Within-host competition and the evolution of malaria parasites

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Submitted for the degree of Doctor of Philosophy
University of Edinburgh
17th September 2004
Declaration

I declare that this thesis is of my own composition, and the research described herein my own work, and of my own conception.

Scientific research, however, is in many cases a collaborative effort, and many of the experiments described would not have been possible without collaboration with others. Details are as follows.

**Chapter 2:** The primers and assays described for the quantification of malaria clones in mixed infections (further used in chapters 3-5 and 7) were developed by Sandra J. Cheesman, in collaboration with Richard Carter.

**Chapter 3:** Riccardo Pansini, a University of Edinburgh zoology honours student, collaborated on data collection and initial analysis.

**Chapters 4 and 7:** Richard Culleton collaborated on experimental design and data collection.

**Chapter 5:** Experiment 1 was carried out by Ali Anwar and Andrew Read; experiment 2 was carried out by Michelle Helinski, an MSc student from the University of Wageningen, the Netherlands, and myself. Michelle Helinski collaborated on data collection and initial data analysis, while I analysed and interpreted both experiments.

**Chapter 6:** Andrew S. Bell developed the PCR assays (further used in chapter 7).

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Edinburgh, 17th September 2004
"When we no longer look at an organic being as a savage looks at a ship, as at something wholly beyond his comprehension; when we regard every production of nature as one which has had a history; when we contemplate every complex structure and instinct as the summing up of many contrivances, each useful to the possessor, nearly in the same way as when we look at any great mechanical invention as the summing up of the labour, the experience, the reason, and even the blunders of numerous workmen; when we thus view each organic being, how far more interesting, I speak from experience, will the study of natural history become!"

Charles Darwin (1859)

"What is true for *E. coli* is true for elephants, but only more so."

*A coli-centric soul, Bruce R. Levin (1996)*

"It's tough to make predictions, especially about the future."

*Yogi Berra*
In a way, this thesis is an unexpected one. Five years ago, I spent eight months in Andrew Read’s lab, working on mixed malaria infections and having a great time both inside and outside of the lab. The world was a simple place then: I liked the research, I liked the people, I liked the city; and there was no doubt I would come back to do my PhD. Back in Holland, however, the world suddenly became complicated with a neurological treatment that, carried out wrongly, left my mother with an incurable, debilitating and progressive disease. Four months in the jungle of Borneo, to finish my degree, were just enough for me to decide that I needed to stay with my family, rather than leave for Scotland. As time passed, what we thought would be a matter of months turned out to be a matter of years, and it took a lot of persuasion from my parents for me to decide to come to Edinburgh after all. This thesis is much indebted to them. I am also extremely grateful to Andrew Read, for his endless patience and understanding, and Kenneth Murray and Alix Fraser of the Darwin Trust, who waited for me, and kept my PhD funding secure for when I was finally ready to start.

I have benefited from the presence, advice, company and assistance of many people. First of all, Andrew Read gave me great support from beginning to the end, was there when I needed him, gave me great confidence, and at numerous occasions suggested just that little difference – changing a few words or changing an angle - that turned half-baked first versions into real manuscripts and chapters. Sandie Cheesman taught me how to do real-time PCR and provided me with the primers and protocols so essential to my work. Richard Carter saw me spend more time in his lab than in my own during the first year, and Richard Culleton was there to help me with carrying out big experiments, even in the middle of the night, and usually to the tune of some weirdo reggae song. I have very much enjoyed the company of my students Riccardo Pansini and Michelle Helinksi; by teaching them about my research, I started to appreciate it myself much more; and the amount of work they did to make this thesis feasible, was incredible. David Walliker has been a great source of assistance, drugging mice, providing parasite clones and giving me ideas; he has also been a great source of inspiration, if only for his judging my findings by saying: ‘but what is new about this?’ Brian Chan was not only a great and entertaining friend, he was also of great importance in helping me with parasite injections, the thing I liked least about this PhD.

Many other people helped me at different stages during my research, for example with developing molecular tools, sampling mice, dissecting mosquitoes or keeping the blood-thirsty creatures alive: Andy Bell, Heather Ferguson, Katrina Grech, Gráinne Long, Ronnie
Mooney, Derek Sim, Les Steven and Andrew Wargo are all thanked for their help. Andy Bell, Lucy Crooks, Andrea Graham, Tom Little, Margaret Mackinnon, Sue Mitchell, Lars Råberg and Sarah Reece provided me with inspiration and scientific discussions, be it great ideas, seemingly simple questions that made me rethink, or remarks à la Lars: ‘Uhm, I have scrutinised your paper...’. Richard Carter, Sandie Cheesman, Margaret Mackinnon, Sean Nee, and Stuart West read manuscripts before they were sent off to journals.

Special thanks go to Meghan Gannon, who started her PhD at the same time as me, but within 2 months decided that ‘shitty worms’ were less disgusting than ‘bloody malaria’: she never really could avoid malaria though, reading about every manuscript or chapter I produced. Andrea Graham supported me throughout: scientifically, emotionally, and even footballly on that weird day that Scotland managed to beat Holland. Kathryn Watt was not only there to help out on long days of dissecting mosquitoes, but was there also to make me put my research, and the whole of science, into a healthy perspective; usually in the early morning when the only other sound was that of the bubbling algae she attended.

Many more people have made my PhD possible. Judi Allen let me use her Lightcycler, a machine that underlies about 87.5% of this thesis. David Walker, Jayne Glendinning, Helen Black, John Stanton and Billy Duff ordered machines and consumables, and transferred money where it was necessary. Loyd Mitchell repeatedly repaired the old Coulter Counter (also known as CeeCee). John Tweedie, Sheena Boath, Evelyn Rutherford and Kerry Thomson fed my mice and kept them alive. Finally, Peter Whelpdale did not switch on the heating in the winter, so that the only way to keep warm was to do yet more experiments; quite a few pages are thus indebted to him.

The last few years have been made fun and happy by many friends, including many from the lab already mentioned above. Most happiness came from Lisa, who has been absolutely fabulous in being there, supporting me, and showing patience with a Jaap that most people in the lab have never seen. I have also had great times with Alex, Arjen, Axel (x2), Carlo, Clare, Duur, Ian, Karlijn, Ludo en Geke, Peter, Steve, and many others.

My dad, Daphne and Mark have always been there, encouraging me when doing a PhD seemed like a banal thing to do. I am most indebted to my mum, who in all her pain and suffering supported and encouraged me. She did not get the chance to see my thesis finished, but I am sure she knew it would be here.
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Abstract

Humans often become co-infected with different malaria strains that differ in virulence, or drug resistance. Theoretical models have shown that competition between co-infecting strains has important medical implications. Virulent parasites (causing substantial damage to their hosts) are assumed to be competitively superior to avirulent parasites, for example, driving the evolution of virulence upwards; competition has also been implicated as a major determinant in the spread of drug resistance. Even though the precise details differ between many theoretical models, they are all based on biological assumptions that remain largely untested experimentally. This thesis therefore tests whether competition between co-infecting malaria strains occurs, and whether more virulent strains have a competitive advantage, assumptions underlying most of these evolutionary models.

I used Plasmodium chabaudi in laboratory mice as a model for human malaria, and developed a real-time quantitative PCR assay to track parasite clones in mixed-clone infections over time. Several experiments were carried out using this molecular tool, showing that intense competition occurs between Plasmodium clones occupying the same host. There was a direct relationship between virulence and in-host competitiveness, thus supporting a second fundamental theoretical assumption. Host genotype was an important determinant as well: competition was more intense in a resistant than a susceptible mouse strain. Timing of infection was even more important: the later a parasite clone was inoculated after another had established, the more it suffered from competition.

How in-host competitiveness related to between-host transmission, was assessed by feeding infected mice to Anopheles stephensi mosquitoes. This showed a linear relationship between in-host competitiveness and mosquito transmission, suggesting that competitive suppression in the mouse host translated into reduced transmission to the mosquito vector. Finally, I found that drug treatment can disrupt competitive interactions between drug-resistant and sensitive clones, resulting in competitive release of drug resistance, with the potential consequence of enhanced spread of resistance.

This thesis confirms that within-host competition importantly affects the evolution of virulence and drug resistance. The results suggest that reducing the number of mixed malaria infections could have beneficial effects by reducing the evolutionary selection for increased virulence. Also, as resistant hosts were shown to cause more intense competition than susceptible hosts, it is suggested that vaccination could enhance selection for increased virulence.
Abbreviations

AMA1: apical membrane antigen 1
IFA: immuno-fluorescent antibody assay
IFN-γ: gamma interferon
IL12: interleukin 12
i.p.: intraperitoneum (as in i.p. injection)
i.v.: intravenous (as in i.v. injection)
MSP1: merozoite surface protein 1
PCR: Polymerase Chain Reaction
PI: post-infection
RBC: red blood cell
TNF-α: Tumour necrosis factor alpha
1. General introduction

1.1. Chocolate cake and the evolution of parasites

Remember that birthday party when you were small; when you were seven years old? Remember that big chocolate cake that your mum had made for you: the one with the icing? And remember that there were ten children and that, in principle, everyone could have had a piece? So did you? No, of course not, because that awful boy of a Patrick Nasty had two pieces! Meaning that, yes, you were left out: on your own birthday!

This thesis is not about chocolate cakes, and anyone with an interest in those is referred to the book by Pamella Asquith (1983). No, this thesis deals with Patrick Nasty, and with a special set of Patricks Nasty in particular. Leaving out all those Patricks that roam the world and rule our governments, this thesis deals with those that lurk in African mosquitoes to strike young children, killing considerate and nice little Patricks in their wake. This thesis is about selfishness; about parasites that eat more cake than they deserve; about parasites that become so obsessed with having a host to themselves that they start selfishly killing it, wiping out their own fellow parasites at the same time. Put in another way: this thesis is about understanding why it is that malaria is so deadly, killing over a million children a year.

‘Nothing in biology makes sense except in the light of evolution,’ titled Theodosius Dobzhansky a beautiful essay, writing that, ‘without that light it becomes a pile of sundry facts, some of them interesting or curious but making no meaningful picture as a whole’ (Dobzhansky 1973, p129). Dobzhansky was certainly not the first one to observe this. In fact, his words strongly echo what Charles Darwin wrote more than a hundred years earlier, in 1859 (Darwin 1859): ‘When we no longer look at an organic being as a savage looks at a ship, as at something wholly beyond his comprehension; when we regard every production of nature as one which has had a history; when we contemplate every complex structure and instinct as the summing up of many contrivances, each useful to the possessor, [...] how far more interesting [...] will the study of natural history become!’

Today, however, the absolute majority of people – and scientists for that matter – still look at disease as something aberrant and something that should not happen (Nesse & Williams 1994): after all, we still go to the doctor saying something is wrong with us, rather than commenting in awe that we suffer from a highly evolved gut parasite with the most exquisite adaptations promoting its own survival in our gut environment, with the – okay – rather unpleasant effect of us having diarrhoea. It’s not that I think that we should be happy to be sick, but objectively, there is just no justification to see disease as something wrong
After all, over fifty percent of the species on earth are parasites (Poulin & Morand 2000; Zimmer 2001), which means that organisms that do not have the potential to cause disease are hard to find! In most, if not all, cases, disease is some highly adapted trait, the result of some exciting history that we ourselves have shared with viruses, bacteria, worms or protozoans.

This thesis tries to contribute to understanding how nasty little Patricks, stealing pieces of chocolate cake, have shaped that history.

1.2. Parasite tragedies

Until the 1980’s it was commonly believed that parasites should evolve to be benign to their hosts (Burnet & White 1972; see Anderson & May 1982; Levin 1996), the idea being that if parasites depend on their hosts for their own survival and transmission, they should leave their hosts alive. Virulent parasites in this view were those recently introduced to new hosts, so that they had not yet evolved benignity. This ‘conventional wisdom’ has been challenged in recent years by researchers who have pointed out that many parasites with a long history of co-evolution with their hosts cause high virulence (e.g. Anderson & May 1982; Ewald 1983; Knolle 1989; Read & Schrag 1991; Lenski & May 1994; Read 1994; Levin 1996).

Over the last few decades, therefore, several theories have been put forward to explain the evolution of virulence (Bull 1994; Read 1994; Levin 1996; Ebert 1999). Some of these theories view virulence as a host adaptation to infection (e.g. fever, Ewald 1980); as a direct parasite manipulation to increase transmission (e.g. rabies virus increasing the biting rate of rabid canids, Ewald 1980); as a coincidental result of parasites being in the wrong environment and therefore causing disease (e.g. gut bacteria causing bladder infections, Levin 1996); or as the result of short-sighted evolution of pathogens in environments where they do not belong (e.g. poliovirus rapidly replicating in central nervous system, causing inflammation, Levin 1996).

No theory has received as much attention, however, as the trade-off model, more satirically known as the ‘enlightened’ view (Levin & Svanborg Edén 1990; Levin 1996). This theory describes two main mechanisms by which high virulence can evolve and be maintained (e.g. Stearns & Ebert 2001).

Following standard usage (e.g. Anderson & May 1982; Frank 1996), virulence is defined here as parasite-inflicted reduction of host fitness, and can be expressed in terms of host mortality or morbidity.
First, virulence is an unavoidable consequence of a parasite’s efforts to optimise its fitness: parasites require extensive host exploitation to obtain transmission to the next host (Figure 1.1A), but at the same time such replication damages host tissues, and uses up host resources (Figure 1.1B), thereby increasing the chances of killing the host (e.g. Anderson & May 1982; Sasaki & Iwasa 1991; Antia et al. 1994; Frank 1996; Ebert 1999; Read et al. 2002). By this logic, parasites that replicate too slowly will be cleared by the host’s immune response before they can transmit (Antia et al. 1994), whereas parasites that replicate too fast will kill their host before they are able to transmit (Anderson & May 1982; Frank 1996). The result is selection for parasites with intermediate levels of within-host replication, so that they grow fast enough to obtain transmission to the next host, but not so fast that they kill their host before realising this; as there is a direct relationship between replication rate and virulence, evolution is thus expected to select for parasites with some kind of intermediate level of virulence (Figure 1.1C).

Much higher virulence can evolve, however, due to within-host competition\(^2\) between parasite strains\(^3\) (e.g. Levin & Pimentel 1981; Bremermann & Pickering 1983; Ewald 1983; Bonhoeffer & Nowak 1994a; 1994b; Nowak & May 1994; Van Baalen & Sabelis 1995; Frank 1996; Mosquera & Adler 1998). Thus, prudent parasites suffer great fitness losses in hosts simultaneously infected with faster-growing (virulent) parasites: this is because virulent parasites kill the host before prudent parasites have realised their fitness potential. Even though host death also reduces the fitness of virulent parasites, prudent parasites suffer disproportionately and are eliminated by natural selection: a process commonly known as ‘the tragedy of the commons’ (Hardin 1968). Thus, even though prudence maximises parasite fitness when alone, in mixed infections selfishness will result in persistence of highly virulent strains that could not survive without within-host competition (Figure 1.1D, e.g. Nowak & Sigmund 2002). This follows exactly the same logic as the chocolate cake mentioned above: one chocolate cake should be enough for 10 children, but due to the selfishness of Patrick Nasty, everyone will try to get more than they strictly need, so that nice children end up with nothing.

\(^2\) Following ecological definitions (Begon et al. 1996), competition is here defined as the reduction of parasite growth, density or transmission, or the increase of parasite death rate, of one parasite strain due to the presence of another.

\(^3\) In this thesis, the words strain, genotype and clone are used interchangeably.
Figure 1.1. Under the trade-off model, both transmission (A) and mortality rate (B) increase with increasing parasite replication rate. As a result, parasites obtain an optimal fitness at some intermediate level of virulence (C). A parasite in a mixed infection, however, has to compete for limited amounts of resources. This will lead to selection for an increased level of virulence, resulting in a lower optimal fitness (D).

1.3. Experimental evidence

Despite the large amount of theory addressed to the evolution of virulence, experimental evidence to substantiate its claims is seriously lacking, so that a real understanding of parasite virulence is still not realised. In a way this is surprising, because the theory hinges on many assumptions and predictions that are quite easily testable. For example, for evolution to occur, there should be heritable variation in parasite growth rates and virulence levels, which selection can operate on (Darwin 1859). The theory then assumes that parasites with higher growth rates should have both more transmission and cause higher virulence.
Also, the theory assumes that there is competition between co-infecting parasite strains, and that given such competition, virulent strains will have a competitive advantage in mixed infections. Here, I briefly summarise experimental evidence supporting theory, showing that there is least support for the assumption that virulent parasites are competitively superior to prudent parasites. This is the assumption tested in this thesis.

1.3.1. Variation in virulence and the trade-off model

Variation in virulence among parasite strains occurs where it is looked at, for example in *Plasmodium*, *Theileria annulata* and *Babesia bovis* (Mackinnon & Read 1999a; Chotivanich et al. 2000; Nevils et al. 2000; Taylor et al. 2002; Taylor et al. 2003; Mackinnon & Read 2004b). That such variation occurs, is even clear from the colloquial language of calling a particular flu nasty or mild: indeed, most influenza viruses hardly cause any symptoms, whereas some of them have been able to wipe out millions of people (Ewald 1994).

Although some authors have argued that a relationship between virulence and transmission does not always exist (Lipsitch & Moxon 1997; Weiss 2002), many empirical studies have now confirmed that it does (Lipsitch & Moxon 1997), for example in pathogenic bacteria in *Daphnia*, *Theileria* in cows, *Plasmodium* in humans and *Schistosoma mansoni*, *Trypanosoma brucei* and *Plasmodium chabaudi* in mice (Diffley et al. 1987; Ebert 1994; Turner et al. 1995; Mackinnon & Read 1999a; Davies et al. 2001; Taylor et al. 2002; Mackinnon & Read 2004b). Indirect evidence for the trade-off model comes from experiments in which parasites are serially passaged from one host to the next, circumventing natural transmission routes. In general these experiments result in increased virulence, and it has been suggested that this is a result of artificially diminishing the negative effects of high virulence on transmission (Levin & Svanborg Edén 1990; Ebert 1998; 1999). The most widely cited example supporting the trade-off model is the case of myxoma virus that was introduced in Australia in 1950 to control excessive rabbit populations. Initially extremely virulent, the virus rapidly evolved to be less virulent, to an intermediate level optimising fitness (see Anderson & May 1982).

1.3.2. Mixed infections and competition between co-infecting strains

With the use of molecular tools, such as polymerase chain reaction (PCR), it is increasingly becoming clear that most, if not all, parasites often share their hosts with other strains of the same species (Read & Taylor 2000; 2001). Such mixed infections are probably most
thoroughly investigated for human malaria (see below), but they also occur with *Crithidia bombi* in bumblebees, schistosomes in freshwater snails and humans, *Theileria annulata* in cattle, *Cryptosporidium parvum* in humans and cattle, and *Toxoplasma gondii*, *Streptococcus pneumoniae* and herpes simplex type 1 in humans (Ben Miled *et al.* 1994; Obaro *et al.* 1996; Davies *et al.* 1999; Brouwer *et al.* 2001; Aspinall *et al.* 2003; Tanriverdi *et al.* 2003; Bowden *et al.* 2004; Schmid-Hempel & Funk 2004; Villena *et al.* 2004; Widmer 2004).

Mixed infections may result in competition between such strains, by which competition is defined as an interaction between the strains, in which the population sizes of one strain are depressed by the presence of the other strain (Begon *et al.* 1996). Competition is well described between different parasite species infecting the same host (Richie 1988; Cox 2001), but it is more likely to occur between different strains of the same species, which almost certainly occupy very similar niches (Read & Taylor 2000; Cox 2001; Read & Taylor 2001).


### 1.3.3. Virulence and within-host competition

There is thus no doubt that competition occurs between co-infecting parasite strains. However, whether in such competition virulent parasites are competitively superior to avirulent parasites, has to my knowledge never been assessed directly, and the only evidence for this is highly indirect. Thus, serial passage of parasites through new host environments generally increases virulence (Ebert 1998; Mackinnon & Read 1999b; Mackinnon & Read 2004a), and attenuated vaccine revertants can arise (Bull 1994; Kew *et al.* 2002). Both can be explained by new mutants arising and increasing in frequency, because they have a competitive advantage. However, other explanations are possible: for example, there could be an increase of genetic diversity of the parasite population, which in itself could cause
more virulence than an infection with a single parasite genotype (e.g. Frank 1996; Taylor et al. 1998; Read et al. 2002).

Other indirect evidence comes from a population-structure analysis of fig wasp species (Herre 1993; Herre 1995; Frank 1996). These wasps have species-specific parasitic nematodes, and where horizontal transmission of the parasites occurred, parasites were more virulent (Herre 1993); in retrospect it was concluded that this was due to the increased frequency of mixed infections due to horizontal transmission and selection for increased virulence (Herre 1995; Frank 1996). A study on *Cucumber mosaic virus* in eastern Spain (Escriu et al. 2000; 2003) also inferred within-host competition to explain the initial increase of virulence during epidemic spread. Finally, Ebert and Mangin (1997) used within-host competition as a post-hoc explanation for the increased virulence of microsporidia in artificial *Daphnia magna* populations.

Where competitiveness and virulence have been directly studied, it has been generally shown that avirulent parasites competitively suppress virulent parasites, contradicting existing theory (Orcutt & Schaedler 1973; Hargreaves et al. 1975; Seed 1978; Duval-Iflah et al. 1983; Allaker et al. 1988; Lenhoff et al. 1998; Read & Taylor 2000; 2001; Franco et al. 2003). Importantly, however, most of these studies involved establishing an avirulent strain in the host before challenging the host with a virulent strain, so that the avirulent strain grew up to considerable numbers before the virulent strain was introduced (Orcutt & Schaedler 1973; Duval-Iflah et al. 1983; Allaker et al. 1988; Barrow & Page 2000). Thus, the fact that the conclusions of these experiments contradict the assumptions of much virulence theory could be a result of researchers searching for protective strains of parasites. To take them as evidence that a virulence-competitiveness relationship does not exist, is almost as meaningless as basing that conclusion on the fact that live attenuated vaccines can be used to protect against disease (Handman 2001; Wareing & Tannock 2001; Hoffman et al. 2002b; Pugachev et al. 2003).

### 1.3.4. A case of common sense?

One possible reason for the lack of experiments testing the relationship between competitiveness and virulence is that many scientists (at least many I have met at conferences and within our own building) find this relationship such common sense that experiments are not deemed necessary. As pointed out above, many experiments have shown that avirulent strains can suppress virulent strains. This could be the result of the exact
experimental designs underlying these results, but it could also be that a relationship between virulence and competitiveness does not exist. Certainly such a relationship need not exist. It depends on the exact mode of competition through which parasites compete.

If parasites could compete for limited amounts of resources, nutrients or space (Smith & Holt 1996), a positive relationship between virulence and competitiveness is expected. This form of competition is the one with closest resemblance to competition for chocolate cake, and most likely paves the way for the tragedy of the commons and the success of Patricks Nasty: this is because parasites will try to use up more resources than their competitors, so that faster growing and more virulent parasites are favoured (Brown et al. 2002).

However, other modes of competition could also occur. First, competition could arise when parasites rely on the cooperative production of extracellular growth molecules, such as those involved in iron uptake. Here, ‘cheating’ parasites can arise (Velicer 2003), which rely solely on others for the production of such molecules, so that they grow at faster rates in mixed infections, but cause lower virulence when alone (Chao et al. 2000; Brown et al. 2002; West & Buckling 2003; Griffin et al. 2004). Similarly, where coinfecting pathogens compete by producing harmful molecules that affect their competitors (Riley & Gordon 1999), competitive ability may not be linked to virulence because competitively superior strains may be directing resources at the production of allelopathic substances rather than maximising host exploitation and virulence (Read & Taylor 2000; Gardner et al. 2004; Kirkup & Riley 2004; Massey et al. 2004).

Second, parasite strains may interact through immunity: in mammals, immune effector molecules include the lymphocyte product IFN-γ, the macrophage product TNF, and nitric oxide and reactive oxygen intermediates. All these molecules act non-specifically, so that when induced by one strain, they will affect not only this strain, but also other strains (Cox 2001). That such competition occurs, is probably best illustrated with vaccines that consist of live-attenuated parasites (Mead & Barrow 1990; Handman 2001; Wareing & Tannock 2001; Hoffman et al. 2002b; Pugachev et al. 2003). An important possibility is that the immune system, when faced with a mixed infection, will focus its attention on a dominant virulent parasite, so that the avirulent parasite in fact has a competitive advantage in such infections (Taylor et al. 1997a; Read & Taylor 2001; Almogy et al. 2002; Franco et al. 2003; Hughes & Boomsma 2004).

One mathematical model has also shown that when parasites exert sub-lethal effects on their hosts, thereby reducing host growth, and when such parasites are at the same time dependent
on host size, competition can favour the evolution of reduced virulence (Schjørring & Koella 2003).

Thus, there is no a priori reason to assume that virulent parasites are competitively superior to prudent parasites; and experiments to test this assumption are badly needed. This thesis focuses on within-host competition between malaria parasites, to understand which factors determine competitive outcome, and to find out whether virulent parasites are indeed competitively superior, as assumed by theory. Only if such a relationship exists, can we confirm that within-host competition is a major mechanism by which parasites can evolve and maintain high virulence, thereby expanding our understanding of parasite evolution. This could not only make us understand why malaria kills over a million people every year, but perhaps even predict the consequences of human interventions on the subsequent evolution of malaria virulence.

