-positive by microscopy. There were 14 instances (3.30%) where gametocytes were detected by microscopy but not with nested RT-PCR, likely to be due to failed RNA extraction. PCR product from DNA was seen in 110 (27%) out of 405 blood samples when RT-PCR for gametocyte RNA was negative. Because both gametocytes and asexual parasites contain DNA but only gametocytes contain Pc230 RNA, we believe these samples represent cases where asexual parasites were present but gametocytes were absent. Additionally, it has been shown that PCR of parasite DNA is not possible after the parasites have been cleared by the host (Jarra and Snounou, 1998).

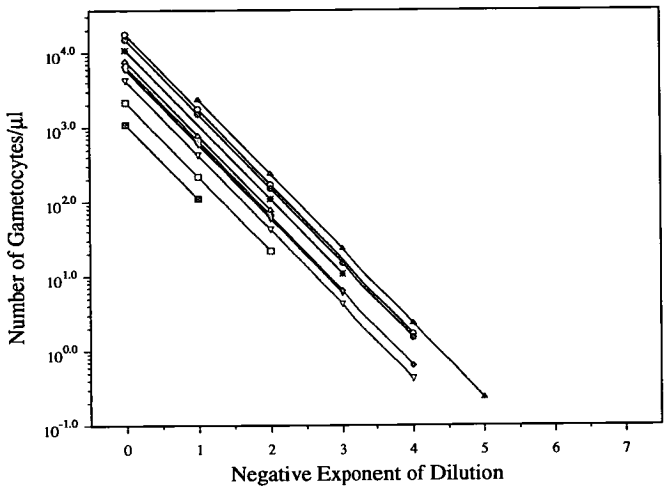

Fig. 1. Detection level of gametocytes with RT-PCR of RNA coding for the *Pcs230* gene. Each line represents a separate dilution series of independent samples. *Y*-axis shows number of gametocytes in the sample estimated by microscopy. *X*-axis shows the position of the sample in the dilution series with 0 being the neat (undiluted) sample and 7 being the seventh dilution in the series (i.e., the neat sample diluted 10^{-7}). A dilution series up to 10^{-7} was performed on seven samples and up to 10^{-6} on four samples. Where symbols are present, positive RT-PCR was obtained. Where symbols are absent, RT-PCR was negative. In one case, RT-PCR on the undiluted sample was negative likely due to pipetting error.

3.2. Persistence of *P. chabaudi* asexuals and gametocytes in infected mice from longevity experiments

Figs. 2A and B show *P. chabaudi* parasite density in chronic infections revealed by microscopy, nested PCR, and RT-PCR for one of the five mice in experiment I and one of the 10 mice in experiment II, respectively. All of the remaining mice in both experiments show the same pattern as those in Figs. 2A and B (see Supplementary data). In both experiments, gametocytes were often detected using RT-PCR several days after they were no longer detectable by microscopy. Mice controlled initial parasitemia after about 3 weeks. Subsequently, the infections showed one or a few transient peaks of patent parasitemia that recrudesced between days 30 and 60, during which time gametocytes were frequently only detectable by RT-PCR. In most mice, parasites were not detected after day 50 PI and none were detectable either by microscopy or molecular methods after day 60. The median time (±1 SE) to clearance of gametocytes (i.e., time for 50% of mice to clear gametocytes) was 36 ± 3 days derived by RT-PCR compared to 18 ± 2 days by microscopy (Kaplan-Meier test).

3.3. qRT-PCR

The qRT-PCR technique for gametocyte-specific *pcs230* RNA in 13 separate serial dilutions of gametocytes detected approximately one gametocyte per reaction, corresponding to ≤1 gametocyte/µl blood (Fig. 3). As expected, the number of gametocytes was highly related to the threshold cycle