1.3.5. **Other implications of within-host competition**

As discussed, within-host competition is a critical determinant of virulence evolution. However, within-host competition is also relevant to the evolution of other traits, such as drug resistance and vaccine escape. Thus, the evolutionary success of a drug-resistant mutant, whether evolved de novo, or acquired through infection, very much depends on its competitiveness relative to the other strains within the same infection (e.g. Hastings & D'Alessandro 2000); assuming that competitive suppression of resistant mutants occurs, administration of drugs could interfere with such competition and artificially enhance the fitness of drug-resistant mutants and thus enhance the spread of resistance (Hastings & D'Alessandro 2000). Also, where vaccines are used to control disease, it is well known that vaccine escape mutants can arise and spread (e.g. Kew et al. 2002); again, how well such mutants survive, evolve and spread through the population, depends importantly on how well they compete with wild-type mutants in non-vaccinated hosts and attenuated forms of the pathogen in vaccinated hosts (e.g. Bull 1994).

Thus, within-host competition has many important medical implications, but the biological factors that determine competition within hosts is at present so poorly understood that predictions are practically impossible (Read & Taylor 2000; 2001).
1.4. Malaria

1.4.1. Introduction

Malaria features in the top ten diseases, with huge medical (Breman 2001) and economic (Sachs & Malaney 2002) burdens in this world. Malaria causes coma, respiratory distress and severe anaemia (Marsh & Snow 1997), killing perhaps one person every 30 seconds, mounting to over at least a million deaths a year (Breman 2001). Most victims are children in sub-Saharan Africa, and most cases are caused by the most virulent of the four species of human malaria parasites, *Plasmodium falciparum*. Another 500 million people suffer from malaria-related anaemia and immune suppression that leave them vulnerable to other diseases, exacerbated by their poverty.

As malaria parasites have become repeatedly resistant to anti-malarial drugs (Wongsrichanalai et al. 2002), and mosquitoes have evolved resistance against widely used insecticides (Spielman & D’Antonio 2001), more people suffer from malaria today than ever before in human history. The parasite has a complex life cycle, and immunity against malaria is so poorly understood that a vaccine is also still not available (Richie & Saul 2002; De Roode 2003 [Thesis Appendix]; Hoffman & Richie 2003). To find new molecular ways of fighting this disease (Hoffman et al. 2002a; Long & Hoffman 2002), the full genomes of both malaria parasite (Gardner et al. 2002) and vector have recently been sequenced (Holt et al. 2002). Intriguingly, although it is evolutionary processes that render malaria parasites so virulent and hard to kill, there are few people working on the evolution of malaria, and most of these are concerned with reconstructing the past (e.g. Hume et al. 2003; Su et al. 2003).

1.4.2. A complex life cycle

The malaria life cycle (Figure 1.2) starts with the bite of a mosquito that injects haploid sporozoites into the human blood stream. These migrate to the liver, where they invade liver cells, replicate, and burst out of the cells: one sporozoite can give rise to no less than 30 thousand merozoites that travel to the blood where they start causing disease. Merozoites invade red blood cells, grow into trophozoites, which mature into schizonts: these may contain between 16 and 32 new merozoites for *Plasmodium falciparum* and around 8 for the rodent malaria *Plasmodium chabaudi*. Merozoites burst out of their red blood cell, ready to invade new red blood cells. This process of asexual replication repeats itself every 48 (*Plasmodium falciparum*) or 24 (*Plasmodium chabaudi*) hours, and, in humans, results in the fevers so characteristic of malaria. Some parasites make the red blood cells stick to blood
vessels (thereby in some cases killing the patient), while others turn into gametocytes, of which male and female forms exist. Gametocytes, when taken up by a new blood-feeding mosquito, turn into gametes that fertilise each other to form an ookinete: this parasite crosses the mosquito’s mid-gut wall, encysting on it to form an oocyst. Meiosis is followed by asexual replication, resulting in hundreds to thousands of new sporozoites. These are released into the mosquito haemocoel, after which they migrate to the salivary glands; there they wait to be injected into the next patient.

Figure 1.2. The malaria life cycle comprises both a mammalian host, in this case a human (a), and a mosquito host (b). Reproduced from Wirth (2002).

1.4.3. Virulence in malaria

Killing at least a million people a year, Plasmodium can be called a virulent parasite, and many factors contribute to disease, including host genetics, parasite genetics, and socio-economics. Several studies have associated parasites with certain alleles of several genes with disease (e.g. Engelbrecht et al. 1995; Al-Yaman et al. 1997; Ofosu-Okyere et al. 2001; Magesa et al. 2002), although other studies have not (e.g. Contamin et al. 1996; Zwetyenga et al. 1998). Moreover, different studies have generally found different allele families to be
associated with disease (Engelbrecht et al. 1995; Al-Yaman et al. 1997; Ofosu-Okyere et al. 2001; Magesa et al. 2002), which could point to geographic variations in host/parasite interactions (Zwetyenga et al. 1998). That host genetics are an important determinant in disease becomes apparent from a study in Mali, where the parasite density that triggered fever strongly varied from one subject to another (Delley et al. 2000), and from a study in Tanzania, where the number of co-infecting malaria clones was host-dependent (Färnert et al. 1999). In addition, different people treated for neurosyphilis had different maximum malaria parasite densities (Molineaux et al. 2002). A lot of this variation probably arises from different people having differences in their immune control of malaria, but also from mutations that confer resistance to malaria infection (e.g. Cockburn et al. 2004; Mockenhaupt et al. 2004).

Many factors are involved in virulence of malaria infections, such as rosetting (adherence of infected to unindected red blood cells, Rowe et al. 1995), parasite replication rate (Chotivanich et al. 2000) and immunopathology (reviewed in Marsh & Snow 1997; Artavanis-Tsakonas et al. 2003; Mackinnon & Read 2004b), but it has recently been argued that all these factors are very likely related to within-host parasite densities (Mackinnon & Read 2004b). Virulence in malaria has been persuasively described in terms of the trade-off model (Mackinnon & Read 2004b), in that both virulence and transmission are caused by within-host replication of malaria parasites. As such, virulence in malaria can be explained as an evolutionary adaptation of parasites to optimise their fitness.

Whether within-host competition between malaria strains also contributes to the evolution of virulence in malaria, is far less clear from existing data.

1.4.4. **Mixed infections in malaria**

Mixed infections in malaria are extremely common, with frequencies of mixed infections higher than 80% in some populations, and the number of co-infecting strains exceeding 10 (Table 1.1). Although there is a strong positive relationship between transmission intensity and clone multiplicity (the number of genetically distinct clones per host) (Babiker & Walliker 1997; Arnot 1998), people in areas with low transmission, such as The Gambia, Sudan and Cameroon, are also commonly infected with several genotypes. Observed within-host diversity is probably an underestimation, because of the limited discriminatory power of genetic tools (Contamin et al. 1995; Arnot 1998; Babiker et al. 1999), and the fact that most studies are based on cross-sectional – not longitudinal – surveys, which makes it easy to miss strains (Snounou 2004).
Table 1.1. Frequency and multiplicity of mixed-genotype infections of the human species *P. falciparum* and *P. vivax* (for different geographic regions and age classes of investigated patients), and the rodent malaria *P. c. chabaudi*. Frequency is the proportion of malaria infections that consist of several distinct genotypes; multiplicity is the mean number of genotypes per malaria infection; also shown is the range of the number of genotypes observed per infection.

<table>
<thead>
<tr>
<th>Country</th>
<th>Village/Area</th>
<th>Age (yr)</th>
<th>EIR</th>
<th>Frequency</th>
<th>Multiplicity</th>
<th>Range</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. falciparum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>The Gambia</td>
<td>all</td>
<td></td>
<td></td>
<td>0.52</td>
<td>2.0</td>
<td>1-4</td>
<td>(Conway et al. 1991)</td>
</tr>
<tr>
<td>The Gambia</td>
<td>Farafenni</td>
<td>1-18</td>
<td></td>
<td>0.96</td>
<td>3.6</td>
<td>1-10</td>
<td>(Sutherland et al. 2002)</td>
</tr>
<tr>
<td>Senegal</td>
<td>Dielmo</td>
<td>0-15</td>
<td>100-220</td>
<td>0.63</td>
<td>1.9</td>
<td>1-4</td>
<td>(Scherf et al. 1991)</td>
</tr>
<tr>
<td>Senegal</td>
<td>Dielmo</td>
<td>&gt;15</td>
<td></td>
<td>0.15</td>
<td>2.0</td>
<td>1-4</td>
<td>(Ntoumi et al. 1995; refs in Babiker et al. 1999)</td>
</tr>
<tr>
<td>Senegal</td>
<td>Dielmo</td>
<td>1-84</td>
<td>120</td>
<td>0.90-0.92</td>
<td>3.4-3.7</td>
<td>1-12</td>
<td>(Konaté et al. 1999)</td>
</tr>
<tr>
<td>Senegal</td>
<td>Ndiop</td>
<td>1-84</td>
<td>17</td>
<td>0.53</td>
<td>1.6</td>
<td>1-4</td>
<td>(Zwetyenga et al. 1998; Konaté et al. 1999)</td>
</tr>
<tr>
<td>Senegal</td>
<td>Dakar</td>
<td>all</td>
<td></td>
<td>0.80</td>
<td>2.0</td>
<td></td>
<td>(Zwetyenga et al. 1998)</td>
</tr>
<tr>
<td>Ghana</td>
<td>Agogo</td>
<td></td>
<td></td>
<td>0.59</td>
<td>2-2-2.4</td>
<td></td>
<td>(Robert et al. 1996)</td>
</tr>
<tr>
<td>Ghana</td>
<td>Dodowa</td>
<td>1-10</td>
<td>30</td>
<td>0.68</td>
<td>2.9</td>
<td>1-12</td>
<td>(Beck et al. 2001)</td>
</tr>
<tr>
<td>Sudan</td>
<td>Asar</td>
<td>&lt;1</td>
<td></td>
<td>0.06-0.52</td>
<td>1.1-1.5</td>
<td>1-3</td>
<td>(Babiker et al. 1991a; 1998)</td>
</tr>
<tr>
<td>Sudan</td>
<td>Daraweesh</td>
<td>9-36</td>
<td>1</td>
<td>0.36</td>
<td>1-1-2.1</td>
<td>1-4</td>
<td>(Roper et al. 1998)</td>
</tr>
<tr>
<td>Cameroon</td>
<td>Yaoundé</td>
<td>&gt;5</td>
<td>3-13</td>
<td>0.57</td>
<td>2.2-2.9</td>
<td>1-7</td>
<td>(Basco &amp; Ringwald 2001)</td>
</tr>
<tr>
<td>Ghana</td>
<td></td>
<td></td>
<td></td>
<td>0.70</td>
<td>2.1</td>
<td>1-4</td>
<td>(refs in Babiker et al. 1999)</td>
</tr>
<tr>
<td>Tanzania</td>
<td>Magoda</td>
<td>1-5</td>
<td>250</td>
<td>1.00</td>
<td>2.7</td>
<td></td>
<td>(Magesa et al. 2002)</td>
</tr>
<tr>
<td>Tanzania</td>
<td>Idate, Kilombero</td>
<td>&lt;1</td>
<td></td>
<td>0.77</td>
<td>2.3-3.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tanzania</td>
<td>Michenga</td>
<td>All</td>
<td>&gt;500</td>
<td>0.85</td>
<td>3.3</td>
<td>1-6</td>
<td>(Babiker et al. 1991)</td>
</tr>
<tr>
<td>Tanzania</td>
<td>Idate, Kilombero</td>
<td>1-5</td>
<td></td>
<td>0.85</td>
<td>3.4</td>
<td></td>
<td>(Beck et al. 1997)</td>
</tr>
<tr>
<td>Guinea Bissau</td>
<td>Antula</td>
<td>1-78</td>
<td></td>
<td>0.64-0.68</td>
<td>2.0-2.5</td>
<td>1-8</td>
<td>(Arez et al. 2003)</td>
</tr>
<tr>
<td>Papua New Guinea</td>
<td>Madang</td>
<td></td>
<td></td>
<td>0.72</td>
<td>1.7-1.9</td>
<td>1-3</td>
<td>(Paul et al. 1995)</td>
</tr>
<tr>
<td>Papua New Guinea</td>
<td>&lt;18</td>
<td></td>
<td></td>
<td>0.51</td>
<td></td>
<td></td>
<td>(Ali-Yaman et al. 1997)</td>
</tr>
<tr>
<td>Colombia</td>
<td>Several</td>
<td>8-51</td>
<td></td>
<td>0.26-0.29</td>
<td>1-3</td>
<td></td>
<td>(Snowin et al. 1991)</td>
</tr>
<tr>
<td><em>P. vivax</em></td>
<td>Mae Sod district</td>
<td></td>
<td></td>
<td>0.36</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>P. c. chabaudi</em></td>
<td>Central African Republic</td>
<td></td>
<td></td>
<td>0.23</td>
<td>1.2</td>
<td>1-2</td>
<td>(Beale et al. 1978)</td>
</tr>
</tbody>
</table>

EIR: entomological infection rate: number of infective mosquito bites/person/year. a Symptomatic patients. b Asymptomatic carriers. c Ranges given when, in the cited study, different genetic markers or villages were used to assess multiplicity. d Children vaccinated with SPf66 vaccine. e Gametocyte positive children after drug treatment. f Pregnant women.
Competition between different human malaria species has been inferred (Boyd & Kitchen 1937; Richie 1988; Bruce et al. 2000; Bruce & Day 2002; Mayxay et al. 2004; Snounou & White 2004). As such competition is most probably the result of limited resources and cross-species immune responses, it is even more likely that competition operates between different strains of the same species, which rely much more on the same resources (e.g. the type of red blood cells they infect), and are probably much more antigenically similar, so that immune responses should be more strain-transcending. Despite this, evidence of within-host competition between strains of the same species can only be indirectly inferred.

First, there is a general straight relationship between transmission intensity and clone multiplicity, but the slope of this relationship is smaller than 1 (Arnot 1998): this implies that not every additional mosquito bite results in a proportionate increase in clone multiplicity, which could be due to competition between strains. Second, although in Tanzanian infants of 1-2 years there was a correlation between parasite density and clone multiplicity, there was no such relationship for older children in the same study (Smith et al. 1999a), or for children in other studies (e.g. Conway et al. 1991; Färnert et al. 1999; Basco & Ringwald 2001; Cattamanchi et al. 2003): thus, in such mixed infections, parasites have to ‘share’ the available number of parasites between them, resulting in at least one of them having lower densities than it would have in a single infection. Third, *P. falciparum* infections in humans were genetically more diverse than the corresponding infections in mosquitoes (Arez et al. 2003), which could be a result of competition for transmission.

Indirect evidence for competition also comes from a large number of studies that have tried to relate malaria morbidity to clone multiplicity. In high-transmission areas, where people are infected many times with different malaria strains, multiplicity increases early in childhood, reaches peak levels at 3-7 years and decreases thereafter (Ntoumi et al. 1995; Mercereau-Puijalon 1996; Konaté et al. 1999; Smith et al. 1999a); at young age clone multiplicity in these areas is associated with clinical disease, whereas at older age it is not (Engelbrecht et al. 1995; Contamin et al. 1996; Robert et al. 1996; Al-Yaman et al. 1997; Beck et al. 1997; Färnert et al. 1999; Magesa et al. 2002). In low-transmission areas, on the other hand, there are no age-effects (Conway et al. 1991; Zwetyenga et al. 1998; Konaté et al. 1999; Basco & Ringwald 2001), and clone multiplicity is associated with disease (Mercereau-Puijalon 1996; Roper et al. 1998; Zwetyenga et al. 1998; Ofosu-Okyere et al. 2001).
These contrasting relationships are most likely caused by different immune mechanisms (Smith et al. 1999b). Thus, with age, subsequent infections with different strains result in a gradual acquisition of strain-specific immune responses: having a broad range of such responses then results in cross-reactive immunity that may prevent superinfection with novel strains and subsequent disease (Smith et al. 1999b; Tanner et al. 1999). However, individuals without such strong immunity - such as infants, primigravid women (Beck et al. 2001) and travellers - become readily infected with new strains against which they have no protective immunity; these strains then grow up and cause disease (Lines & Armstrong 1992; Contamin et al. 1996; Mercereau-Puijalon 1996; Arnot 1998; Babiker 1998; Roper et al. 1998; Felger et al. 1999; Missinou et al. 2001; Ofosu-Okyere et al. 2001; Kun et al. 2002; Cattamanchi et al. 2003). Taken together, these studies thus strongly suggest that there is competition between malaria strains in the same host. In immune individuals, in which infection protects against further superinfection and high parasite densities of novel strains, that competition is strong; in non-immune individuals, on the other hand, novel strains can easily infect and grow up to high enough numbers to cause disease, showing they suffer less from competition. However, it is possible that the patterns observed in non-immune individuals are in fact the result of PCR artefacts: thus the correlation between parasite densities and the number of clones could arise if the detected clones have high parasite densities, obscuring PCR detection of minority clones (Contamin et al. 1995; Arnot 1998; Roper et al. 1998; Smith et al. 1999b).

1.4.6. *Plasmodium chabaudi* as a model system

The only way to demonstrate competition unequivocally is by using experimental malaria infections. For ethical and logistic reasons, however, experimental malaria infections of humans are not possible, so that I use the rodent malaria *Plasmodium chabaudi* in laboratory mice as a model instead. Even though the natural host of *Plasmodium chabaudi* is the thicket rat (*Thamnomys rutilans*), *P. chabaudi* in mice has been widely used as a model system for the human malaria parasite *Plasmodium falciparum*, because it shares many infection characteristics with *P. falciparum* (Cox 1988; Carlton et al. 2001; Mackinnon & Read 2004b).

Both species usually infect normocytes (older red blood cells), rather than reticulocytes, undergo sequestration to endothelial cells in the microvasculature (Cox et al. 1987; Gilks et al. 1990), and undergo rosetting: the adherence of infected to uninfected cells (Rowe et al. 1995; Rowe et al. 1997; Mackinnon et al. 2002a). In resistant mouse strains, *P. chabaudi*
shows recrudescent parasitaemic peaks after recovery from the acute phase (Cox 1988; Taylor-Robinson 1995; Phillips et al. 1997), as does \textit{P. falciparum} in humans. Both species also undergo clonal antigenic variation (Biggs et al. 1991; Roberts et al. 1992; Phillips et al. 1997), and provoke strain-specific immunity (Jarra & Brown 1985; Mackinnon & Read 2004b). Mixed-strain infections of \textit{P. chabaudi} also occur in natural populations (Table 1.1, Beale et al. 1978). The main difference between the two species is that \textit{P. chabaudi} in mice reaches levels of parasitaemia of 30-60%, whereas \textit{P. falciparum} only exceptionally reaches parasitaemia levels higher than 10% (Taylor-Robinson 1995).

\textbf{Figure 1.3.} Parasite density (A) and red blood cell density (B) of one C57Bl/6J mouse infected with an avirulent and one mouse infected with a virulent clone of \textit{P. chabaudi}. Parasite densities are characterised by an exponential increase during the first seven days, after which they collapse. New parasite waves are detected about 12 and 15 days into the infection, after which one or more parasite peaks can occur, consisting of antigenic variants of the parasite (Phillips et al. 1997). The period until the second parasite wave is referred to as the acute phase of the infection; the part of the infection starting with that second wave is the chronic phase of infection. When parasite numbers increase exponentially, red blood cell densities in the mouse decrease rapidly: this is a consequence of both parasite lysis of red blood cells and immune mechanisms clearing infected and uninfected red blood cells. The collapse of parasite densities after the initial peak (also called crisis) is a combination of red blood cell densities becoming low and immunity aimed directly at parasites. Virulent parasites reach higher peak densities and cause more anaemia (lower red blood cell densities) in the mice they infect than do avirulent parasites. Data obtained from Chapter 4.
*P. chabaudi* makes a good model for studying the evolution of virulence. Many different parasite isolates were derived from the wild (Beale *et al.* 1978), and clonal isolates of these differ in their intrinsic virulence (Mackinnon & Read 1999a; Ferguson *et al.* 2003b). Parasite densities can be easily recorded using microscopy (Figure 1.3A), and virulence can be easily measured, for example by counting red blood cell densities of infected mice (Figure 1.3B) or recording mouse live weights (e.g. Mackinnon & Read 1999a; Ferguson *et al.* 2003b). Furthermore, *P. chabaudi* can be readily transmitted to mosquitoes in the lab (e.g. Mackinnon & Read 1999a; Ferguson *et al.* 2003b), so that the whole life cycle can be studied. Finally, *P. chabaudi* infects a range of different mouse strains, so that the effects of host genotype effects on virulence ecology and evolution can also be assessed (Stevenson *et al.* 1982; Mackinnon *et al.* 2002b).

**1.4.7. Competition in Plasmodium chabaudi**

Evidence for competition in human malaria may be indirect, but in *P. chabaudi* several experiments have now unequivocally demonstrated competition between different strains (Snounou *et al.* 1992; Taylor *et al.* 1997a; Read & Taylor 2000; De Roode *et al.* 2003 [Thesis Appendix]); this competition is most likely due to both resource competition and strain-transcending immunity.

Asexual *P. chabaudi* parasites need blood glucose (e.g. Li *et al.* 2001) and red blood cells, and the huge decrease of red blood cell numbers during an infection (Figure 1.3B) results in an enormous decrease in available resources. Anaemia in malaria is caused by a combination of parasite rupture (Cox 1988; Menendez *et al.* 2000; McQueen & McKenzie 2004) and immune mechanisms, such as phagocytosis of parasitised and unparasitised red blood cells (Cox 1988; Jakeman *et al.* 1999; Menendez *et al.* 2000), and decreased red blood cell production (Cox 1988; Menendez *et al.* 2000). That parasite growth is limited by available red blood cells has been shown experimentally: blood transfusion during and after peak parasitaemia prolonged the patency of infection for up to five days after peak parasitaemia (Yap & Stevenson 1994). One theoretical study has also shown that the initial growth of parasites is very much dependent on the availability of red blood cells, that there is a critical threshold red blood cell density for parasite establishment, and that parasite numbers can collapse due to a decrease in red blood cell densities regardless of immunity (Hetzel & Anderson 1996).

Competition for resources such as red blood cells is certainly not the only mode of competition (Hellriegel 1992; Gravenor *et al.* 1995; Haydon *et al.* 2003). Apparent
competition, through a shared immune response, is also likely to occur in *P. chabaudi* infections in mice. Clearance of parasites during the acute phase is predominantly regulated by non-specific Th1 cellular responses. These involve activation of CD4+ cells upon parasite antigen presentation by mainly dendritic cells; CD4+ Th1 cells activate macrophages, with the subsequent induction of interleukins such as Tumour Necrosis Factor alpha (TNF-\(\alpha\)) and Interferon gamma (IFN-\(\gamma\)), stimulation of Natural Killer Cells, and the production of nitric oxide and reactive oxygen species (Taylor-Robinson 1995; Phillips *et al.* 1997; Li *et al.* 2001). As these molecules act non-specifically, they will affect not just one but all the clones present in the blood at that time. This has been confirmed by heterologous-challenge experiments that showed cross-immunity between different *P. chabaudi* strains and even different rodent malaria species (Jarra & Brown 1989; Snounou *et al.* 1989). Parasite clone-specific immunity is also involved in clearance and control of initial *P. chabaudi* parasitaemia, however (Jarra & Brown 1989; Snounou *et al.* 1989), with antibodies targeting parasitised red blood cells for phagocytosis through clone-specific processes (Mota *et al.* 1998). Acquired immunity is also strain-specific (Jarra & Brown 1985), and during the chronic phase of infection Th2 antibody-mediated, and directed at specific strains and antigenic variants (Taylor-Robinson 1995; Phillips *et al.* 1997; Li *et al.* 2001).

The implication is that clones that differ substantially in their immunogenic properties should affect each other less than those that are very similar; also, immune-mediated competition during the chronic phase should be weaker than during the acute phase (De Roode *et al.* 2003 [Thesis Appendix]), not just because of very specific immune responses, but also because of red blood cell densities recovering during the chronic phase (Hetzel & Anderson 1996).
1.5. Thesis aims

Even though competition in *P. chabaudi* has been shown to occur, nothing is known about how virulence relates to within-host competitiveness. This thesis deals with experimentally elucidating whether virulence is directly related to competitive ability and which biological factors affect the outcome of competition between virulent and avirulent parasites.

Besides this, I studied how antimalarial drugs interfere with within-host competition, and how within-host competition affects between-host transmission: this latter question is not only important for virulence studies, but also for studies on drug resistance, vaccine-escape mutants, and any other medically important traits affected by within-host competition. As the quantitative assessment of individual clones in an infection is a crucial prerequisite for all these studies, I first developed a molecular quantitative technique to do this (Chapter 2).

Subsequent chapters then deal with the following related questions.

- Is there a relationship between virulence and within-host competitive ability, as predicted by theory? (Chapter 3)
- What role does host genotype play in the outcome of within-host competition between virulent and avirulent parasites? (Chapter 4)
- What role does the timing of infection play in the outcome of competition; are resident parasite genotypes competitively superior to super-infecting genotypes? As such, can avirulent parasites become competitively superior to virulent parasites? (Chapter 5)
- How does within-host competitive ability relate to between-host fitness? Does competitive superiority within the mammalian host translate into competitive superiority within the vector population? (Chapter 6)
- Can the use of anti-malarial drugs change patterns of competition between virulent and avirulent parasites, and how could this affect the subsequent spread of drug resistance? (Chapter 7)

In the final chapter (Chapter 8), I consider the implications of the findings in chapters 2-7 for the evolution of virulence ('what role does Patrick Nasty play?'), the spread of drug-resistance, and for malaria. I also ask whether these findings could be used to predict the evolutionary consequences of human interventions aimed at reducing the malaria burden in the field.
2. Real-time PCR for analysis of genetically mixed infections of malaria parasites: technique development and validations


2.1. Abstract

Here I report the development and validation of real-time quantitative PCR to analyse mixed-clone *Plasmodium chabaudi* infections. Using allele-specific primers I quantified the relative proportions of two genetically distinct *P. chabaudi* clones over a 100-fold range, from small blood samples that are easily obtained from experimental animals. I demonstrate that the time of day when samples are taken affects the accuracy of the PCR results, showing that best results are obtained between 9:00 and 13:00 hours. As the described PCR assays are based on between-clone sequence differences in the highly polymorphic *mspl* gene, the technique could in principle be expanded to combinations of any two clones that have sequence differences in that or any other gene.

2.2. Introduction

2.2.1. Why we need PCR

Eight genetically distinct *Plasmodium chabaudi* clones have been well characterised for their life-history characteristics such as asexual growth, virulence and infectivity to mosquitoes (Mackinnon & Read 1999a). Ideally, one could compete any of these clones against any other to study within-host competition, thus finding out whether virulent clones indeed out-compete avirulent clones (see Chapter 1). In the past, the choice of clones and experiments has been dictated by available diagnostic tools, rather than those clones' biological properties, such as virulence. Taylor *et al.* (1997a; 1998), used two clones distinguishable by monoclonal antibodies; even though such antibodies can be raised against any clone of choice, antibody assays are extremely tedious and time-consuming in practice. De Roode *et al.* (2003 [Thesis Appendix]) chose three clones for the simple reason that they could be distinguished by size polymorphisms in their *mspl* (merozoite surface protein 1) genes as visualised on agarose gels following PCR amplification. With the advent of more sophisticated and high-throughput PCR techniques, however, clone choice can now be based on biological properties rather than on the availability of antibodies or old-fashioned PCR protocols.
This chapter describes the use of real-time quantitative PCR for the analysis of genetically diverse infections. Real-time quantitative PCR is a recent and highly versatile technique that can in principle be used for quantification of any organism of choice. The method is based on quantifying DNA template concentrations in PCR reactions (Higuchi et al. 1993), and as such concentrations are often directly proportional to the number of parasites in a tested sample, real-time quantitative PCR has wide applications in parasitology (Bell & Ranford-Cartwright 2002). To date, for example, the method has been used to quantify *Toxoplasma gondii* DNA from clinical samples (Lin et al. 2000), *Borrelia burgdorferi* spirochaetes from tick vectors, and the rickettsial organisms *Ehrlichia phagocytophila* and *E. risticii* from their hosts (reviewed in Bell & Ranford-Cartwright 2002). For malaria specifically, real-time PCR has been used to quantify the erythrocytic (Hermsen et al. 2001; de Monbrison et al. 2003), pre-erythrocytic (Bruña-Romero et al. 2001; Witney et al. 2001) and sporozoite stages (Bell & Ranford-Cartwright 2004) of *Plasmodium falciparum*; it is also used as a diagnostic tool to detect absence and presence of parasites (de Monbrison et al. 2003; Fabre et al. 2004).

*P. chabaudi* has several genes, such as the *mspl* and *ama1* (apical membrane antigen 1) genes, that are extremely polymorphic between different clones (Cheesman S.J., pers. comm., Deleersnijder et al. 1991; McKean et al. 1993). As any two clones almost certainly have some sequence polymorphisms in one of these genes, clone-specific real-time PCR assays could be designed to distinguish and quantify these clones in mixed infections.

This thesis deals with only one such combination: mixed-clone infections of AS and AJ parasites. Ironic as this may seem after an argument about the versatility of real-time quantitative PCR, there are good reasons for this. First, this combination can be used to identify those factors that need optimising in terms of the PCR assays and sampling itself. Second, this clone combination is a combination of a virulent (AJ) and an avirulent (AS) clone, and can be used to reveal those biological factors that are most likely to affect within-host competition. These include virulence *per se* (Chapter 3), host genotype (Chapter 4), the timing of infection (co- or super-infection; Chapter 5), transmission to the mosquito vector (Chapter 6), and the absence or presence of anti-malaria drugs (Chapter 7). Only when it has been demonstrated that real-time PCR is suitable to study all of these factors, will it make sense to expand the method to a larger number of clone combinations.

### 2.2.2. Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) is a revolutionising technique to produce a selective enrichment of a specific DNA sequence by a factor exceeding $10^6$, facilitating analyses that
require large amounts of DNA (Saiki et al. 1988). PCR amplification involves two oligonucleotide primers that flank the DNA sequence to be amplified, and repeated cycles of heat denaturation of the DNA, annealing of the primers to their complementary sequences and extension of the annealed primers with DNA polymerase. As the extension products are also complementary to and capable of binding primers, each successive cycle essentially doubles the amount of DNA synthesised in the previous cycle (Saiki et al. 1988), thus giving rise to an exponential increase in DNA product. This exponential phase lasts for many cycles, but eventually the reaction becomes less efficient, and DNA amounts reach a plateau. This may occur because primer concentrations drop, enzymes and nucleotides become limiting, or because re-association between amplified products occurs before primers can anneal (Williams et al. 1998). If the reaction indeed reaches plateau because the concentration of product drives product re-association, samples containing different starting amounts of DNA will reach plateau at the same DNA concentration; at that time they will contain identical amounts of product, masking initial sample differences (Williams et al. 1998; Bell & Ranford-Cartwright 2002). Thus, end products of PCR can be used qualitatively (absence or presence of a certain DNA sequence), but cannot be used to quantify initial DNA template concentrations.

2.2.3. Real-time quantitative PCR

Recent developments have made quantification of initial DNA template concentrations possible, however, and of these, real-time quantitative PCR assays are the most widely used (Higuchi et al. 1993; Heid et al. 1996; Williams et al. 1998; Bell & Ranford-Cartwright 2002). The power of real-time quantitative PCR is that it does not rely on end product analysis, but that it quantifies DNA on the basis of the exponential phase of the PCR reaction. PCR amplification is monitored in real time, using fluorescence that is emitted either by DNA intercalating dyes, such as ethidium bromide (Higuchi et al. 1993), or by fluorogenic probes that specifically bind to the amplified product (Heid et al. 1996). The amount of fluorescence increases when the PCR reaction proceeds, as the amount of amplified DNA increases. Cycle-by-cycle monitoring (Figures 2.1A, E) makes it possible to very precisely identify the cycles in which the PCR is in the exponential phase, with the PCR product doubling every cycle. After the PCR run, a baseline is set that identifies the cycle in which the exponential signal can be distinguished from the background for each sample. The number of cycles it takes for the fluorescence of a sample to be distinguished from the background is called the $C_T$ value, and is inversely related to initial template concentration.
A standard curve is generated, plotting the log concentrations of known standards against the $C_T$ values (Figures 2.1B, F), and concentrations of target template of unknown samples can be extrapolated from this. An alternative approach to quantification is based on the S-shape of the amplification curves. Calculating the second derivative of each amplification curve yields a maximum in the early logarithmic phase of the PCR. This second derivative maximum is then used as a crossing point ($C_T$) in the further calculations to determine initial target concentrations. This approach is used for most of the experiments in this thesis.

One advantage of the fluorogenic probe approach over the intercalating dye approach is that it is more specific (Heid et al. 1996). The reason is that intercalating dyes, such as ethidium bromide, bind to any double-stranded DNA, including primer dimers and non-specific amplification products, and can therefore artificially increase the estimate of the initial template concentration (Higuchi et al. 1993). Despite this disadvantage, I used the intercalating dye approach for the work described in this thesis. The reason is that, at least when I started my PhD, the intercalating dye system was cheaper, had a shorter developing time (in terms of developing primers), was universally applicable to all potential markers, and was most widely used (Bell & Ranford-Cartwright 2002). More recently, however, I started to use the fluorogenic probe assay (Chapter 6).

Figure 2.1. (A) Logplot of fluorescence against cycle number for four AS DNA standards of known concentration (7.2, 0.72, 0.072 and 0.0072 ng/μl: circles, squares, crosses and diamonds respectively). (B) A standard curve based on the logplot in (A): the cycle number at which the reaction enters the log-linear phase is inversely related to the initial template concentration. (C) Melting curve of the four known standards in (A), an AJ DNA sample (dashed line, plusses) and a negative control (dashed line). (D) First negative derivatives of melting curves in (C). The four AS DNA standards show peaks around 88°C; the specific AS PCR product thus has a melting temperature of 88°C. AS 0.0072 ng/μl shows a tiny primer dimer peak at around 76°C. The negative control shows no specific product as expected, but a big primer dimer peak at around 77°C. AJ DNA also shows no specific product; there is thus no cross-reaction between AJ DNA and AS primers. (E) Logplot of fluorescence against cycle number for four AJ DNA standards of known concentration (4.3, 0.43, 0.043, 0.0043 ng/μl: circles, squares, crosses and diamonds respectively). (F) A standard curve based on the logplot in (E). (G) Melting curve of the four known standards in (E), an AS DNA sample (dashed line, plusses) and a negative control (dashed line). (H) First negative derivatives of melting curves in (G). The four AJ DNA standards show peaks around 88°C; the specific AJ PCR product thus has a melting temperature of 88°C. The negative control shows no specific product as expected, but a primer dimer peak at around 82°C. AS DNA also shows no specific product, but a non-specific peak at around 86°C; there is thus no cross-reaction between AS DNA and AJ primers.
2.2.4. The Roche Lightcycler

Even though the first real-time PCR experiments were done with a very basic setup of a traditional thermal cycler and a video camera (Higuchi et al. 1993), commercial systems for real-time quantitative PCR are now widely available (Williams et al. 1998; Bell & Ranford-Cartwright 2002). All these instruments record fluorescence in real time as the samples pass photodetection diodes within the instrument, following fluorophore excitation using an integral light source.

This study makes use of the Roche Molecular Biochemicals Lightcycler system, with a SYBR Green I detection assay (Roche). In this system, blue light from a LED is focused on the tip of a glass capillary (in which the reaction occurs), and excites the SYBR Green I dye. The emitted fluorescent light is then conducted back into the optical unit of the Lightcycler, where a set of filters and mirrors separates the light into different wavelengths that can be detected in one of three detection levels. SYBR Green I fluoresces strongly when bound to the minor groove of double-stranded DNA (dsDNA), but minimally when free in solution. The fluorescence intensity of bound SYBR Green I is relative to the amount of PCR product. Fluorescence is measured every cycle after the elongation phase, when there is only dsDNA, and can thus be used to monitor the increase in PCR product formation.

As mentioned above (§ 2.2.3), the downside of this intercalator system is that it non-specifically binds to any dsDNA, including non-specific PCR products and primer dimers. In the development of SYBR Green I based assays, it is therefore important to optimise primers and reaction conditions, and it is essential to be able to obtain a product melting curve, revealing the presence of specific PCR products, non-target products and primer dimers. The Lightcycler system is capable of providing this: at the end of the PCR run, the temperature of the samples is slowly raised, and the fluorescence monitored at frequent intervals (Figures 2.1C, G). As soon as the dsDNA starts to denature, the SYBR Green I dye is released, and fluorescence decreases.

The melting temperature \( T_m \) of a DNA fragment is defined as the temperature at which half of the DNA becomes single-stranded, and half remains double-stranded. This temperature can be easily visualised by taking the first negative derivative \(-dF/dT\) of the melting curve. It is then visible as a peak when plotting \(-dF/dT\) against temperature (Figures 2.1D, H). This permits easy identification of the fragment-specific \( T_m \). As each DNA fragment has a unique \( T_m \), the melting curve shows whether the targeted fragment has been produced, and whether non-specific products and primer dimers have been produced.
2.3. Parasites and hosts

The parasites I used were three *Plasmodium chabaudi chabaudi* clones denoted AS, AS(pyr1A) and AJ. AS and AJ were originally clonally isolated from different thicket rats in the Central African Republic, and are genetically distinct (Beale *et al.* 1978). AS(pyr1A) was derived through pyrimethamine selection from clone AS (Walliker *et al.* 1975); AS and AS(pyr1A) are thus genetically very similar, and can be quantified using the same PCR primers.

As hosts I used two inbred mouse strains denoted C57Bl/6J and CBA/Ca (Ann Walker, University of Edinburgh). Mice were fed on 41B maintenance diet (Harlan UK) and drinking water supplemented with 0.05% para-amino benzoic acid (Jacobs 1964); they were kept in a 12 L : 12 D cycle. I will refer to these mouse strains as C57 and CBA from hereon. Mice were always at least six weeks old, and were either males or females, depending on availability. Parasite inoculations consisted of $10^6$ parasites, were prepared from donor mice by diluting blood in 0.1 ml calf-serum solution (50% heat-inactivated calf-serum, 50% Ringer's solution [27mM KCl, 27mM CaCl₂, 0.15 M NaCl], 20 units heparin/ml mouse blood), and injected via the intraperitoneum. Alternatively, mice were infected with frozen parasite-infected blood stored in liquid nitrogen, and defrosted in citrate saline (0.9% NaCl, 1.5% tri sodium citrate dihydrate, pH 7.2) prior to inoculation.

2.4. PCR assays

2.4.1. DNA standards for AS and AJ

Seven female C57 mice were infected with AS, and seven were infected with AJ. On day 8 post-infection (PI) these mice were killed and as much blood collected from them as possible. DNA was extracted as described previously (Grech *et al.* 2002). Briefly, mouse white blood cells were removed by column purification using CF11 (Sigma) and Plasmodipur™ filters (Euro-Diagnostica). Parasites were isolated from host erythrocytes by saponin lysis and genomic DNA prepared by phenol-chlorophorm extraction. DNA concentrations were measured using a spectrophotometer. AS DNA and AJ DNA were diluted down to 400 ng/μl and 500 ng/μl respectively and stored as aliquots for future use in real-time PCR. Later on I measured these standards against standards produced by S.J. Cheesman, using real-time quantitative PCR, and found them to be and 72.0 ng/μl for AS and 40.3 ng/μl for AJ. Apparently, as I had not treated my samples with RNase, the RNA
that was present highly skewed my quantifications based on spectrophotometry. Values of
72.0 ng/μl for AS and 40.3 ng/μl for AJ were used in subsequent experiments.

When these standards were running low, I prepared new standards. I infected ten female C57
mice with AS and another ten with AJ. Mice were bled again and DNA extracted as
described above. DNA samples were treated with RNAs before re-extracting with phenol-
chlorophorm. DNA samples were then quantified using the old set of standards and set to
57.5 ng/μl for AS and 49.9 ng/μl for AJ.

For each PCR run, DNA standards were freshly prepared from these stocks by serial dilution
in H2O, and ranged from 0.0072 – 7.2 or 0.00575 – 5.75 ng/μl for AS, and 0.00403 – 4.03 or
0.00499 – 4.99 ng/μl for AJ.

### 2.4.2. PCR assays for AS and AJ

Primers were designed against variable block III of the *msp1* gene (Cheesman et al. 2003
[Thesis Appendix]). For AS they were ACAGTAACACAAAGAAGAAC (forward; f86837) and
GATACTTTGTGAGTTGCTGG (reverse; f86838); for AJ they were ACCAGCACAAGAGCA-
ACA (forward; f86835) and TTGCGGGTTTCTGTTGAGGCT (reverse; f86836). Reaction
conditions were almost similar for AS and AJ, both with 0.4 μM of forward and reverse
primers. The only difference was that in the AS reaction the final MgCl2 concentration was
4.5 mM, whereas with AJ it was 3.5 mM. Both PCR assays started with a denaturation step
at 95°C for 600 s. Amplification consisted of 40 cycles, with 95°C denaturation, 6 s of
annealing at 59°C for AS and 61°C for AJ, and 7 s of elongation at 72°C. Amplification was
followed by a melting curve analysis, with 0.2°C/s increment from 65°C to 95°C. Reaction
volumes were 10μl, with 1μl of DNA per reaction for standards and 2 μl of DNA per
reaction for unknown samples. It was concluded that these reaction conditions are optimal,
as they showed straight standard curves, and no primer dimer formation (Figure 2.1,
Cheesman et al. 2003 [Thesis Appendix]).

### 2.4.3. Cross-reactivity

To check for cross-reactivity, I tried to amplify AJ DNA using AS primers and reaction
conditions, and to amplify AS DNA using AJ primers and reaction conditions. In both my
experiments (Figures 2.1D, H), and others (Cheesman et al. 2003 [Thesis Appendix]) there
was no such cross-reactivity.
2.5. Quantification of AS and AJ parasites in artificially prepared mixtures

2.5.1. The need for artificial mixtures

Before using real-time PCR as a quantification method in real experiments, I wanted to make sure that obtained proportions of AS and AJ parasites reflected the true proportions of the parasites present. Therefore several series of AS and AJ mixtures were prepared and real-time PCR was used to analyse these.

2.5.2. Artificial mixtures

The first series of artificial mixtures described here was prepared by R. Carter (see also Cheesman et al. 2003 [Thesis Appendix]). These mixtures were made from three female CBA mice: one infected with AS, one infected with AJ, and one uninfected. Red blood cell densities were assessed by flow cytometry using a coulter counter (Beckman Coulter), and blood smears were taken to calculate parasitaemia (as the percentage of red blood cells infected with parasites). Mice were killed and blood was collected in 5 ml of citrate saline.

This resulted in a new volume, which was measured. The red blood cell densities of these solutions were calculated, and solutions were diluted with citrate saline to a concentration of $10^9$ red blood cells/ml. The AS solution was then diluted with the control solution to make its parasitaemia identical to that of the AJ solution (7.4%).

The AS and AJ solutions with the same numbers of red blood cells and parasites were mixed to give artificial mixtures with different ratios between the two clones (AS:AJ, 100:0, 99:1, 80:20, 67.5:32.5, 50:50, 32.5:67.5, 20:80, 6:94, 1:99, 0:100). Each mixture had a volume of 200 µl, corresponding to $2.0 \times 10^8$ red blood cells. The original mice had around $1.0 \times 10^{10}$ red blood cells per ml blood, so that $2.0 \times 10^8$ roughly corresponded to 20 µl of blood, which is significantly more than blood samples usually taken for DNA extraction (5 µl).

DNA was extracted from these blood mixtures by S.J. Cheesman, using the Roche High Pure Template Preparation Kit (Roche). Of each extracted DNA sample, 2 µl was used per PCR reaction. Quantifications were done twice for each sample, with two AS quantifications in one run, and two AJ quantifications in another run. The means of the two replicates were averaged and the average proportions of AS per sample were compared to the expected proportions (Figure 2.2). This showed that real-time quantitative PCR works well for quantifying AS and AJ parasites in these mixtures, as the data points fell almost exactly on the theoretically perfect straight line of observed vs. expected values (Figure 2.2).
Artificial mixtures were made from blood from three female CBA mice: one uninfected, one infected with AS and one infected with AJ. Diamonds represent data points, while the dashed line represents the theoretically perfect 1:1 observed:expected relationship.

2.5.3. **Artificial mixtures serially diluted**

The artificial mixtures described above were effectively based on 20 μl of mouse blood per sample. In standard experiments, it would be more ideal to use 5 μl samples, as these are more easily obtained from the tails of the mice sampled. The mixtures were also based on one level of parasitaemia only; for real-time PCR to be useful in experiments, however, it would have to work well over several orders of magnitude of parasitaemia. I therefore prepared a new series of artificial mixtures, using effectively 5 μl of mouse blood per sample, and diluting extracted DNA from these mixtures 10- and 100-fold.

One female C57 mouse was infected with AS, one with AJ and one was left uninfected. Red blood cell densities were assessed and blood smears taken to calculate parasitaemia. When the AS mouse reached a parasitaemia of 42%, mice were killed and blood was collected in 2 ml of citrate saline. This resulted in a new volume, which was measured. Red blood cell densities of these solutions were calculated, and solutions were diluted with citrate saline to the same number of red blood cells/ml. The AJ solution was diluted with the control solution to make its parasitaemia identical to that of the AS solution (42%). The AS and AJ solutions with the same numbers of red blood cells and parasites were then mixed to give artificial mixtures with different ratios between the two clones. Volumes were chosen such that the final mixtures represented 5 μl of mouse blood.
From these mixtures I extracted DNA using Instagene Matrix (BioRad). Subsequently I diluted these DNA samples 10- and 100-fold in sterile H$_2$O. AS and AJ were quantified and the proportion of AS calculated. Observed AS proportions were compared to the expected proportions (Figure 2.3), showing that quantifications of the AS proportions were very close to what they should have been for undiluted (Figure 2.3A), 10-fold diluted (Figure 2.3B) and 100-fold diluted (Figure 2.3C) samples. Thus, real-time quantitative PCR can be used to accurately quantify AS and AJ proportions in small blood samples, and over two orders of magnitude of DNA concentrations.

![Figure 2.3](image)

**Figure 2.3.** Observed versus expected proportions of AS in artificial mixtures of AS and AJ parasites (A), and in those same mixtures diluted 10 times (B) and 100 times (C). Diluted mixtures were obtained by diluting extracted DNA in sterile water. Artificial mixtures were made from blood from three female C57 mice: one uninfected, one infected with AS and one infected with AJ. Open circles represent data points, while the dashed lines represent the theoretically perfect 1:1 observed:expected relationships.
Figure 2.4. Observed versus expected proportions of AS in artificial mixtures of AS and AJ parasites at parasitaemia levels of 30% (A), 3.0% (B) and 0.3% (C). Artificial mixtures were made from blood from three male CBA mice: one uninfected, one infected with AS and one infected with AJ. Solutions of AS- and AJ- infected blood were created and diluted 1, 10 or 100 x with uninfected blood to create mixtures. Triangles represent data points, while the dashed lines represent the theoretically perfect 1:1 observed:expected relationships.

2.5.4. Artificial mixtures at different levels of parasitaemia

The 10- and 100-fold diluted artificial mixtures described in § 2.5.3 were obtained through diluting DNA samples in H\textsubscript{2}O. Here, I will describe mixtures that were based on diluting artificial mixtures using uninfected blood. This is important, as it is theoretically possible
that when parasite numbers are low relative to the blood volume they are in, quantifications could be affected by the presence of relatively many PCR inhibitors, or because PCR template concentration is low compared to non-template DNA (from white blood cells).

One male CBA mouse was infected with AS(pyr1A), one was infected with AJ and one mouse was left uninfected. When the AS mouse reached 31% parasitaemia, and the AJ mouse 33.9% parasitaemia, volumes of respectively 30 and 27 μl of tail blood were diluted into an end volume of 1500 μl of citrate saline/blood. AS and AJ stock solutions were then used to prepare artificial mixtures (AS:AJ, 50:50, 10:90, 1:99, 99:1, 9:1), making sure that the end volume represented a volume of 5 μl of mouse blood. The AS and AJ stock solutions were also diluted 10- and 100-fold using blood from the control mouse. These dilutions were used to prepare mixtures of the same ratios, at levels of 3.0 % and 0.3 % parasitaemia respectively. Quantifications were done twice for each sample, and the observed mean proportions of AS were compared to the expected proportions (Figure 2.4).

As observed AS proportions were again very close to those expected, it is clear that real-time quantitative PCR can be used to accurately estimate AS and AJ proportions in small blood samples, and over a range of 30 down to 0.3 % parasitaemia.

2.5.5. Artificial mixtures prepared from real-life samples

Despite the results described in §§ 2.5.2 - 2.5.4, I became concerned at one point that AS was relatively under-quantified in artificial mixtures, especially at low concentrations (see § 2.6.3.3). At that time I had already analysed one experiment fully (Chapter 4), and was concerned that the results obtained might have been biased towards AJ. To find out if this was indeed the case, I decided to create another set of artificial mixtures. These were based on 5 μl blood samples that I had collected from single AS(pyr1A) or AJ infections in CBA mice as part of the experiment described in chapter 4. These blood samples had been frozen at −70 °C since the experiment, and not been used before.

I prepared 50 mixtures, each consisting of a 5 μl blood sample from an AS(pyr1A) infected mouse and a 5 μl blood sample from an AJ infected mouse. DNA was extracted from these blood mixtures using Instagene Matrix (BioRad) and real-time PCR was performed. Three ranges of parasitaemias were distinguished (>10%, 1.0 – 10% and 0.1 – 1.0%) to find out if the level of parasitaemia itself influenced a bias (if such a bias already existed).