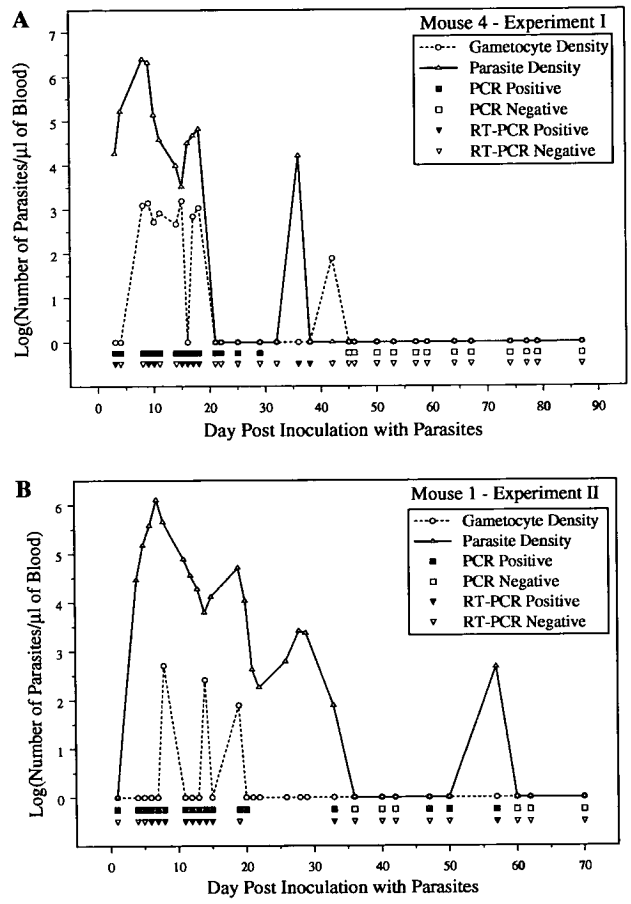

Fig. 2. *Plasmodium chabaudi* asexual forms and gametocytes detected by microscopy, PCR, and RT-PCR in a mouse from experiment I (A) and one from II (B). Red solid lines and blue dotted lines represent asexual stages and gametocytes density, respectively, as determined by microscopy. Squares show qualitative PCR for respective days, filled squares are positive samples, empty squares are negative samples, absence of a square means no sample taken. Triangles represent qualitative RT-PCR samples for *Pcs230* gene on respective days, illustrated in the same manner. PCR and qualitative RT-PCR were more sensitive for detecting asexuals and gametocytes than microscopy as indicated by filled squares and triangles, respectively. No RNA samples showed an RT-PCR product when reverse transcriptase was omitted, thus excluding DNA contamination during RNA extraction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

($R^2 = 0.86$, $F_{1,32} = 174$, $P < 0.0001$), with no significant differences in the slope of the relationship between the 13 dilution series. Specifically, there was no difference in slope between the two dilution series made from samples diluted in whole blood and 11 dilution series made extracted RNA diluted in water ($P > 0.05$ in all cases). The common slope (±1 SE) of the combined 13 dilution series was CT = −3.20(±0.24). Our results are very close to the optimum obtainable slope of −3.3 for PCR amplification during qRT-PCR.

4. Discussion

We have previously developed an RT-PCR technique to detect RNA encoding proteins that are expressed

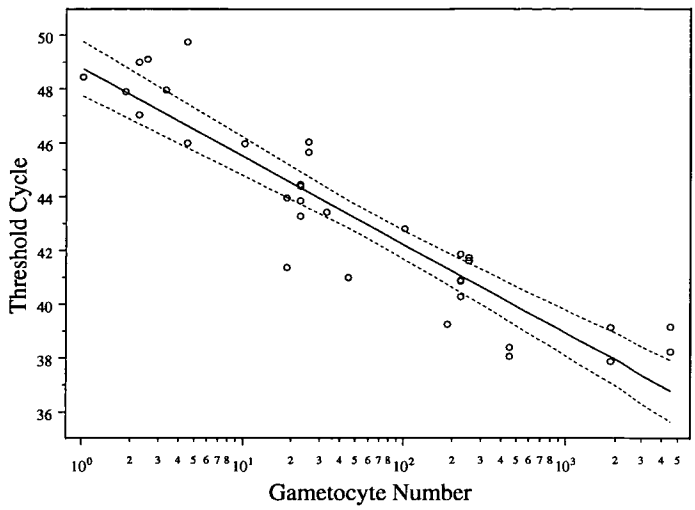

Fig. 3. Threshold cycle (determined by qRT-PCR) and gametocyte number (determined by microscopy) for 13 dilution series (11 from diluted extracted RNA, 2 from dilutions of actual parasites with whole blood). The lines are the least squares regression line (solid) with 95% confidence intervals (dotted). There is a strong correlation between threshold cycle and gametocyte number ($R^2 = 0.86$, $F_{1,32} = 174$, $P < 0.0001$; slope $\pm$ 1 SE $= -3.20 \pm 0.24$). See text for further details.

exclusively in *P. falciparum* gametocytes (Babiker et al., 1999; Menegon et al., 2000). Here, we have extended this protocol and established robust and sensitive RT-PCR and qRT-PCR methods to monitor the persistence of *P. chabaudi* gametocytes throughout infection. The techniques are based on the amplification of *pcs230* mRNA, which is expressed exclusively in gametocytes (Hall et al., 2005; Niederwieser et al., 2000; Thompson et al., 2001).