There turned out to be no bias, however, for any of the three parasitaemia ranges (Figure 2.5). In all cases the observed proportions of AS in these mixtures were scattered around the
perfect observed vs. expected relationship. There was considerable scatter around these lines, but this is not surprising given the fact that obtaining exactly 5 µl of blood from each mouse is not possible, because not all blood is released from the 5 µl glass capillaries that are used. The fact that the data points are around the line, however, and not mostly below it, shows that there was no bias towards AJ in measuring these mixtures.

Figure 2.5. Observed versus expected proportions of AS in artificial mixtures of AS and AJ parasites at parasitaemia levels of >10% (A), 1.0-10% (B) and 0.1-1.0% (C). Artificial mixtures were made by mixing together blood obtained from female CBA mice infected with either AS or AJ. These blood samples had been frozen for more than 12 months. Triangles represent data points, while the dashed lines represent the theoretically perfect 1:1 observed:expected relationships.
2.5.6. **Mixtures analysed straight after DNA extraction or nine months later**

One possible explanation for finding an under-representation of AS in the artificial mixtures described in § 2.6.3.3, is that DNA samples were stored too long at −20 °C before PCRs were done, and that hydrolysis of DNA had to some extent degraded the DNA (S.J. Cheesman and A.S. Bell, pers. comm.). Some evidence for this comes from quantifying artificial mixtures straight after DNA extraction and again later, when they had been frozen for nine months. Leaving the samples frozen for such a long time indeed resulted in an under-representation of AS in some of the artificial mixtures (Figure 2.6). It was therefore decided that to obtain the most reliable results, PCRs would have to be done as soon as possible after DNA extraction. This was achieved for the experiments described in Chapters 3-7.

**Figure 2.6.** Observed versus expected proportions of AS in artificial mixtures of AS and AJ parasites at a level of 0.3% parasitaemia. PCR reactions were done on these samples shortly after DNA extraction (A) and again when DNA samples had been frozen for nine months (B). Artificial mixtures were made as described in Figure 4; note that results in (A) are identical to those shown in Figure 2.4C. Triangles represent data points, while the dashed lines represent the theoretically perfect 1:1 observed:expected relationships.
2.6. Time of day effects in quantification by real-time PCR

2.6.1. Introduction

The time at which blood samples for PCR analysis are taken, could affect the reliability of the analysis. This is because using a DNA-based method for parasite quantification assumes a direct relationship between DNA-concentration and parasite number; such a relationship only exists when every parasite has only one genome (and thus only one PCR template).

A 1:1 relationship between parasites and genome copies certainly does not exist for malaria parasites throughout the entirety of their erythrocytic schizogonic cycle. *Plasmodium* species in laboratory mice undergo a 24-hour asexual replication cycle within the blood, during which several asexual parasitic stages can be distinguished (Cox 1988; Chimanuka et al. 1999). The cycle begins with haploid merozoites invading red blood cells; for *P. chabaudi* in a 12 L : 12 D cycle, this is thought to happen early in the morning, when it is dark (Cox 1988; Chimanuka et al. 1999). Merozoites grow into haploid ring-stage parasites, resembling diamond rings (Figure 2.7A). Ring-stage parasites turn into trophozoites, distinguished by densely stained cytoplasm that is no longer in the shape of a ring, and lacks a vacuole. Trophozoites mature from early trophozoites (still resembling rings; Figures 2.7B, C) into late mature trophozoites (Figure 2.7D). The parasites now undergo nuclear division, and when two or more nuclei are visible, the parasite is called a schizont. Schizonts (Figure 2.7E) may contain between 4 and 8 new merozoites (Cox 1988), which upon red blood cell lysis invade uninfected red blood cells (Figure 2.7F).

As a merozoite contains 1 genome copy, whereas a schizont may contain around 8, it is obvious that at some stage of the erythrocytic cycle DNA is replicated 2 up to 8 times. Several authors have grown malaria parasites in culture in the presence of \[^{3}\text{H}]\text{adenosine} or other isotopes that are incorporated into DNA during DNA synthesis. Quantifying amounts of DNA using scintillation assays, they have shown that DNA synthesis occurs primarily in maturing trophozoites and schizonts, in both *P. falciparum* (Gritzemacher & Reese 1984; Inselburg & Banyal 1984) and *P. chabaudi* (Newbold et al. 1982). Indeed, DNA synthesis inhibitors did block the maturation of trophozoites, but not the differentiation of rings into trophozoites (Inselburg & Banyal 1984). Based on such results, it would be best to sample infections at times when rings are the most abundant life-stages, to ensure as much as possible that concentrations of DNA correspond linearly to numbers of parasites. It would therefore be important that parasites underwent their erythrocytic cycles in synchrony, which means that at any time point there would be predominantly one parasite stage only.
Different parasite clones are known to undergo schizogony at different times during the day (at least in reversed L:D cycles: McLean 1986), and it is thus important to study the erythrocytic cycles of both AS and AJ, so that time points at which both parasites occur in predominantly the ring-stage can be identified.

Here, I analysed the erythrocytic schizogonic life cycles of AS(pyrlA) and AJ parasites over two 24-hour periods during experimental infection. Doing this, I wanted to find out whether AS(pyrlA) and AJ parasites were synchronous, and having such information, to identify the most optimal time during the day at which to take samples. The fact that the artificial mixtures described in §§ 2.5.2 – 2.5.5 gave accurate results, and the fact that they were all prepared from parasites between 08:00 and 12:00 hours, suggested that morning might be the optimal time for doing this.

Figure 2.7. Microscopic images of red blood cells infected with ring-stage parasites (A), early trophozoites (B, C), late trophozoites (D), a schizont with 8 merozoites (E) and newly invaded ring-stage parasites (F). Images were obtained using 1000x light microscopic magnification of Giemsa-stained blood smears.
In this experiment I used AS(pyr1A) and AJ. For simplicity AS(pyr1A) will be referred to as AS from hereon. Hosts were eight weeks old C57 and CBA female mice (Ann Walker, University of Edinburgh), which were kept in a 12 L : 12 D cycle.

The experiment consisted of three treatments for both C57 and CBA mice: infections with AS alone, infections with AJ alone, and mixed AS+AJ infections. Each treatment group had 5 mice, resulting in 30 mice in total. Mice infected with just AS or AJ received $10^6$ parasite-infected red blood cells, whereas mice infected with both clones received $2 \times 10^6$ parasites, made up of $10^6$ AS and $10^6$ AJ parasites. Inoculations were prepared from donor mice by diluting blood in 0.1 ml calf-serum solution (50% heat-inactivated calf-serum, 50% Ringer’s solution [27mM KCl, 27mM CaCl$_2$, 0.15 M NaCl], 20 units heparin/ml mouse blood), and injections were done via the intraperitoneum.

To monitor production of asexual parasites, I took thin blood smears from tail blood. These were fixed with methanol and stained with Giemsa to determine levels of asexual parasitaemia using 1000x microscopy. When asexual parasitaemia was high, I counted 500 red blood cells in at least 4 microscopic fields. With lower parasitaemias I counted at least 20 microscopic fields (corresponding to at least 10,000 red blood cells), and calculated the average number of cells per field. Red blood cells infected with asexual parasites were classified into the following categories: red blood cells infected with rings (ring-stage parasite), early trophs (early trophozoites), late trophs (late trophozoites) and schizonts. Absolute densities of these were calculated as the product of parasitaemias and red blood cell densities (measured using a Coulter Counter) taken on the same day.

Measurements were taken at 4-hourly intervals starting at 09:00 hours on day 5 and ending at 09:00 hours on day 6 PI, and again at 4-hourly intervals starting at 09:00 hours on day 12 and ending at 09:00 hours on day 13 PI. At all these time points I also collected 5 µl blood samples from each mouse. For mixed AS+AJ infections, these samples were DNA extracted as such. For single infections, however, I always pooled the blood sample of an AS infected mouse with that of an AJ infected mouse. Thus, for example, the blood samples from mouse 1 from the AS infected C57 mice were always pooled with those of mouse 1 from the AJ infected group of C57 mice. DNA was extracted using Instagene Matrix (BioRad) and AS and AJ DNA concentrations were measured using real-time quantitative PCR.
Figure 2.8. Parasite densities at 4-hourly intervals from 9:00 hours on day 5 PI (9:00) to 9:00 hours on day 6 PI (09:00). Shown are densities for single AS (A, B), single AJ (C, D) and mixed AS+AJ (E, F) infections in C57 and CBA mice respectively. Left panels for each mouse strain show densities of ring-stage parasites (rings), early trophozoites (early trophs) and total parasite (total) densities, while right panels show late trophozoite (late trophs) densities; the latter were shown in separate panels as they were much lower than the densities of rings, early trophs and totals. All data points are the means (± 1 s.e.m.) of 5 mice, except for AS infections in C57 mice, which are based on 4.

2.6.3. Results and discussion

2.6.3.1. Parasite dynamics over time

Between 09:00 on day 5 and 09:00 on day 6 PI, all infection types (AS, AJ and AS+AJ) increased their parasite densities (grey dashed lines in left panels of Figures 2.8A-F). During most of the 24-hour period, ring-stage parasites were the predominant parasites in all infections (solid lines in left panels of Figures 2.8 A-F), thus showing that *P. chabaudi* infections in this experiment are relatively asynchronous. This in contrast with human
malaria parasites in culture (Inselburg & Bányal 1984), and in vitro synchronised *P. chabaudi* parasites (Chimanuka *et al.* 1999), which showed very distinct and sequential peaks of rings, trophs and schizonts, indicating synchronous development.

Infections of AS in both C57 and CBA mice showed peaks of late trophozoites around 17:00 (Figures 2.8A, B), and in CBA mice, this followed a slight increase of early trophozoites between 9:00 and 13:00 (Figure 2.8B). Total parasitaemias increased mostly between 17:00 and 01:00 (Figures 2.8A, B). Both these observations indicate that AS parasites underwent schizogony sometime between 17:00 and 01:00. In AJ and AS+AJ infections, there was an increase in the numbers of early trophozoites between 9:00 and 17:00 (Figures 2.8C-F). This increase in early trophozoites was followed by large peaks of late trophozoites in AJ and AS+AJ infections at around 21:00, and increases in total parasitaemias around 01:00, indicating that schizogony in AJ and AS+AJ infections happened between 21:00 and 01:00. Thus, AS and AJ parasites seemed to undergo schizogony about 4 hours apart; the fact that AS+AJ infections resembled AJ single infections more than AS single infections is probably a result of AJ being the dominant clone in those infections. It must be noted, however, that AS infections had much lower parasite densities than AJ and AS+AJ infections, and that especially late trophozoite counts were sometimes based on a single trophozoite only. Some caution must therefore be taken in interpreting these results.

For all infections, hardly any schizonts were detected (results not shown), probably because most schizonts sequestered in mouse organs such as the liver (Gilks *et al.* 1990; Chimanuka *et al.* 1999) and spleen (Gilks *et al.* 1990). The fact that I did not observe a dip in total parasitaemias prior to schizogony (Gilks *et al.* 1990; Chimanuka *et al.* 1999), and that there was not a marked and quick increase in parasitaemia (Chimanuka *et al.* 1999), again confirms that the infections studied here were desynchronised.

On days 12 and 13, parasite densities were much lower than on days 5 and 6 PI, and it was therefore harder to obtain reliable parasite counts, and to interpret patterns of parasite dynamics. Nonetheless, it appeared that, in general, early trophozoites increased in density between 09:00 and 17:00 (dashed black lines in left panels of Figures 2.9B-F), after which late trophozoites peaked at around 21:00, even for AS infections in CBA mice (right panels of Figures 2.9B-F). Thus, even though early trophozoite production seemed to have shifted to an earlier time of day since days 5-6 PI, parasite replication still happened at the same time (following the late trophozoite peaks at around 21:00). Densities for AS infections in C57 were so low (most counts based on 1 or a few parasites; late trophozoites not observed), that interpretations for them are not possible.
Figure 2.9. Parasite densities at 4-hourly intervals from 9:00 hours on day 12 PI (9:00) to 9:00 hours on day 13 PI (09:00). Annotations are as in Figure 2.8. All data points are the means (± 1 s.e.m.) of 5 mice, except for AS infections in C57 mice, which are based on 4.

2.6.3.2. DNA synthesis over time

To find out when DNA replication occurs in these *P. chabaudi* infections, I analysed blood samples taken at 4-hourly intervals using real-time PCR. The AS and AJ DNA concentrations obtained in that way were then divided by the total density of parasite-infected red blood cells to obtain an estimate of the DNA content per infected cell.

For the samples collected on days 5 and 6 PI, the DNA content per infected cell increased throughout the day (Figure 2.10). For AS infections in C57 mice, this generally happened between 09:00 and 17:00 (Figure 2.10A), whereas for AJ and AS+AJ infections this happened mostly between 13:00 and 21:00 (Figures 2.10C-F). AS infections in CBA mice did not show much variation through time, although a slight increase was observed between 21:00 and 01:00 (Figure 2.10B). In general, however, it can be concluded that DNA content
per parasite increased when numbers of early and late trophozoites increased (see § 2.6.3.1; Figure 2.8). This has also been observed in highly synchronous *P. chabaudi* infections (Newbold *et al.* 1982).

![Figure 2.10. DNA content per infected red blood cell at 4-hourly intervals from 9:00 hours on day 5 PI (9:00) to 9:00 hours on day 6 PI (09:00). Shown are means for single AS (A, B), single AJ (C, D) and mixed AS+AJ (E, F) infections in C57 and CBA mice. All data points are the means (± 1 s.e.m.) of 5 mice, except for AS infections in C57 mice, which are based on 4. RBC: red blood cell.](image)

PCR analysis was not done on samples from C57 mice collected on days 12 and 13 PI, as parasitaemias were too low for accurate quantification. They were done for CBA mice, however, revealing different patterns than those seen on days 5 and 6 PI (Figure 2.11). Highest DNA contents per parasite were now observed at 09:00 on day 12 for AS and AJ, with peaks again at 01:00 on day 13 PI (Figures 2.11A, B). For AS+AJ infections, a peak was seen at 17:00 on day 12 PI. Whether these results are truly indicative of what happened
in these infections, is questionable, as overall parasite densities were far lower than those on
days 5 and 6 PI (see also § 2.6.3.1). If they are, then the most likely explanation for the
differences between days 5-6 and days 12-13 PI is that infections became less synchronised.
This would mean that at any one time point more maturing trophozoites were present, thus
obscuring the patterns as seen on days 5-6. This in itself would not be surprising; the
infections studied here all peaked around day 7/8 PI, and parasites may be less synchronous
after than before their peak parasitaemias (M.J. Mackinnon, pers.comm.).

Figure 2.11. DNA content per infected red blood cell at 4-hourly intervals from 9:00 hours on day 12
PI (9:00) to 9:00 hours on day 13 PI (09:00). Shown are means for single AS (A), single AJ (B) and
mixed AS+AJ (C) infections in CBA mice only; C57 mice had too low parasitaemias to perform real-
time quantitative PCR. All data points are the means (± 1 s.e.m.) of 5 mice, except for AS infections
in C57 mice, which are based on 4.
Figure 2.12. Observed versus expected proportions of AS in artificial mixtures made from blood samples obtained from C57 and CBA mice infected with either AS or AJ. Mixtures were made at 09:00 (A), 13:00 (B), 17:00 (C) and 21:00 (D) on day 5 PI and at 01:00 (E), 05:00 (F) and 09:00 (A) on day 6 PI. Circles and triangles represent data points, while the dashed lines represent the theoretically perfect 1:1 observed:expected relationships.
2.6.3.3. Observed and expected AS proportions at 4-hourly intervals

From the above paragraphs, it would seem best to analyse mixed infections early in the morning, when not many mature trophozoites are present and DNA content per parasite is low. Here I analyse the observed proportions of AS in the artificial mixtures made by pooling AS and AJ samples together, and compare them to those as expected on the basis of the total numbers of parasite-infected red blood cells that should have been present in these mixtures. It is important to note here that PCRs were not done straight after DNA extraction, and that hydrolysis of DNA might therefore have occurred (§ 2.5.6). Indeed, it was found that AS proportions were slightly lower than expected. I therefore corrected these proportions for the 09:00 time-points to use these as a reference point to analyse all the other time points.

Doing this, I found that the observed proportions of AS at 09:00 (Figure 2.12A) were much closer to the expected proportions, and showed less scatter around the observed:expected line than did the observed proportions of AS at 13:00, 17:00, 21:00 and 01:00 (Figures 2.12B-E). Proportions at 05:00 also were very close to their expected values, except for two outlying values (Figure 2.12F). On days 12 – 13 PI, AS observed proportions were closest to expected proportions at 13:00 (Figure 2.13B), while the rest of the time points showed considerable deviation from and scatter around the 1:1 observed:expected line (Figures 2.13A, C-F).

2.6.3.4. AS proportions over time

The observed AS proportions in the real AS+AJ mixed infections were analysed over time. Despite the fact that time of day affected the relationship between observed and expected proportions of AS in artificial mixtures (§ 2.6.3.3), there was hardly any evidence that the observed AS proportions in the real AS+AJ mixed infections changed between 09:00 and 17:00 on day 5 PI (Figures 2.14A, B for C57 mice and 2.14C, D for CBA mice respectively), although they did decrease afterwards (Figures 2.14A-D). On days 12-13 PI, AS proportions were considerably less constant over time (Figures 2.14E, F; CBA mice only), although they were still constant between 09:00 and 13:00.

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Figure 2.13. Observed versus expected proportions of AS in artificial mixtures made from blood samples obtained from CBA mice infected with either AS or AJ. Mixtures were made at 09:00 (A), 13:00 (B), 17:00 (C) and 21:00 (D) on day 12 PI and at 01:00 (E), 05:00 (F) and 09:00 (A) on day 13 PI. Circles and triangles represent data points, while the dashed lines represent the theoretically perfect 1:1 observed:expected relationships.
Figure 2.14. Proportions of AS in mixed AS+AJ infections over a 24-hour period. Shown are AS proportions at 4-hourly intervals from 09:00 hours on day 5 PI (9:00) to 09:00 hours on day 6 PI (09:00) for C57 (A, B) and CBA mice (C, D) respectively, and AS proportions at 4-hourly intervals from 09:00 hours on day 12 PI (9:00) to 09:00 hours on day 13 PI (09:00) for CBA mice only (E, F). Panels A, C and E show data for individual mice, while panels B, D and F show means (± 1 s.e.m.) for the same mice.
2.6.4. **Conclusions: time of day effects in quantification by real-time PCR**

This experiment showed that, in general, parasites mature between 09:00 in the morning and 21:00 at night. During this time DNA synthesis takes place, increasing the DNA content per parasite. This was especially true before peak parasitaemia, but to a lesser extent after peak parasitaemia, when infections seemed to be less synchronised.

It was shown that time of day affected the results of real-time PCR analysis, showing that, at least on days 5 and 6 PI, most accurate results were obtained at 9:00 in the morning. This, together with the fact that observed AS proportions in real mixed AS+AJ infections were rather stable between 09:00 and 17:00 on days 5-6 PI and between 09:00 and 13:00 on days 12-13 PI, shows that the best sampling time would be between 09:00 and 13:00 in the morning. The other artificial mixtures described in this chapter were made in exactly that time interval, and were accurately quantified for AS and AJ parasites, supporting this assertion.

The reason that less accurate results were obtained at certain time points, is probably due to the different parasite clones undergoing different erythrocytic cycles. AS started producing trophozoites before AJ, and the DNA content per parasite also increased earlier for AS than for AJ. That could explain why an overquantification of AS was observed in artificial mixtures prepared at 13:00 and 17:00 on day 5 (Figures 2.12B, C). An overquantification of AJ in artificial mixtures was observed at 21:00 and 01:00 (Figures 2.12D, E), when AJ had started to produce trophozoites, thus increasing its DNA content per parasite.

The infections in this experiment were not synchronous. There was no rapid increase of parasitaemia over a short time, lifting parasitaemia from one plateau to the next (Chimanuka et al. 1999), there was no clear succession of parasite stages (Newbold et al. 1982; Gilks et al. 1990; Chimanuka et al. 1999), and there was no dip in parasitaemia prior to schizogony (Cox et al. 1987; Gilks et al. 1990). This is possibly the result of a build-up of asynchrony of parasite populations that have been serially passaged through mice, as happened with the parasites used here. It would be interesting to test this possibility by comparing the synchrony of these parasites with infections of the same parasites initiated with mosquito inoculations or initiated with *in vitro* synchronised parasites (Chimanuka et al. 1999).
2.7. **Overall conclusions**

All the experiments described in this chapter were designed to find out whether real-time quantitative PCR can be used to accurately quantify AS and AJ parasites in mixed AS+AJ infections. On the basis of the presented results, the following conclusions can be drawn.

- AS- and AJ-specific real-time PCR assays were designed and found to be highly accurate. Furthermore, the AS assay did not amplify AJ DNA and the AJ assay did not amplify AS DNA.

- The real-time PCR assays for AS and AJ can be used to accurately quantify AS and AJ proportions in artificially prepared parasite mixtures from very low (~0.1 %) to very high (>30 %) parasitaemias.

- AS and AJ proportions can be accurately quantified with real-time PCR, using small volumes of blood (5 µl), which are easily obtained from the tails of mice.

- The best time for sampling blood from mice for subsequent PCR analysis is between 09:00 and 13:00.

As the DNA sequences for the *msp1* gene and other genes for more *P. chabaudi* clones are available, these real-time PCR assays could be expanded for analysing mixed-clone infections consisting of different clones from the ones described here (Cheesman *et al.* 2003 [Thesis Appendix]).
3. Virulent parasites are competitively superior in genetically diverse malaria infections

A shorter version of this chapter is submitted as: De Roode, J. C., Pansini, R., Cheesman, S. J., Walliker, D. & Read, A. F. Virulent parasites are competitively superior in genetically diverse malaria infections.

3.1. Abstract

Humans and animals often become co-infected with microparasite strains that differ in virulence. The outcome of the ensuing interaction can determine how sick the host becomes, and the direction of selection on virulence genes. Serial passage experiments and the reversion to virulence of attenuated live vaccines imply that virulent variants are competitively superior. However, direct evidence that this is the case is seriously lacking. I show here that in a rodent malaria model virulence is positively associated with competitive ability. Thus, measures to reduce malaria transmission should, by also reducing multiplicity of infection, impose evolutionary pressure on malaria for reduced virulence.

3.2. Introduction

Genetically diverse infections are common (Read & Taylor 2000; 2001) and in malaria are the rule rather than the exception (e.g. Beale et al. 1978; Snewin et al. 1991; Paul et al. 1995; Daubersies et al. 1996; Babiker et al. 1999; Felger et al. 1999; Bruce et al. 2000). Mixed genotype infections are of medical significance, because a large body of evolutionary theory asserts that competition between co-infecting pathogen genotypes can select for increased virulence, and thus more severe disease (Levin & Pimentel 1981; Bremermann & Pickering 1983; Van Baalen & Sabelis 1995; Frank 1996; Gandon 1998; Gandon 1998; Ebert 1999). This theory assumes that parasites that prudently exploit their hosts, and are thus avirulent, will be out-competed by more aggressive parasites, which rapidly exploit their hosts. Even if this means that host life expectancy is reduced so that all parasites do worse, prudent parasites will do disproportionately worse, and be eliminated by natural selection: a process commonly referred to as 'the tragedy of the commons' (Hardin 1968).

However, evidence that parasites with higher virulence are indeed competitively superior is currently circumstantial and anecdotal. Serial passage of pathogens through new host environments typically increases virulence (Ebert 1998), and live attenuated vaccines, such as polio, can revert to wild type virulence (Bull 1994; Kew et al. 2002). Both phenomena could be due to virulent variants rising in frequency because they have a competitive
advantage (Bull 1994, Ebert 1998). Some in vitro evidence supports this argument (Ebert 1998), but some direct in vivo competition experiments between avirulent and virulent parasites in a range of different disease models contradict this view: avirulent genotypes have been shown to overgrow virulent strains in some cases (Orcutt & Schaedler 1973; Hargreaves et al. 1975; Duval-Iflah et al. 1983; Allaker et al. 1988; Lenhoff et al. 1998; Franco et al. 2003, reviewed in Taylor and Read 2000). This could be because experimental effort is directed at the search for protective lines of parasites, rather than at directly determining the competitive ability of virulent strains: some of these experiments indeed first established an avirulent strain to protect against infection with a virulent one. Regardless, these current in vivo data contradict standard models of virulence evolution, and the explanations of why virulence increases following serial passage and reversion of live attenuated vaccines.

Malaria parasites vary in virulence (summarised in Mackinnon & Read 2004b; Read et al. 2004). Determining whether there is a relationship between intrinsic virulence and competitive ability in malaria parasites is important for predicting the consequences for disease severity of interventions that reduce clone multiplicity in malaria infections, such as bed-nets and strain-specific vaccines. Here, I test whether virulence and competitive ability are linked in the rodent malaria Plasmodium chabaudi, a parasite often used as a model for human malaria (Cox 1988).