We found that the qRT-PCR detection limit ranged from 1 to 10 gametocytes/μl of blood making it 100–1000 times more sensitive than microscopy, with similar sensitivity levels to those found using QT-NASBA for *P. falciparum* gametocytes (Schneider et al., 2004). This very low limit of detection was reproducible in 11 independent sets of serial dilutions of gametocytes. Such very low gametocytemia would require an experienced microscopist to examine more than 1000 fields of a well-stained thick blood smear, which could take at least 50 min (Babiker et al., 1999). Additionally, quantification by microscopy is often inaccurate when gametocytes persist at the low levels (<0.01 gametocytes/red blood cell) common in natural malaria infections (L. Crooks, *Transmission Investment in Malaria*, Ph.D. Thesis, University of Edinburgh, 2004; Taylor and Read, 1997). Thus, comparatively, the qRT-PCR technique for gametocytes presented here provides a specific and highly sensitive estimate to gametocyte density, especially at low levels of gametocytemia. It is theoretically possible that different stages of gametocytes may express varying levels of *Pcs230* RNA. However, even when using blood samples from mice on different days post-inoculation, our results showed a strong correlation between expected and observed gametocyte values, showing that expression level is not a source of a large degree of error.

Overall, our protocol is time efficient and capable of processing 96 samples in 8 h. It, therefore, has great epidemiological potential, and can easily be adapted to study human malaria parasites. This method differs from current quantification protocols in that it is, to our knowledge, the first to quantify *P. chabaudi* gametocytes. Since this technology only requires the design of probes and primers and does not require the use of gene constructs as internal standards, assays can rapidly and cheaply be developed. In addition, by utilizing polymorphic gametocyte-specific protein genes, the presented method can easily be adapted for tracking an individual gametocyte genotype when the infection is composed of multiple genotypes. Through the application of newly described sex specific gametocyte protein genes (e.g., Khan et al., 2005), the method can also be developed for the individual quantification of male or female gametocytes.

We used the developed RT-PCR technique to monitor longevity of gametocyte production of two *P. chabaudi* clones. Our results demonstrated that *P. chabaudi* persisted at sub-patent levels, and continued to produce gametocytes for several weeks as chronic infections. This result agrees with that seen in natural *P. chabaudi* infections in the natural host *Thomnomys rutilans*, where chronic infections lasting up to 2 years are often observed (Landau et al., 1999). In the present study, *P. chabaudi* infection persisted for up to 60 days post-infection, however, the duration of our study was limited to 87 days, and therefore it is possible that additional parasite recrudescence phases may have been missed. In addition, all infections were composed of a single *P. chabaudi* clone, whereas, in a recent study of *P. chabaudi*, infections consisting of three clones were found to persist longer than did single-clone infections (De Roode et al., 2003).

The techniques presented here allow a range of hypotheses to be tested. For instance, chronic persistence of malaria infections in the face of sustained immune attack is probably due to antigenic variation by parasites (Phillips et al., 1997). Antigenic variation is often viewed as an adaptation to enhance transmission (e.g., Antia et al., 1996). Techniques such as that presented here make it possible to determine when transmission stages are present in chronic infections, and hence the extent to which antigenic variation can actually contribute to parasite fitness. Of greater practical significance to malaria epidemiology are the findings that chemotherapy led to increased rates of gametocytogenesis in acute infections of *P. chabaudi* and increased gametocyte density in infections of *P. falciparum* (Ali et al., 2005; Buckling et al., 1997, 1999a,b); if the same occurs in chronic asymptomatic infections, inappropriate drug use could enhance malarial transmission. Moreover, multiclone infections are the rule rather than the exception in malaria (Awadalla et al., 2001; Babiker and Walliker, 1997). The dynamics of individual clones in mixed infections is complex (Bruce et al., 2000; De Roode et al., 2005; Read and Taylor, 2001) and the transmission consequences of these dynamics are very poorly understood. We are currently developing allele-specific gametocyte qRT-PCR,