### 3.3. Material and methods

#### 3.3.1. Parasites and hosts

Four *P. chabaudi* clones were used: 3 lines derived clonally from the same ancestral parasite, denoted AS(pyr1A), AS and AS(pyr1B), and an unrelated clone AJ (see Thesis Appendix). AS and AJ were originally isolated from thicket rats of the species *Thamnomys rutilans* (Beale et al. 1978); AS(pyr1A) was then obtained from AS through drug selection with pyrimethamine (Walliker et al. 1975), and AS(pyr1B) was derived from AS(pyr1A) by passage through 11 generations of mice, 6 generations of thicket rats and 4 generations of mosquitoes (not in this order). I will refer to these clones as AVirulent, AModerate, and AVirulent for AS(pyr1A), AS and AS(pyr1B) respectively.

Hosts were six weeks old C57Bl/6J inbred female mice (Harlan, England), which were fed on 41B maintenance diet (Harlan, England) and drinking water supplemented with 0.05% para-amino benzoic acid (Jacobs 1964), and kept in a 12 L : 12 D cycle.
3.3.2. Experimental design

The experiment consisted of 7 treatments with 5 mice each: single infections of $ASS_{avirulent}$, $ASS_{moderate}$, $ASS_{virulent}$ and $AJ$, and mixed infections of $AJ$ with either of the 3 AS clones. Thus, I used $AJ$ as a reference strain against which to compare the different AS clones. Infections were initiated with $10^6$ parasites for single and $2 \times 10^6$ parasites ($10^6$ AS and $10^6$ AJ) for mixed infections. I used a double dose of parasites to initiate mixed infections, because I wanted to compare the performance of an individual clone in a mixed infection to its performance in a single infection, requiring equal numbers of parasites at inoculum; a twofold difference in parasite numbers has a negligible effect on parasite dynamics and virulence (Timms et al. 2001). I prepared inoculations from donor mice by diluting blood in 0.1 ml calf-serum solution (50% heat-inactivated calf-serum, 50% Ringer’s solution [27mM KCl, 27mM CaCl2, 0.15 M NaCl], 20 units heparin/ml mouse blood), and injected them via the intraperitoneum.

3.3.3. Virulence and parasite dynamics

To monitor virulence, red blood cell densities were recorded (using flow cytometry; Beckman Coulter): red blood cell densities decrease dramatically during infection, and these decreases correlate with host mortality and thus virulence (Mackinnon et al. 2002b).

To monitor production of asexual parasites and gametocytes (the sexual transmission stages) thin blood smears were prepared from tail blood. These were fixed with methanol and stained with Giemsa to determine levels of asexual parasitaemia and gametocytaemia as the percentage of infected red blood cells using 1000x microscopy. When asexual parasitaemia was high, 500 red blood cells in at least 4 microscopic fields were counted; with lower parasitaemias and gametocytaemias at least 20 microscopic fields were counted (corresponding to at least 10,000 red blood cells). Parasite densities and gametocyte densities were calculated as the product of parasitaemias or gametocytaemias and red blood cell densities taken on the same day.

Tail blood samples of 5 µl were taken from mice harbouring mixed infections in citrate saline. After 1 minute’s centrifugation at 13,000 rpm, the supernatant was removed and pelleted blood cells stored at -70°C for subsequent DNA extraction using Instagene Matrix (BioRad). I used real-time quantitative PCR to measure the DNA concentrations of both AS and AJ in these samples (Chapter 2, Cheesman et al. 2003 [Thesis Appendix]), and calculated the proportion of AS and AJ of the total parasite population. Absolute numbers of
AS and AJ were then calculated by multiplying these proportions by the overall parasite density on the same day.

PCRs were performed when parasitaemias were higher than 0.1%, which is the lowest level at which AS and AJ proportions can be accurately estimated (Chapter 2). The real-time quantitative PCR protocols cannot distinguish between asexual parasites and gametocytes, and so they estimate the densities of all parasites. In the data analyses, I treated these as estimates of asexual densities. Gametocyte densities were 2-3 orders of magnitude lower than asexual parasite densities, and are thus a negligible component of overall parasite numbers.

Measurements were taken every day from day 0 to day 22 post-infection (PI), then every 2 days from day 24 to day 28 PI, when the experiment was terminated. Measurements were taken between 9.00 and 10.30 a.m., when peripheral blood almost exclusively harboured haploid ring stages of the parasites (Chapter 2).

3.3.4. Trait definition and statistical analysis

To measure virulence I determined red blood cell loss as the difference between a mouse’s initial red blood cell density and the minimum it reached during the infection.

Mice generally experienced several waves of parasites, with the first being substantially larger and longer. For each mouse, I calculated the period until the end of the first wave, and defined this as the acute phase of the infection. The subsequent period, the chronic phase, was defined as starting the day parasite numbers began to recover after the collapse of the first wave. To calculate the number of parasites present during the whole infection, and during the acute and chronic phases, I calculated the area under the relevant parasite density by day PI curves. As the limit of detecting parasites was around 100/μl, I treated observations of zero parasites as being 100 parasites/μl. I could accurately quantify AS and AJ proportions of 0.01 at levels of 0.1% parasitaemia or higher (Chapter 2); below that I set the clone densities at the limit of detection (100/μl). Parasite densities in single infections were set to the same level when parasitaemias were lower than 0.001%. Persistence was defined as the number of days until AS or AJ disappeared below detectible levels.

For gametocytes, I calculated the total densities over the whole infection. I was able to analyse overall numbers of gametocytes only, as the real-time quantitative PCR protocol does not distinguish between AS and AJ gametocytes.
Analysis was carried out using ANOVAs and ANCOVAs in Minitab (Version 13.30, Minitab Inc.). For all the models I first fitted the maximal model including covariate (when relevant) and interactions between explanatory variables. I then minimised the models by removing non-significant terms (p>0.05), beginning with interactions. Where necessary, data were log transformed to meet the necessary normality and homogeneity of variance assumptions.

3.4. Results

In each of the treatments AS_{avirulent}+AJ and AS_{moderate}+AJ, one mouse had delayed and low parasite densities, suggesting that these mice had accidentally received a lower dose of parasites than all the other mice (Timms et al. 2001). They were removed from the analysis.

AJ was the most virulent of the 4 parasite clones (causing most anaemia), and AS_{avirulent} the least virulent, with AS_{moderate} and AS_{virulent} causing intermediate levels of virulence (Figure 3.1).

![Figure 3.1. Virulence (anaemia) of the three related AS clones and the unrelated clone AJ in single clone infections. Red blood cell (RBC) loss is the difference between red blood cell densities of mice at the start of the infections and the minimum they subsequently reached at around day 10 PI. For clarity AS_{avirulent} is labelled as AS(a), AS_{moderate} as AS(m) and AS_{virulent} as AS(v). Bars are the means of 5 replicate mice (+ 1 s.e.m.). All four clones differ in virulence (F_{3,15}=16, P<0.001), as do the three AS clones (F_{2,12}=13, P<0.001).](image)

Clone AJ competitively suppressed all three AS clones, prematurely truncating infections and reducing parasite densities (Figure 3.2). However, the extent of the competitive
suppression was related to intrinsic virulence. Alone, all AS clones persisted for at least 30 days, but in competition with AJ, AS\textsubscript{avirulent} disappeared below PCR-detection after just 10 (± 0) days (mean ± 1 s.e.m.), AS\textsubscript{moderate} after 11.8 (± 0.8) days, and AS\textsubscript{virulent} after 20 (± 2.5) days (F\textsubscript{2,12}=9.7, p=0.005). In all three cases, persistence was less than in the corresponding single-clone infections (F\textsubscript{1,24}=42, p<0.001). Persistence differed between the three AS clones in mixed infections (F\textsubscript{2,24}=7.09, p=0.004), and the persistence of clone AJ was unaffected by the presence of any of the AS clones (Figures 3.2G-I; F\textsubscript{3,14}=0.85, p=0.49).

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**Figure 3.2.** Parasite densities over time (mean ± 1 s.e.m.), comparing AS and AJ clones in mixed infections (A-C), AS clones in mixed and single infections (D-F) and AJ clones in mixed and single infections (G-I). All data points are based on 5 replicate mice, except for mixed AS(a)+AJ and AS(m)+AJ infections (4 mice). The limit of detection was 100 parasites per μl blood, so that y-axes start at 2.
During both the acute and chronic phases of infections, the total numbers of parasites produced by all the AS clones were reduced in the presence of clone AJ (Figures 3.3A, B; \( F_{1,24}=39, p<0.001 \) and \( F_{1,22}=30, p<0.001 \) respectively), but the densities that clones reached varied across the AS clones (\( F_{2,24}=26, p<0.001; \ F_{2,22}=21, p<0.001 \) respectively). Parasites with higher intrinsic virulence achieved higher densities in competition with AJ, so that there was a direct relationship between virulence and competitiveness (Fig. 3.4).

![Graph showing parasite densities during acute and chronic phases of infections](image)

**Figure 3.3.** Total numbers of AS (A, B) and AJ (C, D) parasites produced during the acute (left panels) and chronic (right panels) phases of infections (mean ± 1 s.e.m.). *P. chabaudi* parasites have a 24 hour replication cycle, so that the sum of the daily parasite densities gives the total number of parasites present in an infection. Data points are based on 5 replicate mice, except for mixed AS(a)+AJ and AS(m)+AJ infections (4 mice). * Value denotes a density of 0, as AS(a) disappeared below detection levels in mixed AS(a)+AJ infections during the acute phase.
The tester clone AJ produced similar numbers of parasites during the acute phase when in mixtures with $AS_{avirulent}$ and $AS_{moderate}$, but it was competitively suppressed in the presence of $AS_{virulent}$ (Figure 3.3C; $F_{3,14}=7.9$, $p=0.002$). It produced more parasites during the chronic phase when mixed with $AS_{virulent}$ than when alone or in mixture with the other AS clones (showing facilitation rather than competition; Figure 3.3D; $F_{1,16}=6.9$, $p=0.019$). Summarised over the whole infection, AJ produced as many parasites in single and mixed infections ($F_{3,14}=2.0$, $p=0.16$).

Thus, not only did the most virulent AS clone grow best in competition with AJ, it also competitively suppressed AJ during the acute phase. It seems likely that this arose because the total number of parasites of both clones that could be present in an infection was constrained. Overall parasite numbers ($AS+AJ$) in mixed infections were the same as those produced by AJ alone ($F_{1,16}=0.01$, $p=0.94$; $F_{1,16}=1.17$, $p=0.30$ for acute and chronic phases respectively), and the implication is that where a clone achieves substantial densities, the other clone has to suffer.

Gametocyte densities over time in $AS_{avirulent}+AJ$ and $AS_{moderate}+AJ$ infections mostly resembled patterns of gametocyte densities seen in AJ infections, while those in $AS_{virulent}+AJ$ infections were intermediate between $AS_{virulent}$ and AJ (Figure 3.5); gametocyte numbers summarised over the whole infection in mixed infections were not different from those in AJ.
single infections ($F_{1,16}=3.10, p=0.10$). On the basis of this, it seems likely that AJ was the dominant clone in terms of transmission in $AS_{\text{virulent}}+AJ$ and $AS_{\text{moderate}}+AJ$ infections, whereas both $AS_{\text{virulent}}$ and AJ had transmission potential from $AS_{\text{virulent}}+AJ$ infections. This would not be surprising as $AS_{\text{virulent}}$ and $AS_{\text{moderate}}$ disappeared from mixed infections much more quickly than did $AS_{\text{virulent}}$. However, to really confirm this assertion, AS and AJ transmission to mosquitoes would have to be studied (see Chapter 6).

![Figure 3.5. Gametocyte densities over time (mean ± 1 s.e.m.). (A) Gametocyte densities for AS(a), AJ and AS(a)+AJ infections. (B) Gametocyte densities for AS(m), AJ and AS(m)+AJ infections. (C) Gametocyte densities for AS(v), AJ and AS(v)+AJ infections. For mixed infections, overall (AS+AJ) numbers of gametocytes are shown only, as the real-time quantitative PCR does not distinguish between AS and AJ gametocytes. All data points are based on 5 replicate mice, except for mixed AS(a)+AJ and AS(m)+AJ infections (4 mice). As the limit of detection was 100 gametocytes per μl blood, y-axes start at 2. X-axes terminate at day 20, as gametocyte production after that day was negligible.](image-url)
3.5. Discussion

These results show that in *P. chabaudi*, in-host competitiveness increases with virulence, thus supporting a large body of theory that predicts selection for increased virulence due to competition in mixed infections (Levin & Pimentel 1981; Bremermann & Pickering 1983; Van Baalen & Sabelis 1995; Frank 1996; Gandon 1998; Mosquera & Adler 1998). To my knowledge this is the first *in vivo* evidence for such a relationship in a medically relevant model of disease. These results are consistent with standard explanations of why serial passage of *P. chabaudi* (Mackinnon & Read 1999b; Mackinnon & Read 2004a) and many other organisms (Ebert 1998) increases virulence, and why vaccine revertants arise (Bull 1994): newly arisen virulent parasites can have a competitive advantage.

The fact that experiments involving several different organisms have found the reverse (avirulent parasites outgrowing virulent ones: Read & Taylor 2001) could be because experimentalists actively search for strains that protect the host from infection by virulent strains, or because a relationship between virulence and competitiveness does not always exist. The latter is possible if faster growing parasites attract more attention from the immune system (Bruce et al. 2000; Franco et al. 2003; Hughes & Boomsma 2004), or when parasites rely on the cooperative production of extracellular growth molecules. Here, ‘cheating’ parasites can arise, which rely solely on others for the production of such molecules, so that they grow at faster rates in mixed infections, but cause lower virulence when alone (Chao et al. 2000; Brown et al. 2002; West & Buckling 2003). Similarly, where coinfecting pathogens compete by interference competition, competitive ability might not be linked to virulence because competitively superior strains may be directing resources at the production of allelopathic substances rather than maximising host exploitation (virulence) (Read & Taylor 2000; Kirkup & Riley 2004; Massey et al. 2004). These types of considerations imply that the biological details governing interactions between pathogens will determine the qualitative nature of relationships between virulence and competitive ability in any particular disease situation.

In malaria, competition most likely results from competition for limited resources (such as red blood cells or blood sugar), and competition through strain-transcending immunity (Snounou et al. 1989; Hellriegel 1992; Snounou et al. 1992; Taylor et al. 1997a; Taylor & Read 1998; Read & Taylor 2001; De Roode et al. 2003 [Thesis Appendix]). Immunity is certainly important, as there is some evidence that resistant hosts cause stronger competition than susceptible hosts (Chapter 4, De Roode et al. 2004b [Thesis Appendix]). Indeed, immune mechanisms may also be responsible for the facilitation observed here in the chronic
phase (Figure 3.3D). During this phase, immunity is largely specifically directed against
strains and antigenic variants produced by those strains (Jarra & Brown 1985; Phillips et al.
1997; Buckling & Read 2001), and the presence of several clones rather than one during that
phase could make it harder for the immune system to clear the infection effectively (Chapter
4, De Roode et al. 2003; De Roode et al. 2004b [Thesis Appendix]).

An important implication of this study is that actively reducing clone multiplicity in malaria
infections in human populations could have public health benefits. Besides the immediate
protective benefits of interventions such as drugs, bed-nets and in due course (hopefully)
vaccines, reducing clone-multiplicity could have beneficial evolutionary effects: by reducing
the number of competing clones per human, the evolutionary selection for increased
virulence could be reduced, thus selecting for less virulent parasites. As such, reducing
clone-multiplicity may be an elegant example of evolutionary medicine, where human
actions can be used to direct pathogen evolution in for us favourable directions (Zimmer
2003).
4. Host genotype affects the outcome of within-host competition between malaria parasites


4.1. Abstract

During an infection, malaria parasites compete for limited amounts of food and enemy-free space. Competition affects parasite growth rate, transmission, and virulence, and is thus important for parasite evolution. Much evolutionary theory assumes that virulent clones outgrow avirulent ones, favouring the evolution of higher virulence. I infected laboratory mice with a mixture of two *Plasmodium chabaudi* clones: one virulent, the other avirulent. Using real-time quantitative PCR to track the two parasite clones over the course of the infection, I found that the virulent clone overgrew the avirulent clone. However, host genotype had a major effect on the outcome of competition. In a relatively resistant mouse genotype (C57Bl/6J), the avirulent clone was suppressed below detectible levels after ten days, and apparently lost from the infection. In contrast, in more susceptible mice (CBA/Ca), the avirulent clone was initially suppressed, but it persisted, and during the chronic phase of infection, it did better than it did in single infections. Thus, the qualitative outcome of competition depended on host genotype. I suggest that these differences may be explained by different immune responses in the two mouse strains. Host genotype and resistance could therefore play a key role in the outcome of within-host competition between parasite clones, and the evolution of parasite virulence.

4.2. Introduction

According to a large body of theory, competition within hosts generates selection for pathogens that do more damage to their host (virulence: Levin & Pimentel 1981; Bremermann & Pickering 1983; Frank 1992, 1996; Van Baalen & Sabelis 1995; Gandon 1998; Mosquera & Adler 1998; Ebert 1999; Read et al. 2002). Parasite fitness in singly infected hosts is assumed to be maximised when the benefits of host exploitation (increased transmission rate) balance the costs (increased host mortality) (Levin & Pimentel 1981; Anderson & May 1982; Bremermann & Pickering 1983; May & Anderson 1983; Sasaki & Iwasa 1991; Frank 1992, 1996; Antia et al. 1994; Van Baalen & Sabelis 1995; Antia & Lipsitch 1997). But in genetically diverse infections, parasites that slowly exploit hosts will
be out-competed by those that exploit hosts more rapidly. Even if host life expectancy is reduced so that all parasites do worse, prudent parasites do disproportionately worse and are thus eliminated by natural selection.

The majority of mixed-infection models of virulence assume that more virulent strains have a competitive advantage. Logical as this may seem, there is hardly any experimental evidence to suggest that this is indeed the case, and some evidence that the opposite is true (Read & Taylor 2001). Within-host competition is certainly widespread, and is mediated by limited resources, strain-transcending immune responses (apparent competition) or direct interference between competing genotypes (Read & Taylor 2001). In rodent malaria, for example, both resource and apparent competition probably play a role (Snounou et al. 1989; Hellriegel 1992; Snounou et al. 1992; Taylor et al. 1997a; Taylor & Read 1998; Read & Taylor 2001; De Roode et al. 2003 [Thesis Appendix]). But while strains with a higher growth rate probably will do better in resource competition, they could also become a preferred target for strain-specific immune responses, giving less abundant genotypes an advantage (Bruce et al. 2000; Read & Taylor 2000).

If there is a lack of experimental evidence to suggest that virulent clones are competitively superior to avirulent clones, there is even less evidence that any superiority is consistent in genetically different hosts. Numerous studies have shown that disease virulence varies with host genotype, with some host types being more susceptible to severe disease than others (e.g. Stevenson et al. 1982; Ebert & Hamilton 1996; Ebert et al. 1998; Imhoof & Schmid-Hempel 1998a; 1998b; Carius et al. 2001; Mackinnon et al. 2002b). This variation in resistance is likely to be reflected in variation in the strengths of resource- or immune-mediated competition between parasites within hosts. Indeed, one study has now shown that this is the case: an experiment on the endophyte *Epichloe bromicola* parasitic on the grass *Bromus erectus* showed that parasite strains that competitively excluded some strains on one host genotype, were excluded themselves on another (Wille et al. 2002).

Here, I ask whether host genotype is also an important determinant of competitive outcome in a model of human disease. I studied mixed infections of a virulent and an avirulent clone of the rodent malaria parasite *Plasmodium chabaudi* in two different strains of laboratory mice. Based on the theory outlined above, I predicted that the virulent parasite clone would rapidly outgrow the avirulent clone in both mouse strains. This did occur in one mouse strain, but in the other, competitive suppression was transient, with the avirulent clone persisting to do better in the chronic phase of infection than it would have done on its own.
4.3. Material and methods

4.3.1. Parasites and hosts

I used two genetically distinct *Plasmodium chabaudi chabaudi* clones, denoted AS(/pyr1A) and AJ. AS(/pyr1A) was derived through pyrimethamine selection from clone AS (Walliker et al. 1975). Both AS and AJ were originally isolated from thicket rats (Beale et al. 1978). For simplicity AS(/pyr1A) will be referred to as AS from hereon. I chose AS and AJ clones because they differ in their growth rates and virulence (Mackinnon & Read 1999a), with AS producing fewer parasites and causing less virulence than AJ.

Hosts were eight weeks old C57Bl/6J and CBA/Ca inbred female mice (Ann Walker, University of Edinburgh). They were fed on 41B maintenance diet (Harlan, England) and drinking water supplemented with 0.05% para-amino benzoic acid to enhance parasite growth (Jacobs 1964). They were kept in a 12:12 hour light-dark cycle. I will refer to these mouse strains as C57 and CBA from hereon.

4.3.2. Experimental design and inoculation of mice with parasites

The experiment consisted of three treatments for both C57 and CBA mice: infections with AS alone, infections with AJ alone, and mixed AS+AJ infections. Each treatment group had 5 mice, resulting in 30 mice in total. Mice infected with just AS or AJ received $10^6$ parasite-infected red blood cells, whereas mice infected with both clones received $2 \times 10^6$ parasites, made up of $10^6$ AS and $10^6$ AJ parasites. The latter was done because in the analysis I wanted to compare the performance of a clone on its own to its performance in a mixed infection, requiring equal numbers of each parasite clone at inoculation. Although mice that were infected with both clones received a double dose of parasites, previous work has shown that a two-fold difference in parasite numbers has a negligible effect on parasite dynamics and virulence (Timms et al. 2001).

I prepared inoculations from donor mice by diluting blood in 0.1 ml calf-serum solution (50% heat-inactivated calf-serum, 50% Ringer's solution [27mM KCl, 27mM CaCl2, 0.15 M NaCl], 20 units heparin/ml mouse blood), and injected them via the intraperitoneum.
4.3.3. Monitoring of virulence and infections

To monitor virulence, I recorded mouse live weights and red blood cell densities (using flow cytometry; Beckman Coulter). Both body mass and red blood cell densities decrease dramatically during infection, and these decreases correlate with host mortality and thus virulence (Mackinnon et al. 2002b).

To monitor production of asexual parasites and gametocytes (the sexual transmission stages) I took thin blood smears from tail blood. These were fixed with methanol and stained with Giemsa to determine levels of asexual parasitaemia and gametocytæmia using 1000x microscopy. When asexual parasitaemia was high, I counted 500 red blood cells in at least 4 microscopic fields. With lower parasitaemias and gametocytæmias I counted at least 20 microscopic fields (corresponding to at least 10,000 red blood cells), and calculated the average number of cells per field. Gametocyte numbers were counted using polarised light. I calculated parasite densities and gametocyte densities as the product of parasitaemias or gametocytæmias and red blood cell densities taken on the same day.

I took measurements every day from day 0 to day 23 post-infection (PI), three times a week from day 23 to day 48 PI, once a week from day 48 to day 69 and on day 83 PI, when the experiment was terminated. Measurements were taken between 9.00 and 10.30 a.m., when peripheral blood almost exclusively harboured haploid ring stage parasites (see Chapter 2).

4.3.4. Monitoring of individual clones in mixed infections

I collected 5 μl samples of tail blood from mice harbouring mixed infections in citrate saline. After 1 minute’s centrifugation at 13,000 rpm, I removed the supernatant and stored the pelleted blood at -70°C for subsequent DNA extraction using Instagene Matrix (BioRad). I used real-time quantitative PCR to measure the DNA concentrations of both AS and AJ in these samples (Chapter 2, Cheesman et al. 2003 [Thesis Appendix]), and calculated the proportion of AS and AJ of the total parasite population. Absolute numbers of AS and AJ were then calculated by multiplying these proportions by the overall parasite density on the same day.

I took blood samples on the same days that we took our other measurements, but performed PCRs only when parasitaemias were higher than 0.1%, which is the lowest level at which we can accurately estimate AS and AJ proportions (Chapter 2). The real-time quantitative PCR protocols cannot distinguish between asexual parasites and gametocytes, and so they estimate the densities of all parasites. In the data analyses, I treated these as estimates of
asexual densities. Gametocyte densities were 2-3 orders of magnitude lower than asexual parasite densities, and are thus a negligible component of overall parasite numbers.

4.3.5. Trait definition

Prior to statistical analysis I defined and constructed the following traits that described part of or whole of the infection. For measures of virulence I determined the 'minimum weight' and 'minimum red blood cell density' that mice reached. Mice that died were included in the analysis of minimum weights and red blood cell densities, using weight or red blood cell density on the day of death as the minima they reached. For all other traits, these mice were excluded from the analyses.

Mice generally experienced several waves of parasites, with the first being substantially larger and longer. For each mouse, I calculated the period until the end of the first wave, and defined this as the acute phase of the infection. The subsequent period, the chronic phase, was defined as starting the day parasite numbers began to recover after the collapse of the first wave (day 15.6 ± 0.55 PI; mean ± 1 s.e.m.). To calculate the number of parasites present during the whole infection, and during the acute and chronic phases, I calculated the area under the relevant parasite density by day PI curves. As the limit of detecting parasites was around 100/μl, I treated observations of zero parasites as being 100 parasites/μl. I could accurately quantify AS and AJ proportions of 0.01 at levels of 0.1% parasitaemia or higher (Chapter 2); below that I set the clone densities at the limit of detection (100/μl). Parasite densities in single infections were set to the same level when parasitaemias were lower than 0.001%.

For gametocytes, I chose slightly different ways of calculating densities, as they showed different dynamics to asexual parasites, having a first wave before day 9 and one or several thereafter. I calculated total gametocyte densities over days 0-9 and days 10-83 PI, and total densities over the whole infection. I could analyse overall numbers of gametocytes only, as the real-time quantitative PCR protocol does not distinguish AS and AJ gametocytes.

4.3.6. Statistical analysis

I analysed all traits mentioned above using ANOVAs and ANCOVAs in Minitab (Version 13.30, Minitab Inc.). The explanatory variables used were mouse 'Strain' and infection 'Treatment'. Strain had two factor levels (C57 and CBA); Treatment had up to three factor levels (AS, AJ and AS+AJ), depending on the analysis. For all models I first fitted the
maximal model including covariate (when relevant), Treatment, Strain and an interaction between Treatment and Strain. I then minimised the models by removing non-significant terms (p>0.05), beginning with the interaction. I log transformed initial and minimum red blood cell densities as well as all parasite and gametocyte densities prior to analysis, to meet the necessary normality and homogeneity of variance assumptions.

4.3.7. Follow-up experiment

To confirm the most important findings of this study, I compared the results obtained here with those obtained in the experiment described in Chapter 2 (§ 2.6.2). This experiment was exactly as the one described here, except that mice were sampled on days 6 and 13 PI only.

4.4. Results

One C57 mouse infected with AJ died on day 12, one infected with AS+AJ on day 7 and two CBA mice infected with AS+AJ died on day 10 and 11 PI. AJ infections induced greater weight loss and lower minimum red cell densities than AS infections (Treatment: F_{1,17}=25.4, p<0.001; Treatment: F_{1,18}=25.3, p<0.001 respectively), regardless of mouse genotype (Strain and Treatment x Strain n.s.). Thus, as expected, AS was the less virulent clone.

4.4.1. AS and AJ parasite densities

In mixed infections in both C57 and CBA mice, there were substantially more AJ than AS parasites during the first 10-14 days (Figures 4.1A, B). After day 10, AS disappeared below detectible levels in C57 mice, and never reappeared. It persisted, however, in the three CBA mice that survived the first two weeks. One of these experienced separate AS and AJ parasite waves around day 35 and 55 p.i. (Figure 4.2A). In the other two mice, AS started to overgrow AJ around days 22 and 18 respectively (Figures 4.2B, C). Later in the infection AJ overgrew AS again in one of these (Figure 4.2C).

Formal analysis confirmed this picture. During the acute phase in both mouse strains, AS was competitively suppressed by AJ, producing far fewer parasites in mixed infections than it did alone (Figures 4.1C, D and 4.3A; Treatment: F_{1,13}=155, p<0.001). This suppression was greater in CBA than in C57 mice (Treatment x Strain: F_{1,13}=9.1, p=0.01). In C57 mice, AS completely disappeared below detectible levels before the end of the acute phase, showing competitive exclusion.
In CBA mice, AS was also competitively suppressed during the acute phase, but it was not excluded. During the chronic phase, it produced more parasites than it would have done alone (Figures 4.1D, 4.3B; Treatment: $F_{1,13}=6.8, p=0.022$; Treatment x Strain: $F_{1,13}=34, p<0.001$), showing that AS actually benefited from the presence of AJ (i.e. facilitation, not competition). During both acute and chronic phases, AS produced more parasites in CBA than in C57 mice, whether alone or in mixture (Fig. 4.3A, B; Strain: $F_{1,13}=125, p<0.001$; $F_{1,13}=52, p<0.001$ respectively).

Figure 4.1. Log parasite densities over time (mean ± 1 s.e.m.) for C57 mice (left) and CBA mice (right). The top row (A and B) shows AS and AJ parasite densities in mixed AS+AJ infections, the middle row (C and D) compares AS parasite densities in single AS infections and mixed AS+AJ infections, and the bottom row (E and F) compares AJ parasite densities in single AJ and mixed AS+AJ infections. All data points are based on 5 replicate mice, except for single AJ infections in C57 mice (4 mice after day 12), mixed AS+AJ infections in C57 mice (4 mice after day 7) and mixed AS+AJ infections in CBA mice (4 mice on day 11 and 3 mice from day 12 onwards). As the limit of detection was 100 parasites per μl blood, y-axes start at 2.
During the acute phase, AJ produced roughly the same numbers of parasites in mixed and single infections, in both C57 and CBA mice (Figures 1E, F and 3C; Treatment: F_{1,12}=1.42, p=0.25), thus showing that, unlike AS, AJ did not suffer from competition. Like AS, it produced more parasites in CBA than in C57 mice (Strain: F_{1,14}=27, p<0.001). During the chronic phase, AJ produced slightly fewer parasites in C57, but more in CBA mice than it did on its own (Figure 4.3D; Treatment x Strain: F_{1,12}=8.1, p=0.015). When analysing these numbers for CBA mice only, however, there was no difference between single and mixed
infections (Treatment: $F_{1,6}=2.7$, $p=0.15$), thus showing that, unlike AS, AJ did not experience facilitation during this phase.

**Figure 4.3.** Total numbers of parasites produced during the acute phase (left) and chronic phase of the infection (right) for C57 and CBA mice (mean ± 1 se.m.). Plotted points are AS parasite densities in single AS and mixed AS+AJ infections (A and B), AJ parasite densities in single AJ and mixed AS+AJ infections (C and D), and overall parasite numbers in single AS, single AJ, and mixed AS+AJ infections (E and F). All data points are based on 5 replicate mice, except for single AJ infections in C57 mice (4 mice), mixed AS+AJ infections in C57 mice (4 mice) and mixed AS+AJ infections in CBA mice (3 mice). * Value denotes a parasite density below detection.
In the follow-up experiment, I infected another five of both C57 and CBA mice with AS+AJ, and sampled these on day 6 and day 13 PI. In both mouse strains AS was present on day 6. By day 13, however, AS had disappeared below detectible levels in the peripheral blood of C57 mice (Figure 4.4A), but was still present in CBA mice (Figure 4.4B), actually overgrowing AJ at that time. These results thus confirmed the qualitatively different dynamics of mixed infections in C57 and CBA mice as observed in the main experiment.

![Figure 4.4](image)

**Figure 4.4.** Proportions of AS and AJ (mean + 1 s.e.m.) in mixed AS+AJ infections in C57 (A) or CBA mice (B) on days 6 and 13 post-infection. Data are based on five replicate mice in a follow-up experiment. * Value denotes a proportion of 0.

### 4.4.3. Overall parasite densities

During the acute phase, infections in C57 mice consisted of fewer parasites than those in CBA mice (Figure 3E; Strain: $F_{1,20}=110$, $p<0.001$). In both mouse strains, AJ and AS+AJ infections produced more parasites during the acute phase than did AS infections (Treatment: $F_{2,20}=74$, $p<0.001$).

During the chronic phase, AJ and AS+AJ infections produced more parasites than AS (Figure 4.3F; Treatment: $F_{2,20}=28$, $p<0.001$). AJ and AS+AJ infections also differed from each other, but differently in the two mouse genotypes: in C57 mice AS+AJ infections produced slightly fewer parasites than AJ infections, whereas in CBA mice they produced more (Figure 4.3F; Treatment x Strain: $F_{2,20}=11$, $p=0.001$). Due to these higher numbers in CBA mice during the chronic phase, AS+AJ infections produced more parasites in CBA mice over the whole of the infection than did AJ infections (Treatment x Strain: $F_{2,24}=26$, $p<0.001$).
4.4.4. Gametocyte densities

In C57 mice, most gametocytes were produced during the first 9 days of the infection, with AS having lower densities than AJ and AS+AJ (Figure 4.5A; Treatment: $F_{2,27}=11.71$, $p<0.001$), whereas in CBA mice most gametocytes were produced after day 9 PI, when AJ produced fewer gametocytes than AS and AS+AJ (Figure 4.5B; Treatment: $F_{2,20}=5.0$, $p=0.017$; Strain: $F_{1,20}=6.04$, $p=0.023$; Treatment x Strain: $F_{2,20}=6.1$, $p=0.009$). In C57 mice, gametocyte peaks in mixed infections mostly resembled those of AJ infections, the numerically dominant clone during that period (Figures 4.5A, 4.1A). In CBA mice, mixed infections produced gametocyte dynamics that did not resemble gametocyte production in either AS or AJ single infections, but which peaked during a period when both clones were present in high numbers (Figures 4.5B, 4.1B). Summarised over the whole infection, AJ produced most gametocytes in C57 mice, but fewest in CBA mice, in which AS produced most (Strain: $F_{1,20}=8.6$, $p=0.008$; Treatment x Strain: $F_{2,20}=8.3$, $p=0.002$).

![Graphs showing log gametocyte densities over time for C57 (A) and CBA (B) mice infected with single AS, single AJ, or mixed AS+AJ infections. Shown are overall (AS+AJ) numbers of gametocytes only, as the real-time quantitative PCR cannot be used to distinguish AS and AJ gametocytes. All data points are based on 5 replicate mice, except for single AJ infections in C57 mice (4 mice after day 12), mixed AS+AJ infections in C57 mice (4 mice after day 7) and mixed AS+AJ infections in CBA mice (4 mice on day 11 and 3 mice from day 12 onwards). As the limit of detection was 100 gametocytes per µl blood, y-axes start at 2.]

Figure 4.5. Log gametocyte densities over time (mean ± s.e.m.) for C57 (A) and CBA mice (B) infected with single AS, single AJ, or mixed AS+AJ infections. Shown are overall (AS+AJ) numbers of gametocytes only, as the real-time quantitative PCR cannot be used to distinguish AS and AJ gametocytes. All data points are based on 5 replicate mice, except for single AJ infections in C57 mice (4 mice after day 12), mixed AS+AJ infections in C57 mice (4 mice after day 7) and mixed AS+AJ infections in CBA mice (4 mice on day 11 and 3 mice from day 12 onwards). As the limit of detection was 100 gametocytes per µl blood, y-axes start at 2.
4.5. Discussion

The results presented here show that host heterogeneity affects the outcome of in-host competition between pathogen strains. In C57 mice, the avirulent clone disappeared below detectible levels from the peripheral blood after ten days of the infection and produced far fewer parasites than it would have done on its own. This competitive suppression almost certainly reduced the clone's transmission potential: the period of greatest gametocyte production in single infections occurred after day 10 p.i. (Figure 4.5A). As the avirulent clone never reappeared over the course of the study, it seems that it was competitively excluded, something that has not been observed before in this species (Taylor et al. 1997b; Taylor & Read 1998; Read et al. 2002). In contrast, the virulent clone did not suffer at all from competition in mixed infections.

In CBA mice, the avirulent clone was also competitively suppressed during the acute phase, but it persisted and went on to produce more parasites during the chronic phase than it would have done on its own. Thus, in CBA mice, competitive suppression gave way to facilitation. This may have resulted in enhanced transmission: the density of the avirulent clone peaked around day 20 p.i. and coincided with a large gametocyte peak (Figures 1D and 5B). Whether the presence of the more virulent clone actually enhanced the overall fitness of the avirulent clone would depend on the host mortality rate induced by the virulent clone, and on how many of the gametocytes produced around this time were of the virulent clone.

This experiment, with just two parasite clones and two mouse strains, generated the whole spectrum of outcomes currently captured in a range of different mixed-strain models of virulence evolution. In extreme co-infection models, clone dynamics are unaffected by the presence of other clones (e.g. May & Nowak 1995; Leung & Forbes 1998); in these experiments, this was so for clone AJ in both mouse strains. Coexistence with competitive suppression of at least one clone, as envisaged in other models (e.g. Sasaki & Iwasa 1991; Frank 1992, 1996; Herre 1995; Van Baalen & Sabelis 1995) occurred within CBA mice. Finally, the competitive exclusion of AS from C57 mice before much transmission stage production occurred, bears substantial resemblance to superinfection models (which are perhaps better called superseding infection models: Van Baalen & Sabelis 1995). These models allow no coexistence and postulate that competitive suppression instantly reduces transmission to zero (e.g. Levin & Pimentel 1981; Bremermann & Pickering 1983; Bremermann & Thieme 1989; Knolle 1989; Nowak & May 1994; Leung & Forbes 1998; Gandon et al. 2002). Thus, it may prove difficult to capture the real world complexities of a
disease like malaria (and others: Hood 2003) in models that assume that the outcome of competition is independent of environmental conditions, such as host genotype.

A general conclusion of the above models is that mixed-clone infections will generate selection for increased virulence. However, the precise details of competition will affect the transient evolutionary dynamics, and magnitude of standing virulence in a population, which is what is relevant to animal and human health. Competitive exclusion of avirulent clones, for instance, will affect potential evolutionary trajectories, rates of evolution and average levels of virulence in a population.

Several recent studies have shown that parasite virulence depends on host genotype (e.g. Ebert & Hamilton 1996; Ebert et al. 1998; Imhoof & Schmid-Hempel 1998a; 1998b; Carius et al. 2001; Mackinnon et al. 2002b), and theory has shown that such host-genotype dependence of virulence can explain why polymorphisms in growth rate and virulence are maintained (Regoes et al. 2000). If the outcome of competition is as dependent on host genotype as the expression of virulence itself, this could contribute to the maintenance of growth rate and virulence polymorphisms in the parasite population as well. Wille et al. (2002) also suggested this when they found that some strains of the endophyte Epichloë bromicola competitively excluded other strains on one genotype of its host Bromus erectus, but not on another.

Clone AS was competitively suppressed by AJ, except during the chronic phase of infections in CBA mice, where AS did better than it did on its own. This could be due to a combination of strain-specific immunity and antigenic variation. In two of the surviving CBA mice AS overgrew AJ after it had been suppressed earlier (Figures 3B, C). This might suggest that the immune system had been focusing on the clone that had produced the highest number of parasites up till then (AJ), giving AS some advantage. Strain-specific immunity is certainly well known in P. chabaudi (e.g. Jarra & Brown 1985; Buckling & Read 2001, R. Carter unpublished results), and facilitation of one pathogen due to the immune response focusing on another has also been suggested for fungal species infecting leaf-cutter ants (Hughes & Boomsma 2004). P. chabaudi is also known to produce antigenic variants at high rates (McLean et al. 1982; McLean et al. 1990; Brannan et al. 1994; Phillips et al. 1997). Novel antigenic variants escape variant-specific host responses, and when there are more clones in an infection, each generating variants, it seems likely that the immune response may be less efficient at controlling the infection. A combination of strain-specific immunity and antigenic variation has also been suggested to play a role in mixed-species and mixed-strain infections of human Plasmodia (Bruce et al. 2000).
Why did C57 and CBA mice show such different dynamics and outcomes of competition? One important possibility is a strain difference in the efficacy of immune control. Mouse strains differ considerably in their resistance to *P. chabaudi* infections (Stevenson *et al.* 1982). Resistance is associated with reduced parasite densities, and appears to be a complex genetic trait, involving many genes affecting immunity and red blood cell production and characteristics (Stevenson *et al.* 1982; Yap *et al.* 1994; Fortin *et al.* 2002). Parasite densities were higher in CBA mice (Figure 4.3E), and such higher densities could reduce the chance of stochastic loss and increase the chance of producing antigenic escape variants.

If differences in overall immune control do explain the strain differences in the outcome of competition, one would expect to find coexistence of the two parasite clones in C57 mice that were made more susceptible, for example by administration of anti-IL12 monoclonal antibodies (Yap *et al.* 1994). Conversely, clone AS would be expected to disappear in CBA mice that were more resistant, for example by artificially increasing their IL12 levels (Yap *et al.* 1994).

If overall resistance does affect the outcome of within-host competition, then differences are not only expected between host genotypes, but also between individuals that differ in their sex, nutritional or health status, vaccination status, or any other factors that influence resistance. Semi-immune mice are much more resistant to *P. chabaudi* infection than naive mice (Buckling & Read 2001; Mackinnon & Read 2003). Extrapolating from the results presented here, it would be expected that only the virulent clone would survive in such vaccinated hosts. It is important to test this, because this implies that vaccination could increase the frequency of virulent clones in a population, thus leading to unforeseen consequences of vaccination (Gandon *et al.* 2001; Gandon *et al.* 2003; Read *et al.* 2004).
5. Timing of infection affects strength of competition and competitive dominance in genetically diverse malaria infections

To be submitted as: De Roode, J. C., Helsinki, M., Anwar, M. A. & Read, A. F. Timing of infection affects strength of competition and competitive dominance in genetically diverse malaria infections.

5.1. Abstract

Parasite infections often consist of different strains of the same species. Within-host competition between such strains shapes the evolution of drug resistance and disease severity. Although such evolution has been thoroughly investigated using mathematical models, biological factors that cause competition and determine competitive dominance of competing clones are still poorly understood. This study investigates how relative timing of infection of parasite strains affects competition between them. Experiments were carried out using two pairs of genetically distinct clones of the rodent malaria *Plasmodium chabaudi* in laboratory mice: these clones were either infected simultaneously or 3 or 11 days apart. When clones infected mice first, they generally did not suffer from competition, whereas they did when infecting mice at the same time as, or days after, their competitor. When infecting mice 11 days after their competitor, competitive suppression was even greater than when infecting them after 3 days. These results show that timing of infection is a major determinant of the outcome of within-host competition: relative timing does not only change the strength of competition, but also relative competitive dominance, so that competitively inferior clones can be competitively superior when infecting hosts first. The results are discussed in the light of strain-specific immunity, with implications for virulence evolution theory.

5.2. Introduction

Humans and animals often become infected with different strains of the same parasitic species (Read & Taylor 2001), so that parasite strains have to compete for limited amounts of resources and interact through strain-transcending immunity. Such competition can be intense (Read & Taylor 2001), and has many important medical consequences. Within-host competition is a major determinant in the evolution and epidemiology of drug resistance, for example (Hastings & D'Alessandro 2000; De Roode et al. 2004a [Chapter 7, Thesis Appendix]), because the evolutionary success of drug-resistant mutants crucially depends on whether it is able to out-compete drug-sensitive wild-type strains. The same is true for
vaccine-escape mutants (e.g. Kew et al. 2002): their evolution and spread through the population will depend on their competitiveness with attenuated pathogen forms in vaccinated hosts and wild-type strains in others (Bull 1994). Finally, it is asserted that competition between parasite strains selects for increased virulence and thus more severe disease: this is because virulent genotypes are assumed to out-compete prudent genotypes, resulting in a selective advantage of high virulence (e.g. Levin & Pimentel 1981; Bremermann & Pickering 1983; Van Baalen & Sabelis 1995; Frank 1996).

The epidemiological and evolutionary consequences of within-host competition have been thoroughly investigated theoretically, but which biological factors exactly cause competition and determine the success of competing clones, is mostly unknown. Thus, in many models it is assumed that parasites with higher growth rates, and inflicting more damage to the host, are competitively superior to less harmful parasites, yet experimental evidence to support this claim is practically absent (but see Chapter 3). Experiments on mixed infections generally show that competitive success of competing clones cannot be easily predicted from their growth rates in single infections (Nakamura et al. 1992; Hodgson et al. 2004), and that host genotype (Wille et al. 2002; De Roode et al. 2004b) and timing of infection (Orcutt & Schaedler 1973; Morrison et al. 1982; Duval-Iflah et al. 1983; Allaker et al. 1988; Barrow & Page 2000) also play an important role. Colonisation of a host before a competitor (of the same or a different species) can prevent (Hood 2003) or drastically reduce the growth of the competitor, even if that competitor would be competitively superior in simultaneous infections (e.g. Ishii et al. 2002; Thomas et al. 2003).

In human malaria, mixed infections are extremely common, with prevalence reaching over 80% in high-transmission areas (e.g. Babiker et al. 1999; Konaté et al. 1999; Magesa et al. 2002). These mixed infections occur because hosts become infected with different strains simultaneously (i.e. co-infection) or sequentially (i.e. super-infection). Co-infection occurs because meiotic recombination and random mating occur frequently in malaria populations (Babiker et al. 1994; Babiker et al. 1995; Paul et al. 1995; Conway et al. 1999), so that one mosquito bite can contain a range of genetically distinct genotypes (Conway et al. 1991; Druilhe et al. 1998). Super-infection is also common, and occurs because people are bitten by numerous mosquitoes injecting different malaria strains (Ntoumi et al. 1995; Daubersies et al. 1996; Arnot 1998; Basco & Ringwald 2001; Arez et al. 2003).

Whether malaria strains infect their host simultaneously or sequentially, will very likely affect the extent to which they suffer from competitive suppression. This is because malaria parasites replicate exponentially during an infection, causing severe anaemia (Cox 1988;
Jakeman et al. 1999; Menendez et al. 2000), and inducing strong anti-parasitic immune responses (Taylor-Robinson 1995; Li et al. 2001; Artavanis-Tsakonas et al. 2003); therefore strains infecting a host already infected with another strain will have fewer resources and encounter a stronger immune response than strains infecting naive hosts (e.g. Hellriegel 1992).

To study whether sequential infection indeed causes strong competitive suppression, two experiments were carried out using the rodent malaria Plasmodium chabaudi. In the first experiment, two clones denoted AS and CB were used, and mice were infected with them simultaneously or 3 days apart. It was also studied how the initial ratio affected competition: thus, another group of mice were infected with an inoculum consisting of 100CB:1AS. The second experiment used clones AS(pyr1A) and AJ: mice were infected with these clones simultaneously or 3 or 11 days apart. Thus, this experiment was designed to test whether super-infection causes stronger competition than co-infection, and also whether competitive suppression becomes greater the later the second clone infects the host after the first clone.

5.3. Material and methods

5.3.1. Parasites and hosts

Two main experiments were conducted, each making use of a different pair of Plasmodium chabaudi clones. Experiment 1 used clones AS and CB, while experiment 2 used AS(pyr1A) and AJ. AJ, AS and CB were originally cloned from wild parasite isolates from thicket rats in the Central African Republic (Beale et al. 1978); AS(pyr1A) was then derived from AS by selection for resistance against the drug pyrimethamine (Walliker et al. 1975).

Hosts were nine weeks old C57Bl/6J female inbred mice (Harlan, England), kept in a reversed (experiment 1) or normal (experiment 2) 12 L : 12 D cycle; they were kept and fed as described (De Roode et al. 2004b [Thesis Appendix]).

5.3.2. Experiment 1: AS and CB

The first experiment, studying competition between clones AS and CB, consisted of three sub-experiments. The first sub-experiment (‘co-infection’) tested whether parasite clones suffer from competition when infecting the same host simultaneously: 6 mice were infected i.v. with $10^4$ AS parasites, 6 mice with $10^4$ CB parasites, and 24 mice with $10^4$ AS + $10^4$ CB. The second sub-experiment (‘super-infection’) then asked whether competition is stronger
for clones that infect mice already infected with another clone: 2 groups of 7 mice were infected with either $10^4$ AS or CB parasites alone; 11 mice were infected with $10^4$ AS on day 0 and then with $10^4$ CB on day 3, and another 10 mice were infected with $10^4$ CB on day 0 and $10^4$ AS on day 3. The third sub-experiment ('100CB:1AS') asked whether the initial ratio of co-infecting clones affects the strength of competition, with mixed infections consisting of $10^4$ CB and $10^2$ AS parasites: thus, 3 mice were infected with $10^2$ AS parasites, 3 with $10^4$ CB and 3 with $10^4$ CB and $10^2$ AS parasites.

Mouse red blood cell densities were determined by counting the number of cells in a volume of 5 μl tail-blood using flow cytometry (Beckman Coulter). Thin blood smears were taken to determine parasitaemias (here defined as the percentage of red blood cells infected with parasites) using 1000x microscopy; parasite densities were then calculated as the product of red blood cell densities and parasitaemias taken on the same day. For mixed infections, numbers of AS and CB parasites were determined using immuno-fluorescent antibody assays (IFAs). The monoclonal antibodies used were H3, reacting specifically to CB, and B4 and B15, reacting specifically to AS (Boyle et al. 1982; McLean et al. 1991); IFA protocols were as described elsewhere (Taylor et al. 1997a). These assays give ratios of the two infecting clones, which – multiplied by the overall parasite density – give the clonal parasite densities on a given day. As IFAs can give biased estimates of ratios (Taylor et al. 1997a), it was necessary to carry out controlled experiments to compare observed AS:CB ratios with those expected in artificial mixtures, and to correct the estimated ratios before using them to calculate AS and CB densities (see Thesis Appendix). Measurements were taken three times a week until day 35 ('co-infection'), 57 ('super-infection') or 41 ('100CB:1AS') post-infection.

5.3.3. Experiment 2: AS(pyr1A) and AJ

Using a different pair of P. chabaudi clones, the second experiment asked whether clones suffered more from competition the later they infected a host already infected with another clone. On days 0, 3 and 11, groups of 5 mice were infected i.p. with either $10^6$ AS(pyr1A) or $10^6$ AJ parasites alone, while another group of mice were infected with both $10^6$ AS(pyr1A) and $10^6$ AJ parasites simultaneously. Mice in two further treatment groups were infected with $10^6$ AS(pyr1A) parasites on day 0 and then with $10^6$ AJ parasites on days 3 or 11, while the final two groups of mice were infected with $10^6$ AJ parasites on day 0 and then with $10^6$ AS(pyr1A) parasites on days 3 or 11.
Mouse red blood cell densities were determined using flow cytometry (Beckman Coulter) of 2 µl of tail blood. Thin blood smears were taken and parasitaemias calculated using microscopy (Chapter 4, De Roode et al. 2004b [Thesis Appendix]): parasite densities were then calculated as the product of red blood cell densities and parasitaemias taken on the same day. In mixed infections, AS(pyr1A) and AJ were distinguished and quantified using strain-specific real-time quantitative PCR (Chapters 2, 4, Cheesman et al. 2003; De Roode et al. 2004b [Thesis Appendix]). Measures were taken daily up till 12 days, after which they were taken every 2 days until day 35 post-infection (for mice infected with one clone or with a mixture simultaneously), or until 35 days after infection with the second clone (for the other mixed infections).