which will make it possible to track gametocyte densities of individual clones in multiple-clone infections. Such studies will shed more light on the dynamics of malaria parasites and assess biological and environmental factors that influence their gametocytogenesis in nature (Bousema et al., 2004; Price et al., 1999). Gametocyte-specific quantification thus makes it possible to conduct detailed studies of malaria transmission biology and should help to improve predictions of malaria parasite responses to control measures.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.exppara.2005.08.013.

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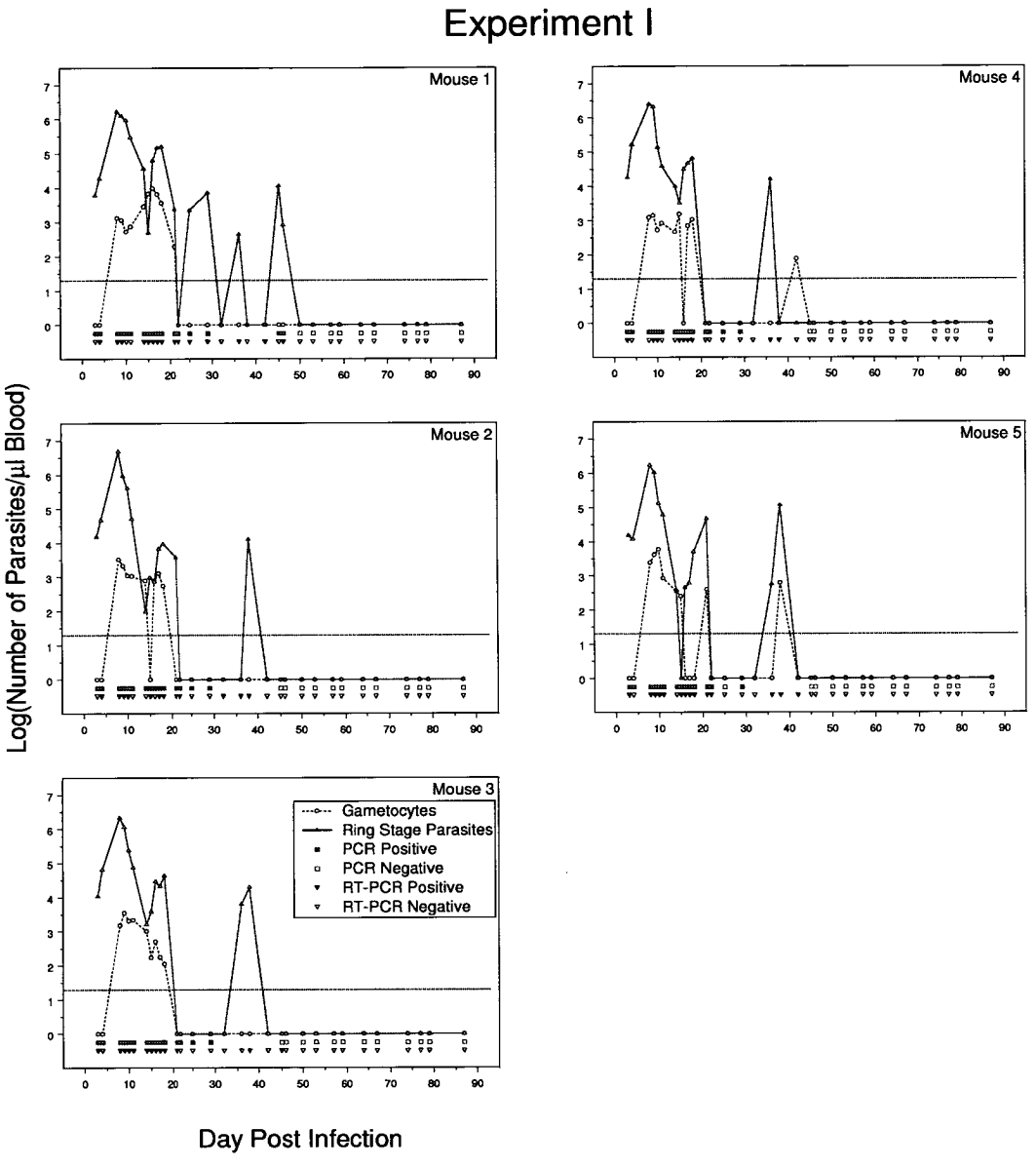

Figure 2a.2: All Mice From experiment 1. Legend as in figure 2 within the manuscript.