5.3.4. Statistical analysis

All analyses were carried out using ANOVA’s in Minitab (v. 13.30, Minitab Inc.), log transforming the data when necessary to meet normality and homogeneity of variance assumptions. Total numbers of parasites of specific clones produced over the whole infection were calculated: *P. chabaudi* parasites have a 24 hour replication cycle, so that the sum of the daily parasite numbers gives the number produced over the entire infection. When mice were monitored for different numbers of days, and it was necessary to compare these mice, I calculated parasite densities over the shortest number of days: for example, even though mice in the super-infection experiment (Experiment 1) were monitored for 57 days, I only used data over the first 35 days when comparing them to mice in the co-infection experiment (monitored for 35 days only).
5.4. Results

Several mice died, while others had delayed and substantially reduced parasite densities, suggesting that they received lower parasite densities than anticipated (Timms et al. 2001). All these mice were excluded from the analysis; numbers of mice on which the analysis was based, are shown in Table 5.1.

Table 5.1. Numbers of mice that were analysed from each treatment group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Experiment 1</th>
<th>Experiment 2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>co-infection exp.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS alone</td>
<td>5</td>
<td>AS(pyr1A) alone day 0</td>
</tr>
<tr>
<td>CB alone</td>
<td>3</td>
<td>AS(pyr1A) alone day 3</td>
</tr>
<tr>
<td>AS(pyr1A)+CB</td>
<td>19</td>
<td>AS(pyr1A) alone day 11</td>
</tr>
<tr>
<td><strong>super-infection exp.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS(pyr1A) alone</td>
<td>6</td>
<td>AJ alone day 0</td>
</tr>
<tr>
<td>CB alone</td>
<td>3</td>
<td>AJ alone day 3</td>
</tr>
<tr>
<td>AS(pyr1A) day 0, CB day 3</td>
<td>7</td>
<td>AS(pyr1A)+AJ day 0</td>
</tr>
<tr>
<td>CB day 0, CB day 3</td>
<td>5</td>
<td>AS(pyr1A) day 0, AJ day 3</td>
</tr>
<tr>
<td><strong>100CB:1AS(pyr1A) exp.</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AS(pyr1A) alone</td>
<td>2</td>
<td>AJ day 0, AS(pyr1A) day 3</td>
</tr>
<tr>
<td>CB alone</td>
<td>2</td>
<td>AJ day 0, AS(pyr1A) day 11</td>
</tr>
<tr>
<td>AS(pyr1A)+CB</td>
<td>3</td>
<td></td>
</tr>
</tbody>
</table>

5.4.1. Experiment 1: AS and CB

5.4.1.1. AS and CB dynamics through time

In mice simultaneously infected with AS and CB, both parasite clones produced about half of the total parasite population: AS had a slightly higher peak than CB around day 10, while CB had a higher peak than AS around day 16 (Figure 5.1A). Compared to single infections, both AS and CB suffered from competition, producing about 3 million fewer parasites in mixtures than they did alone (Figures 5.1B, C).

Compared to infections initiated with AS and CB simultaneously, parasite dynamics were markedly different in mice infected sequentially. Thus, in mice infected with AS on day 0 and CB 3 days later, AS dominated until day 16 (Figure 5.1D), and competitively suppressed CB (Figure 5.1F) during this time. AS parasite numbers then plummeted and were suppressed (Figure 5.1E), while CB densities rose sharply, so that CB started to dominate the infection: it remained the dominant clone for the rest of the infection (Figure 5.1D).
Figure 5.1. Log AS and CB parasite densities over time (mean ± 1 s.e.m.) in experiment 1. The left column shows AS and CB densities in mixed infections, the middle column compares AS densities in single and mixed infections, and the right column compares CB densities in single and mixed infections. (A-C): mice were infected with AS and CB simultaneously; (D-F): mice were infected with CB 3 days after AS; (G-I): mice were infected with AS 3 days after CB; (J-L): mice were infected with AS and CB simultaneously, with an inoculum of 100CB:1AS. The threshold density at which parasites could be detected was around 100/μl mouse blood, so that y-axes start at 2. Curves in panels J-L are interrupted, because no samples were taken between days 22 and 35. See table 5.1 for the numbers of mice on which data-points are based.
In mice infected with CB first (Figure 5.1G), AS was competitively suppressed for almost the entirety of the infection (Figure 5.1H), whereas CB was not (Figure 5.1I). In contrast with CB, which managed to overgrow AS in infections where AS was inoculated first, AS never managed to overgrow CB in infections where CB was inoculated first. In fact, AS parasite numbers followed the same up- and downward dynamics as CB in these infections, so that whenever CB numbers decreased, AS numbers decreased with them (Figure 5.1G).

Mixed infections initiated with a 100CB:1AS inoculum showed very similar dynamics as those initiated with CB 3 days before AS. CB was the dominant clone in these infections (Figure 5.1J), and whenever CB densities decreased, AS densities decreased with them. The result was that AS suffered greatly from competition in these infections (Figure 5.1K), whereas CB did not (Figure 5.1L).

5.4.1.2. AS and CB parasite densities

The extent to which AS and CB suffered from competition in mixed infections depended on when they infected their host: when infecting mice first, neither AS nor CB suffered a reduction in parasite numbers, but when infecting the same host simultaneously or 3 days after their competitor, they did (Figure 5.2). AS suffered a 37 (±24) % (mean and 95%
confidence interval) reduction in parasite numbers in mice simultaneously infected with CB ($F_{1,22}=10$, $p=0.004$), and an even greater reduction (95±35%) when infecting mice 3 days after CB (Figure 5.2A; single/mixed x day interaction: $F_{1,31}=68$, $p<0.001$). CB suffered a 40 (±30) % reduction in parasite numbers in mice simultaneously infected with AS ($F_{1,20}=7.9$, $p=0.011$), but did not suffer significantly more when infecting mice 3 days after AS (58±28% reduction; Figure 5.2B; single/mixed x day interaction: $F_{1,28}=1.3$, $p=0.26$).

In mixed infections initiated with a ratio of 100CB:1AS, AS suffered greatly from competition (a 97±88% reduction of parasite numbers), whereas CB did not (Figure 5.3; single/mixed x clone interaction: $F_{1,7}=13.2$, $p=0.008$). The competitive suppression of AS in these infections was as high as in infections in which AS was inoculated 3 days after CB.

![Figure 5.3. Total numbers of AS and CB produced over the whole infection in single and mixed infections (100CB:1AS) in experiment 1 (mean ± 1 s.e.m.). See table 5.1 for the numbers of mice on which data-points are based.](image)

### 5.4.1.3. Overall parasite densities

Mice infected with AS and CB simultaneously experienced as many parasites as infections consisting of AS or CB alone (Figure 5.4A; $F_{2,24}=1.1$, $p=0.36$), implying that competition was a direct effect of these clones having to share the 'available number of parasites' between them. Mice infected with CB alone also had as many parasites as those infected with AS and CB 3 days apart (Figure 5.4C; Tukey: $p>0.05$); but mice infected with AS alone had fewer parasites than those infected with AS first and then CB. Thus, because CB was able to overgrow AS, these mice experienced heavier parasite burdens than mice infected with AS only (Figure 5.4C; Tukey: $p=0.02$). Finally, infections initiated with 100CB:1AS
produced as many parasites as AS single infections (Tukey: p>0.05), explaining that AS suffered from competition in these mixed infections in which the available number of parasites had to be shared with CB.

![Graph showing parasite numbers](image)

**Figure 5.4.** Overall parasite numbers over the whole infection (mean ± 1 s.e.m.) for infections in experiments 1 and 2. In experiment 1, mice infected with AS and CB simultaneously had as many parasites as those infected with AS or CB alone (A; p>0.05); mice infected with CB 3 days after AS had higher parasite densities than those with AS alone (C; p<0.05), but as many as those with CB alone (C; p>0.05). In experiment 2, mice infected with AS(pyrlA) and AJ simultaneously had higher parasite densities than mice infected with AS(pyrlA) alone (B; p<0.05) but as many as those with AJ alone (B; p>0.05); mice infected with AJ 3 or 11 days after AS(pyrlA) had lower parasite densities than mice infected with AJ alone (D; p<0.05) and as many as those infected with AS(pyrlA) alone (D; p>0.05). Numbers of mice on which data-points are based, are given in Table 5.1.
5.4.2. Experiment 2: AS(pyr1A) and AJ

Among the single-clone infections, mice infected with AJ lost more red blood cells than those infected with AS(pyr1A) (Clone: $F_{1,24}=21$, $p<0.001$), confirming that AJ was more virulent than AS(pyr1A) (Chapters 3, 4, De Roode et al. 2004b [Thesis Appendix]).

5.4.2.1. AS(pyr1A) and AJ dynamics through time

With a different set of clones, and different techniques for distinguishing them, the second experiment showed that the longer the time between infection and super-infection, the more the super-infecting clone suffered from competition. As found previously (Chapters 3 and 4, De Roode et al. 2004b [Thesis Appendix]), in mice infected simultaneously with AS(pyr1A) and AJ, AJ was the dominant clone for the entirety of the infection, with AS(pyr1A) disappearing below detectible levels within 11 days (Figure 5.5A). Compared to single infections, AS(pyr1A) produced far fewer parasites in these mixed infections than in single infections (Figure 5.5B), whereas AJ did not (Figure 5.5C).

AJ did suffer from competition only when it infected mice 3 or 11 days after AS(pyr1A) (Figures 5.5F, I). However, even when inoculated after AS(pyr1A), it always became the dominant clone later on in the infections, overgrowing AS(pyr1A) around day 10 in mice which it infected 3 days after AS(pyr1A) (Figure 5.5D), and around day 25 in mice which it infected 11 days after AS(pyr1A) (Figure 5.5G). Because AJ was always able to overgrow AS(pyr1A) in these infections, AS(pyr1A) was competitively suppressed by AJ, even when it was inoculated 3 days before AJ (Figure 5.5E).

In contrast with AJ, AS(pyr1A) never became the dominant clone in mice infected with AJ 3 or 11 days before AS (Figures 5.5J, M), and it suffered greatly from competitive suppression (Figures 5.5K, N). Competitive suppression was so strong that AS(pyr1A) was only detected in 3 out of the 5 mice which it infected 11 days after AJ. AJ did not suffer at all from competition when it was the first clone to infect mice (Figures 5.5L, O).
Figure 5.5. Log parasite densities over time for infections in experiment 2 (mean ± 1 s.e.m.). The left column shows AS(pyr1A) and AJ densities in mixed infections, while the middle column compares AS(pyr1A) densities in single and mixed infections and the right column compares AJ densities in single and mixed infections. (A-C): mice were infected with AS(pyr1A) and AJ simultaneously; (D-F): mice were infected with AJ 3 days after AS(pyr1A); (G-I): mice were infected with AJ 11 days after AS(pyr1A); (J-L): mice were infected with AS(pyr1A) 3 days after AJ; (M-O): mice were infected with AS(pyr1A) 11 days after AJ. The threshold density at which parasites could be detected was around 100/µl mouse blood, so that y-axes start at 2. See table 5.1 for the numbers of mice on which data-points are based.
5.4.2.2. AS(pyr1A) and AJ parasite densities

AS(pyr1A) produced as many parasites in mixed infections when inoculated 11 days before AJ as it did alone, but it produced fewer parasites when it infected mice 3 days before AJ (38±34% reduction), on the same day as AJ (79±37% reduction) or 3 or 11 days after AJ (99±44% and 99±45% reduction respectively; Figure 5.6A). Thus, the later AS(pyr1A) infected mice with respect to AJ, the more it suffered from competition (single/mixed x day interaction: F_{4,37}=4.8, p=0.004).

In contrast with AS(pyr1A), AJ produced as many parasites in mice that it infected 11 or 3 days before AS(pyr1A) or on the same day (Figure 5.6B); it did suffer from competition, however, when infecting mice after AS(pyr1A), and more so when infecting 11 days (97±12% reduction) than 3 days (88±34% reduction) later (single/mixed x day interaction: F_{4,28}=11.5, p<0.001). Thus, both clones suffered more from competition the later they infected mice already infected with the other clone, but over the whole range of infections, AS(pyr1A) suffered more than AJ.

![Graph showing parasite densities for AS(pyr1A) and AJ in single and mixed infections](image)

**Figure 5.6.** Total numbers of AS(pyr1A) (A) and AJ (B) produced over the whole infection in single and mixed infections in experiment 2 (mean + 1 s.e.m.). See table 5.1 for the numbers of mice on which data-points are based.
5.4.2.3. Overall parasite densities

Among the infections initiated on day 0, there were considerable differences in the overall parasite numbers produced per infection (Treatment: $F_{6,20}=11.7$, $p<0.001$), with single infections of AS(pyr1A) producing fewer parasites than single infections of AJ (Figure 5.4B; Tukey: $p=0.02$). Mice infected with AS(pyr1A) and AJ simultaneously and those with AJ first and then AS(pyr1A) had as many parasites as those infected with AJ alone (Figures 5.4B, D; Tukey: $p>0.05$ for all), while those infected with AS(pyr1A) first and then with AJ had as many as mice infected with AS(pyr1A) alone (Figure 5.4D; Tukey: $p>0.05$ for both); these latter mice thus experienced fewer parasites during an infection than those infected with AJ alone (Tukey: $p<0.001$ for day 3 and $p=0.02$ for day 11), showing that prior infection with AS(pyr1A) reduced the number of parasites in these mice (Figure 5.4D).

5.5. Discussion

These results show that timing of infection is a major determinant of the outcome of within-host competition. Clones did generally not suffer from competition when they infected mice first, but did when they infected mice simultaneously with, or after, their competitor. The later a clone was inoculated after another, the more it suffered from competition. Relative timing of infection could as such turn around competitive hierarchies, so that clones that were competitive inferior in simultaneous co-infections, became competitively dominant when inoculated first. The degree to which clones suffered from competition, however, was also dependent on genetic background: thus, in experiment 2, AJ was always able to overgrow AS(pyr1A) in mice which it infected later, whereas AS(pyr1A) never became dominant in mice already infected with AJ. Finally, it was shown that an initial ratio of 100CB:1AS resulted in similar clone dynamics and competition as seen in infections where AS infected mice 3 days after CB.

Why did clones generally suffer more from competitive suppression by an already resident clone than by one inoculated simultaneously or later? Within-host competition between malaria clones is likely the result of a combination of resource competition and strain-transcending immunity (e.g. Richie 1988; Hellriegel 1992; Snounou et al. 1992; Read & Taylor 2000; 2001; De Roode et al. 2003 [Thesis Appendix]; Haydon et al. 2003). During an infection, malaria parasites replicate exponentially, reaching parasite densities exceeding millions of parasites per ml blood between 8 and 10 days into the infection (Figures 5.1, 5.5). This exponential growth is followed by an exponential collapse of parasite numbers, which is the result of both parasite destruction of red blood cells and immune clearance of infected
and uninfected red blood cells (e.g. Cox 1988; Hellriegel 1992; Yap & Stevenson 1994; Taylor-Robinson 1995; Jakeman et al. 1999; Menendez et al. 2000; Li et al. 2001). Thus, parasite clones infecting mice simultaneously must divide the available red blood cells and many other resources, such as blood glucose, between them and will be affected by cross-reactive immune responses induced against them. Parasites inoculated 3 days after their competitor should then have less time to grow up to high numbers before conditions become unfavourable; this was also confirmed by mixed infections initiated with 100CB:1AS, in which AS did not have enough time to replicate extensively before resources ran out and immunity came into place. Clones inoculated 11 days after their competitor should suffer even more competitive suppression, as they infect a host severely depleted of red blood cells and other resources and with strong immune responses in place (Hellriegel 1992).

The degree to which clones suffer from competition will also depend on how specific immune responses are. Clearance of parasites during the acute phase of infection (i.e. the period until parasites have collapsed after their initial exponential rise) probably involves interferon-gamma (IFN-γ), nitric oxide and reactive oxygen species (Taylor-Robinson 1995; Li et al. 2001). All these molecules act non-specifically, so that when induced by one clone they will also affect another. Strain-specific clearance mechanisms (Jarra & Brown 1989; Snounou et al. 1989), dependent on specific antibodies (Mota et al. 1998), are also involved, however, so that clones that are controlled by very similar immune responses should affect each other more than clones that are controlled by very different responses.

The current experiments suggest that this is indeed the case. Several studies have shown that clones AS and CB are relatively antigenically distinct: some of these (Jarra & Brown 1989; Snounou et al. 1989) transfused AS-infected mice with CB-infected red blood cells, showing that CB parasites maintained patent levels when AS parasites were cleared (and vice versa); others immunised mice with AS or CB and then challenged these mice with AS or CB as the hetero- or homologous clone (Jarra et al. 1986), showing that protection was stronger against homologous rather than heterologous challenge. A general conclusion from these experiments is that immunity elicited against AS is much better at clearing AS than CB, and vice versa. In the infections described in the current study, CB densities rose while AS densities went down (Figure 5.1D), probably because the immune response was directed against AS, the first clone experienced by these mice; the fact that CB densities levelled off before increasing (between days 10 and 15: Figure 5.1D) was probably a result of a strong decrease of red blood cells and other resources induced by the presence of AS; with resources rising again, and immunity focusing on AS, CB could then grow up until it itself became the focus of strain-specific immunity. Because these mice, first infected with AS,
produced immune responses that were not protective against CB, they experienced more parasites overall than mice infected with AS alone (Figure 5.4C). Interestingly, similar effects were not seen in infections in which AS followed CB (Figure 5.1G): in these infections AS dynamics almost exactly followed CB dynamics, suggesting that immune responses focusing on CB also affected AS. This is in accordance with studies that have shown that immunity elicited by CB has a stronger effect on AS than immunity elicited by AS has on CB (Jarra et al. 1986). The result was that AS in these infections suffered a lot more from competition than it did in simultaneous co-infections with CB.

Unlike AS and CB, AS(pyr1A) and AJ are thought to be rather antigenically similar, so that immune responses directed against AS(pyr1A) or AJ are to a large degree cross-reactive; this was found in experiments in which mice were immunised with AS(pyr1A) and then challenged with AJ, or vice versa (S.J. Cheesman, pers. comm.). In the infections in the current study, where AJ came 3 days after AS(pyr1A), AJ managed to overgrow AS(pyr1A), but not to the same degree as CB did in mice already infected with AS (cf. Figures 5.1D and 5.5D); the result was that AJ suffered a lot more from competition in mice which it infected after AS(pyr1A) than in mice which it infected simultaneously. Also, mice that initiated immune responses against AS(pyr1A) were protected against AJ to the extent that they experienced lower parasite burdens than mice infected with AJ alone (Figure 5.4D). The asymmetry in these infections – with AJ being able to overgrow AS(pyr1A) when inoculated after AS(pyr1A), but not vice versa – could have been because immune responses against AJ had a stronger effect on AS(pyr1A) than immune responses against AS(pyr1A) had on AJ: hence the fact that AS(pyr1A) suffered most from mixed infections and AJ was able to become the dominant clone even in infections in which it arrived 11 days after AS(pyr1A). Alternatively, however, it is possible that AS(pyr1A) is more affected by low amounts of resources than AJ.

This study confirms the widely held belief that many malaria cases are caused by newly inoculated parasite clones against which there is no effective immunity (e.g. Lines & Armstrong 1992; Mercereau-Puijalon 1996; Smith et al. 1999b; Beck et al. 2001; Ofosu-Okyere et al. 2001; Kun et al. 2002; Cattamanchi et al. 2003). In malarious areas, people are very commonly infected with antigenically different malaria clones, and it is thought that these contribute to a build-up of protective immunity. Occasionally, however, a clone is encountered against which there is minimal immunity, so that this novel clone can grow up exponentially and cause disease. This situation is very similar to the mice infected with AS, which upon super-infection with the antigenically different clone CB experienced higher parasite burdens than those infected with AS alone. In case of the antigenically more similar
clones AS(pyr1A) and AJ, however, such effects were not seen: in fact mice infected with AS(pyr1A) were protected against AJ, so that they experienced lower parasite burdens than mice infected with AJ alone.

Virulence evolution theory generally assumes that in mixed parasite infections, virulent clones are competitively superior to prudent clones. Although some of these models envisage coexistence of different clones with competitive suppression of at least one of the clones (e.g. Sasaki & Iwasa 1991; Van Baalen & Sabelis 1995; Frank 1996), other models assume that infected hosts cannot be re-infected at all (Bremermann & Thieme 1989), or that virulent clones take over hosts already infected with less virulent clones, out-competing these clones instantly (e.g. Levin & Pimentel 1981; Nowak & May 1994). Clearly, these latter two scenarios receive little empirical support on the basis of this study: in experiment 2, where AJ was more virulent than AS(pyr1A), causing more anaemia in the mice it infected, it suffered greatly from competitive suppression. Even though it was the dominant clone in the end, it is very unlikely that it obtained more transmission from these mixed infections than the avirulent clone AS(pyr1A), because AJ parasite densities were between 2 and 3 orders of magnitude lower than they were in single AJ infections (Figures 5.5F, I); furthermore, most mosquito transmission in *P. chabaudi* infections occurs over the first 2 weeks (Chapter 6), so that the competitive suppression early on undoubtedly resulted in huge transmission losses for the virulent clone. Thus, where the avirulent parasite came first, it was competitively superior to the virulent clone, a result flatly contradicting evolutionary theory.

Such reversal of competitive dominance could explain how virulence polymorphisms are maintained in the field, and why avirulent parasites could persist (Mackinnon & Read 2004b). It must be noted, however, that although AS(pyr1A) was dominant in infections in which it was inoculated first, it suffered more from competition overall than AJ: thus, even in mice infected with AS(pyr1A) 3 days before AJ, and in mice simultaneously infected, did it suffer from competitive suppression, so that it did relatively worse over the whole range of infections that were studied, as such supporting evolutionary theory. How general this phenomenon is, however, remains to be seen. As this study clearly demonstrated, it is not only virulence and timing of infection that determine the outcome of competition, but also how antigenically diverse competing clones are. Thus, it is well possible that avirulent parasites could be so antigenically different from their virulent competitors, that they actually transmit at a higher rate from mixed infections when the immune system focuses primarily on the virulent clone (e.g. Almogy *et al.* 2002; Read *et al.* 2002; Hughes & Boomsma 2004).
6. Within-host competition between malaria parasites affects between-host fitness

6.1. Abstract

Mixed infections in malaria are common, and result in competition between co-infecting strains. Such competition has important implications for the evolution of virulence, the spread of drug resistance and the medical consequences of vaccine escape mutants. Evolutionary theory generally assumes that within-host competitiveness is directly related to transmission success, but experimental evidence for this claim is severely lacking. I studied the relationship between within-host competitiveness and transmission to the mosquito vector for parasites in mixed-clone infections of the rodent malaria Plasmodium chabaudi in laboratory mice. I tracked individual clones throughout the infection using real-time quantitative PCR and assessed transmission by feeding infected mice to mosquitoes. I present results that suggest a linear relationship between within-host competitiveness and relative transmission to the mosquito, so that competition reduces transmission. In addition, parasites transmitted at a much lower absolute rate from mixed than single infections. I suggest that this reduced transmission is due to transmission blocking immunity.

6.2. Introduction

Genetically diverse parasite infections are common (Read & Taylor 2001), and in malaria are the rule rather than the exception (e.g. Arnot 1998; Babiker et al. 1999). Mixed infections result in competition between co-infecting strains of the same parasite species, and such competition has at least three important medical consequences. First, competition is predicted to generate selection for increased virulence (Bremermann & Pickering 1983; Sasaki & Iwasa 1991; Van Baalen & Sabelis 1995; Frank 1996; Gandon 1998; Mosquera & Adler 1998). This is due to a process analogous to the 'the tragedy of the commons' (Hardin 1968), whereby competition favours virulent parasites that rapidly outgrow prudent parasites, even if this means that host life expectancy is reduced so that all parasites do worse. Second, competition between drug-resistant and -sensitive malaria parasite strains has been implicated as a main determinant in the spread of drug resistance (Hastings & D'Alessandro 2000), and it has been shown that interfering with such competition could artificially enhance the fitness of drug-resistant mutants and thus enhance the spread of resistance (Hastings & D'Alessandro 2000; Chapter 7, De Roode et al. 2004a [Thesis Appendix]). Third, where vaccines are used to control disease, it is well known that vaccine
escape mutants can arise and spread (e.g. Kew et al. 2002), and it is generally assumed that this is because of a competitive advantage of such mutants in vaccinated hosts (e.g. Bull 1994).