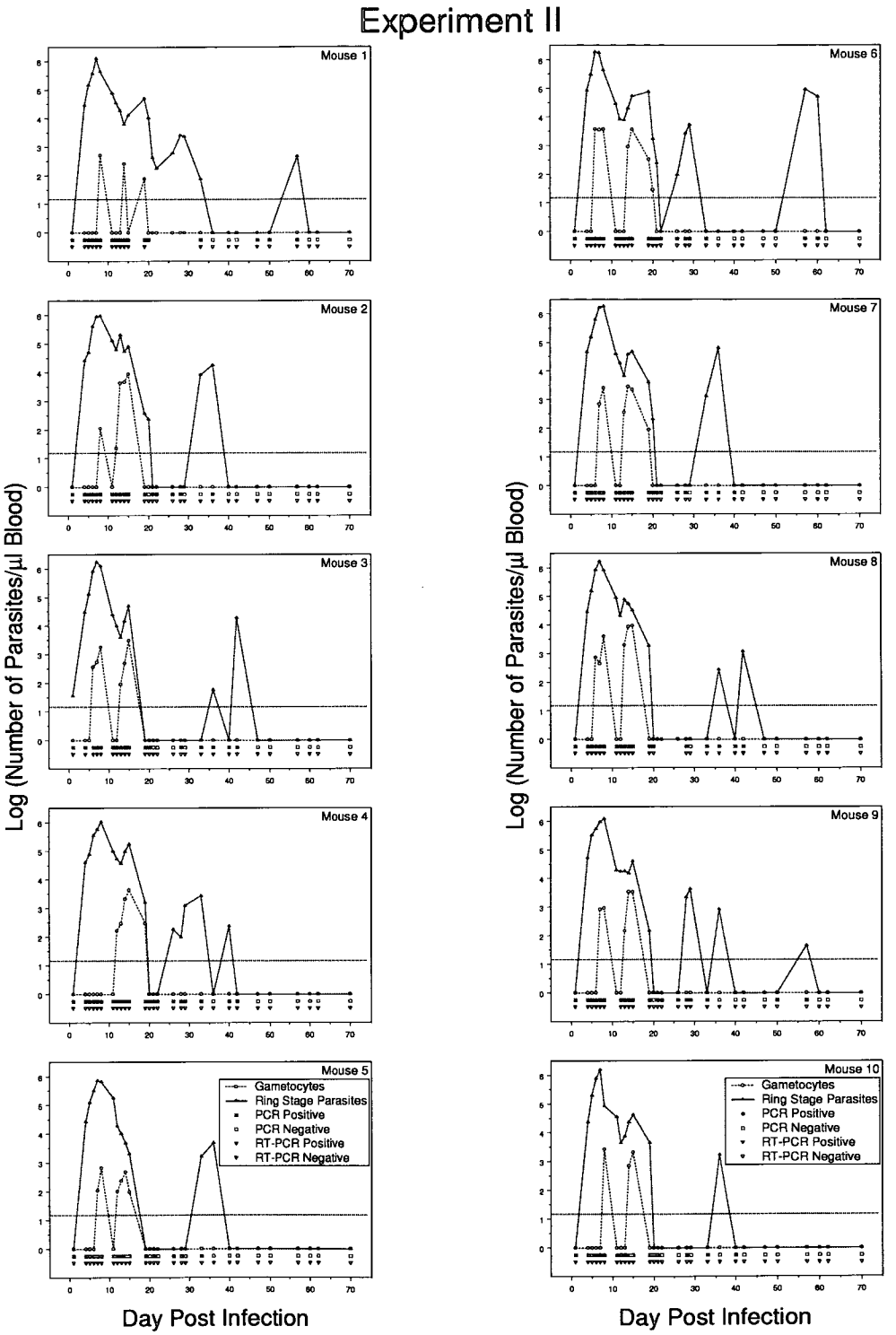

Figure 2b.2: All Mice From experiment 2. Legend as in figure 2 within the manuscript.