Even though these medical consequences of within-host competition have been thoroughly investigated mathematically, experimental evidence to support them is extremely scanty. One of the most important assumptions these models make is that there is a direct relationship between within-host competitiveness and transmission to the host population (i.e. fitness). Logical as this relationship may seem, experimental evidence to support this claim is scarce; and where there is experimental evidence, such a relationship is not at all obvious (e.g. Nakamura et al. 1992).

There are many biological mechanisms of parasite-host systems that could negate a direct relationship between within-host competitiveness and transmission. First, parasites could facultatively increase their relative investment in transmission in the presence of competitors, a mechanism well recognised in some theoretical studies (e.g. Van Baalen & Sabelis 1995). If this occurs, it is possible that competitively suppressed genotypes may transmit as well as, or even better than, dominant genotypes. Malaria parasites, for example, are known to increase their investment in transmission when conditions for asexual replication worsen (Paul et al. 2003) - for instance in response to sub-curative levels of anti-malaria drugs (Buckling et al. 1997) - and it is possible that they do likewise in response to the presence of competitors.

Second, immune responses are complex, and generating immunity against a mixture of parasite genotypes could be either more or less effective than generating immunity against an infection consisting of a single parasite. If the immune response generated against one genotype also affects other parasites, transmission of individual clones could be reduced. On the other hand, if immunity against a mixture of parasites is less effective (Taylor et al. 1998), or when immune responses against the different parasites occupying a mixed infection down-regulate each other (Cox 2001), overall transmission from mixed infections could be enhanced. It is an interesting possibility that under the latter scenario competitively suppressed clones could actually have an advantage if the immune system focuses on the more abundant dominant clone (De Roode et al. 2004b [Chapter 4, Thesis Appendix]; Hughes & Boomsma 2004), leaving the suppressed parasite to transmit freely for at least some period of time.

Third, vector-borne parasites could face trade-offs between competitiveness in different hosts: thus they may be the better competitor in their mammalian host, but the worse in the
invertebrate vector. Such between-host trade-offs certainly exist for virulence in *Schistosoma mansoni*, where strains with high virulence in the mouse host caused low virulence in their molluscan host (Davies *et al.* 2001); there was also no relationship between the virulence of a range of *Plasmodium chabaudi* clones in their mouse and mosquito hosts (Ferguson *et al.* 2003b). It is thus clear that a direct relationship between within-host competitiveness and transmission, as assumed by theoretical models, might not exist.

Here, I study the relationship between within-host competitiveness and transmission for *Plasmodium chabaudi* in laboratory mice. I study mixed infections consisting of a virulent and an avirulent parasite clone, in two different mouse strains. Asexual parasites were tracked throughout the mouse infection with clone-specific real-time quantitative PCR, while transmission was assessed by feeding mice to mosquitoes. In malaria, asexual haploid parasites circulate and replicate in the blood, and only a small subset of these form gametocytes, the stages that are transmitted to mosquitoes. In the mosquito mid-gut gametocytes mature into gametes, which fuse to form diploid zygotes (e.g. Sinden *et al.* 1996). These develop into oocysts on the mid-gut wall, in which genomic replication results in haploid sporozoites that travel to the salivary glands, from where they can infect a new mammalian host. Although parasite fitness is commonly defined as the number of secondary infections resulting from an infection (e.g. Anderson & May 1982; Frank 1996; Levin 1996), I used two surrogate measures of fitness: for each parasite clone, I determined both the proportions of mosquitoes that it infected and the numbers of parasites per infected mosquito.

### 6.3. Material and methods

#### 6.3.1. Parasites and hosts

I used two genetically distinct *Plasmodium chabaudi chabaudi* clones, denoted AS(pyr1A) and AJ, AS(pyr1A) being less virulent than AJ (De Roode *et al.* 2004b [Chapter 4, Thesis Appendix]). For simplicity AS(pyr1A) will be referred to as AS from hereon.

Vertebrate hosts were ten weeks old C57Bl/6J and CBA/Ca inbred female mice (Harlan, England), which were kept as described (De Roode *et al.* 2004b [Chapter 4, Thesis Appendix]), and which will be referred to as C57 and CBA from hereon.
Mosquito hosts were 2-5 days old female *Anopheles stephensi* mosquitoes, maintained on a glucose solution (10% glucose, 0.05% 4-Aminobenzoic acid) in an insectary at 24°C and 80% humidity and a 12 L : 12 D cycle.

### 6.3.2. Experimental design

The experiment consisted of 3 treatments for both C57 and CBA mice: infections with AS alone, infections with AJ alone, and mixed AS+AJ infections. Each treatment group had 10 mice at the start of the experiment, resulting in 60 mice in total. Mice infected with just AS or AJ received $10^6$ parasite-infected red blood cells, whereas mice infected with both clones received $2 \times 10^6$ parasites, made up of $10^6$ AS and $10^6$ AJ parasites (De Roode *et al.* 2004b [Chapter 4, Thesis Appendix]). On days 7, 14 and 21 post-infection (PI), 3 mice of each treatment group (2 mice for AJ in CBA mice on day 21 PI) were fed to 30 mosquitoes each; these days were chosen because in a previous experiment (De Roode *et al.* 2004b [Chapter 4, Thesis Appendix]) I found high gametocyte numbers on these days.

### 6.3.3. Virulence, asexual and sexual parasites in mice

I recorded mouse live weights and red blood cell densities as measures of virulence (Mackinnon *et al.* 2002b; De Roode *et al.* 2004b [Chapter 4, Thesis Appendix]), and monitored parasite asexual densities and gametocyte densities as described (De Roode *et al.* 2004b [Chapter 4, Thesis Appendix]). Individual clones in mixed infections were tracked using real-time quantitative PCR (see below); DNA was extracted from 5 µl blood samples using the BloodPrep® kit (Applied Biosystems) on the ABI Prism® 6100 Nucleic Acid PrepStation according to manufacturer’s instructions. Measurements were taken on days 2, 4, 6, 7, 9, 11, 13, 14, 16, 18, 20 and 21 PI, between 9.00 a.m. and 12.00 noon, when peripheral blood almost exclusively harboured haploid ring stage parasites.

### 6.3.4. Transmission to mosquito

On each of the days 7, 14 and 21 PI, 3 mice from each treatment group were fed to mosquitoes: those mice were chosen that had considerable gametocyte numbers the day before (range: 133 – 11888 gametocytes/µl). Mice were anaesthetised using Rompun/Vetalar, injected intramuscularly at a dose of 2.5 µl per g mouse, and put on top of a carton container covered with net and containing 30 mosquitoes. Mosquitoes were given
20 minutes to feed, after which mice were killed. Mosquitoes that had not taken a blood meal were removed from the pots, and those that died during the following 8 days were likewise removed.

Mid-guts were excised from mosquitoes at 8 days post-feed. The infection status (presence/absence of oocysts) of individual mosquitoes was determined by 40x-100x phase contrast microscopy and, when present, numbers of oocysts were counted. Individual oocyst-bearing mid-guts were placed into microtubes containing cetyltrimethylammonium bromide (CTAB) buffer and Proteinase K at a final concentration of 1 mg/ml, and allowed to digest for at least an hour at 56°C. DNA was extracted using a standard phenol/chloroform extraction, precipitated with propan-2-ol, washed twice in 70% ethanol and air-dried. The DNA was then re-suspended in 10 μl of sterile H2O, boiled for 5 min and stored at −80 °C until required for PCR. Numbers of AS and AJ genomes present on mosquito mid-guts were determined using real-time quantitative PCR.

6.3.5. Real-time quantitative PCR

In collaboration with A.S. Bell, two clone-specific real-time quantitative PCR assays were developed, utilising hydrolysis probe detection and the ABI Prism™ 7000 Sequence Detection System (Applied Biosystems). These assays exploited sequence polymorphisms in the ama1 gene of the two P. chabaudi clones (S.J. Cheesman, unpublished results); the ama1 gene codes for the red blood cell invasion protein apical membrane antigen 1. AS-specific primers had the sequences 5’GGAAAAGGTATAACTATTCAAAAATCTAAGGT 3’ (forward) and 5’AATTGTTATAGGAGAAATGTTTACATCTGTTTG 3’ (reverse), and AJ-specific primers 5’GGAAAAGGTATAACTAATCTACTTAAATCTACTAAAA 3’ (forward) and 5’GTTGTTATAGGAGAAATGTTACATCTGTTTT 3’ (reverse) (polymorphisms in bold). Amplicon sequences, internal to the primer sites, were identical for the two clones, with the AS amplicon 129 bp and the AJ amplicon 127 bp in length. A fluorescent probe (5’ 6-FAM-ATCCTCCTTCTCTTTACTTTCC-MGB 3’) was used for quantification, hybridising to the non-polymorphic region within the ama1 gene amplicon of both clones.

Standard reaction conditions for hydrolysis probes on the ABI Prism™ 7000 SDS platform (initial denaturation 95°C/10 min and 45 cycles of denaturation 95°C/15 s and annealing/extension 60°C/1 min) were employed for both assays. Concentrations of the clone-specific primers (0.3 μM) and common probe (0.2 μM) were the same for both assays.
and each utilised TaqMan® Universal PCR Master Mix (hot start) in a total reaction volume of 25 μl incorporating 2 μl of DNA.

Absolute quantification of AS and AJ genome numbers was realised by comparing the cycle thresholds of the unknown samples against a standard curve. DNA standards, covering 5 orders of magnitude, were generated from duplicate DNA extractions of mouse blood samples bearing known numbers of haploid parasites: \(8.55 \times 10^5 / 2 \mu l\) down to \(8.55 \times 10^0 / 2 \mu l\) for AS, and \(2.14 \times 10^5 / 2 \mu l\) down to \(2.14 \times 10^0 / 2 \mu l\) for AJ. Standards were extracted from the same volume of blood using the ABI Prism® 6100 Nucleic Acid PrepStation as described above. Duplicates of each standard (or triplicates for lower template numbers) were included in each quantitative PCR run. The assays were tested for specificity, sensitivity and repeatability. Each clone-specific assay amplified only its target-template with no amplification of non-target clone DNA, mouse or mosquito DNA. Both assays were found to be able to detect fewer than 5 genome copies per 2 μl DNA sample (corresponding to fewer than 100 parasites per μl mouse blood or 25 genome copies per mosquito mid-gut). Using AS primers, fewer than 5 AS parasites could be detected in the presence of 10,000 AJ parasites, and using AJ primers fewer than 5 AJ parasites were detectible in the presence of 10,000 AS parasites, thus showing that the assay was both highly sensitive and specific.

As gametocytes and asexual parasites have exactly the same genes, these real-time quantitative PCR assays cannot distinguish between asexual parasites and gametocytes, but instead reveal the total number of all blood-stage parasites of the target clone. In data analyses, however, I treated these total counts as estimates of asexual numbers: gametocyte densities were 2-3 orders of magnitude lower than asexual parasite densities, thus forming a negligible component of overall parasite numbers.

6.3.6. Trait definition

6.3.6.1. Virulence

I determined the minimum weights and minimum red blood cell densities that mice reached; mice fed to mosquitoes and killed on day 7 were excluded from the analysis, as minimum weights and red blood cell densities were mostly reached after day 7.

6.3.6.2. Asexual and sexual parasite densities

I expected most gametocytes to be produced around days 7, 14 and 21 PI (De Roode et al. 2004b [Chapter 4, Thesis Appendix]). Gametocytes take about 36-54 hours to mature.
(Gautret et al. 1996): as mice were fed to mosquitoes around 16.00 hours in the afternoon on days 7, 14 and 21, I analysed the corresponding asexual densities as monitored around 9.00 hours the day before (days 6, 13 and 20).

6.3.6.3. The relationship between fitness in mouse and mosquito host

I analysed the proportions of mosquitoes infected with AS or AJ and the numbers of AS and AJ parasites per PCR-positive mosquito: in these analyses asexual densities of AS and AJ in the mouse a day before the feeds were included as covariates, to assess whether asexual density in the mouse host was a good predictor of transmission to mosquitoes. I also determined the proportion of AS in the parasite population in mosquitoes (either as the average of all mosquitoes that fed on a single mouse, or as the total number of AS parasites divided by the total number of AS and AJ parasites in the total mosquito population) and compared these to the proportions of AS in the asexual parasite populations in mice. This analysis was aimed at assessing whether the gene frequency of a clone in the mouse host was a good predictor for its gene frequency in the mosquito host. I also analysed the overall transmission from mixed infections as the proportions of mosquitoes infected, or the numbers of oocysts per infected mosquito to find out if overall transmission of a parasite population was affected by its genetic constitution. In these analyses gametocyte densities were included as covariates.

6.3.7. Statistical analysis

All analyses were carried out using ANOVAs, ANCOVAs and generalised linear models in R (V. 1.6.2). Where necessary, data were log transformed to meet the normality and homogeneity of variance assumptions of these statistical models. Proportions of infected mosquitoes and numbers of genomes and oocysts were analysed using models with binomial and poisson error distributions respectively. For most analyses, quasi-binomial, quasi-poission or negative binomial error distributions were necessary to correct for overdispersion (Crawley 2002). Because mouse, and not mosquito, was the proper replication unit in this experiment, numbers of genomes or oocysts were averaged over the total number of infected mosquitoes that fed on one mouse to get rid of pseudo-replication (Crawley 2002; Grafen & Hails 2002).

Maximal models were fitted first, including all possible interactions and covariates when appropriate. Models were minimised by removing non-significant terms (p>0.05), beginning
with the interactions. As p-values based on F-tests in ANOVA tables are inaccurate in analyses with binomial and poisson error distributions (Crawley 2002), I based significance on deletion of model terms followed by model comparison as described (Crawley 2002).

Figure 6.1. Log parasite densities over time (mean ± 1 s.e.m.) for C57 mice (left panels) and CBA mice (right panels). The top row shows AS and AJ parasite densities in mixed AS+AJ infections (A and B), the middle row compares AS parasite densities in single AS infections with those in mixed AS+AJ infections (C and D), and the bottom row compares AJ parasite densities in single AJ infections with those in mixed AS+AJ infections (E and F). Data points on days 1, 3, 5, 6 and 7 are based on 10 replicate mice (except for AJ and AS+AJ in C57 mice: 9 mice each); data points on days 9, 11, 13 and 14 are based on 7 replicate mice (except for AJ and AS+AJ in C57 mice: 6 mice each; AS+AJ in CBA mice: 6 mice on days 11, 13 and 14; AJ in CBA mice: 5 mice on days 13 and 14); data points on day 16, 18, 20 and 21 are based on 4 replicate mice (except for AJ and AS+AJ in C57 mice and AS+AJ in CBA mice: 3 mice each; AJ in CBA mice: 2 mice). As the limit of detection was 100 parasites per μl blood, y-axes start at 2.
6.4. Results

One C57 mouse infected with AJ and one infected with AS+AJ had delayed parasite peaks; they probably received a lower dose than the other mice in their respective treatment groups (Timms et al. 2001); as timing was important in this experiment, these mice were removed from the experiment and subsequent analysis. One CBA mouse infected with AS+AJ was euthanised on day 9, when it was extremely anaemic. One infected with AJ died on day 13, and another was removed from the experiment on day 16, as its tail’s heavy scar tissue made obtaining blood from it impossible.

6.4.1. Virulence

As expected, AS was less virulent than AJ, causing less anaemia in both mouse strains ($F_{1,24} = 15, p=0.001$); in contrast with previous results (De Roode et al. 2004b [Chapter 4, Thesis Appendix]), however, AS did not cause less weight loss than AJ ($p>0.05$). Mixed infections were as virulent as the virulent clone AJ ($F_{1,21} = 2.75, p=0.11$ for anaemia; $F_{1,21} = 0.94, p=0.34$ for weight loss); on the mosquito feed days 7, 14 and 21 there were no differences in anaemia between either of the 3 treatments (Treatment: $p>0.05$).

CBA mice suffered more anaemia (Strain: $F_{1,24} = 12.6, p=0.002$) and weight loss (Treatment: $F_{1,24} = 13.9, p=0.001$) than C57 mice; on day 14, CBA mice were more anaemic than C57 mice (Strain: $F_{1,21} = 18.2, p=0.001$), but this was not the case on days 7 and 21 ($p>0.05$ for both).

6.4.2. Dynamics of asexual and sexual parasites

In both mouse strains, AJ produced higher numbers of parasites in mixed infections than AS during the first two weeks of infection (Figure 6.1A, B). In C57 mice, AS then disappeared below detectible levels, whereas in CBA mice it started to overgrow AJ, and became the numerically dominant clone up until day 21 when the experiment was terminated (Figure 6.1B). Compared to single infections, AS produced fewer parasites in mixed infections in C57 mice for the entirety of the infection (Figure 6.1C), thus showing it suffered from competition. In CBA mice, however, this was true only for the first 16 days, after which AS produced more parasites in mixed infections than it did on its own (Figure 6.1D), thus showing facilitation at that time, not competition. AJ also suffered from competition, though to a lesser degree than AS. In C57 mice, it produced fewer parasites in mixtures than on its
own between days 6 and 13, after which it produced about as many in single and mixed infections (Figure 6.1E). In CBA mice, AJ also suffered from competition between days 6 and 13, and again between days 16 and 21 (Figure 6.1F), when AS was the dominant clone.

As expected, gametocytes in C57 mice were produced with peaks on days 7 and 14, after which they disappeared below detectible levels (Figure 6.2A). In CBA mice there was also a large peak of gametocytes on day 7 for all 3 infection types; AS infections produced many more gametocytes on day 14 than AJ and AS+AJ infections (Figure 6.2B); AS+AJ infections then went on to produce considerable numbers of gametocytes with a slight peak on day 21 (Figure 6.2B).

*Figure 6.2.* Log gametocyte densities over time (mean ± 1 s.e.m.) for C57 mice (A) and CBA mice (B) infected with single AS, single AJ or mixed AS+AJ infections. For mixed infections gametocyte densities reflect overall numbers of AS and AJ gametocytes, as the real-time quantitative PCR assay used cannot distinguish between AS and AJ gametocytes (see text). As the limit of detection was 100 parasites per μl blood, y-axes start at 2. See Figure 6.1 for the number of mice on which each data point is based.
6.4.3. **Transmission success to mosquito vector**

6.4.3.1. Proportions of mosquitoes infected with AS or AJ

Summarised over the whole infection (thus taking data from days 7, 14 and 21 together), AS and AJ infected fewer mosquitoes from mixed than from single infections (Figure 6.3A, B). This was a result of two effects. First, clones had lower asexual densities due to competition, which led to lower transmission (Figure 6.3A, B; p<0.001 for AS, p=0.003 for AJ). Second, transmission from mixed infections was lower *per se*, so that for a given asexual density both clones infected fewer mosquitoes from mixtures than from single infections (p=0.04 for both AS and AJ). AS infected much fewer mosquitoes from mixed than from single infections on day 7 in both mouse strains (Figure 6.3C, D; p=0.008). On day 14, it also infected fewer mosquitoes from mixtures in CBA mice, but slightly more from mixtures in C57 mice (Figure 6.3E, F; Treatment x Strain interaction: p=0.004). Analysed for days 7 and 14 separately, AJ infected slightly fewer mosquitoes from mixed infections (Figure 6.3E, F), but this reduction was not significant (p=0.15, p=0.11 for days 7 and 14 respectively).

6.4.3.2. Numbers of parasites per infected mosquito

Over the whole experiment 359 out of 1289 mosquitoes contained oocysts and were thus clearly infected, but only 268 of these gave positive PCR results (Table 6.1). Moreover, observations of AS and AJ genomes and the genome numbers themselves were generally very low (Figure 6.4), so that analysis of these numbers in some cases resulted in problems with normality of error distributions. Average parasite numbers for AS and AJ were based only on mosquitoes that showed positive PCR results for AS, AJ or both.

Over the whole infection, both AS and AJ had lower parasite numbers in mosquitoes that fed on mixed infections than in those that fed on single infections (Figure 6.4A, B). As with the proportions of infected mosquitoes, this was not due to lower asexual densities in mixed infections in mice alone: AS certainly had lower numbers in mosquitoes when asexual densities were low (p<0.001), but for a given asexual density AS and AJ obtained lower parasite numbers in mosquitoes from mixed than from single infections (Figure 6.4A, B; p=0.002 for AS, p=0.03 for AJ). On day 7 AS had marginally lower parasite numbers in mosquitoes from mixed infections than single infections (Figure 6.4C, D; p=0.09); on day 14 it had far lower numbers in mosquitoes that fed on mixed than single infections in CBA mice, whereas in C57 mice there was no such difference (Figure 6.4E, F; Treatment x Strain interaction: p=0.006). AJ produced fewer parasites in mosquitoes fed on mixtures than those.
fed on single infections on day 7 (Figure 6.4C, D; p=0.04), but not so on day 14 (Figure 6.4E, F; p=0.14).

Figure 6.3. Relationships between the proportions of mosquitoes infected with AS or AJ and their corresponding asexual parasite densities in mice a day before mosquito feeding. Graphs show the proportions of mosquitoes infected with AS or AJ when fed on mice with single AS or AJ ('AS alone'; 'AJ alone') or mixed AS+AJ ('AS in mixed'; 'AJ in mixed') infections, summarised over days 7, 14 and 21 (A, B), or for day 7 (C, D), 14 (E, F) and day 21 (G, H) separately. Presence of AS and AJ in mosquitoes was assessed with real-time PCR. When no symbols are present in a graph, it means that no parasites were detected in any of the mosquitoes.
Table 6.1. Numbers of dissected mosquitoes that fed on mice infected with AS, AJ or a mixture of AS+AJ, and in which parasites were detected by the presence of oocysts or by PCR detection. Non-italicised numbers refer to individual mice, whereas italicised numbers are subtotals and totals.

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<td>Dissected</td>
<td>Oocyst positive</td>
<td>PCR positive (both clones)</td>
<td>Dissected</td>
</tr>
<tr>
<td>Day 7</td>
<td></td>
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<td>18</td>
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<td></td>
<td>24</td>
<td>15</td>
<td>15</td>
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<td>20</td>
<td>13</td>
<td>12</td>
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<tr>
<td></td>
<td>70</td>
<td>54</td>
<td>52</td>
<td>45</td>
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<tr>
<td>AJ</td>
<td>20</td>
<td>19</td>
<td>4</td>
<td>30</td>
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<td></td>
<td>20</td>
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<tr>
<td></td>
<td>57</td>
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<td>20</td>
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</tr>
<tr>
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<td>8</td>
</tr>
<tr>
<td></td>
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<td>27</td>
<td>10</td>
<td>8 (7)</td>
<td>24</td>
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<td>Total</td>
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<td>1</td>
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<td>53</td>
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</tr>
<tr>
<td>AS+AJ</td>
<td>31</td>
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<td>AJ</td>
<td>25</td>
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<tr>
<td>AS+AJ</td>
<td>22</td>
<td>0</td>
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<td>28</td>
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<tr>
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<tr>
<td>Total</td>
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<td>212</td>
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<tr>
<td>Overall</td>
<td>Total</td>
<td>671</td>
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<td>167</td>
</tr>
<tr>
<td>Over CS7 and CBA</td>
<td>1289</td>
<td>359</td>
<td>268</td>
<td></td>
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</table>
Figure 6.4. Relationships between numbers of AS and AJ parasites per infected mosquito and their corresponding asexual parasite densities in mice a day before mosquito feeding. Graphs show the numbers of AS and AJ parasites in mosquitoes fed on mice with single AS or AJ ('AS alone'; 'AJ alone') or mixed AS+AJ ('AS in mixed'; 'AJ in mixed') infections, summarised over days 7, 14 and 21 (A, B), or for day 7 (C, D), 14 (E, F) and day 21 (G, H) separately. AS and AJ numbers in mosquitoes were quantified using real-time PCR, and plotted points are the averages of all PCR-positive mosquitoes fed on single mice. When no symbols are present in a graph, it means that no parasites were detected in any of the mosquitoes.
6.4.3.3. Relative proportions of clones in mice and mosquitoes

I compared the relative proportions of AS of the total parasite populations in mosquitoes and compared them with the relative proportions in mice the days before the feeds. Relative proportions were calculated in two ways. The first way determined the proportion of AS in each PCR-positive mosquito and then averaged these per mouse. The second way summed the AS parasites over all the mosquitoes fed on a single mouse and then divided this number by the overall AS+AJ parasites summed over those mosquitoes. As there were not many mosquitoes for which I obtained positive genome counts, I decided to pool all the results over host strain and day together, and do the analysis on those pooled results. For both ways of calculating, the observed proportions of AS in mosquitoes were linearly related to the AS proportions in the mice a day before mosquito feeds (Figure 6.5A, B). Thus, these results suggest that a clone present in a mixed infection in the mouse transmitted to the mosquito at the same relative frequency.

![Graph](Figure 6.5. The relationship between the proportions of AS parasites in the mosquito, and mouse one day before a feed day. Shown are the proportions of AS in mosquitoes based on the average per mosquito (A) and based on the overall numbers of AS parasites over all the mosquitoes fed on a single mouse (B). In both cases the observed least squares regression line does not differ from the expected line based on the hypothesis that the proportions in the mosquito are linearly related to the proportions in the mouse. Regression lines: (A) proportion in mosquito = 0.15 (±0.21) + 0.89 (±0.35) * proportion in mouse; \( R^2 = 0.50, p=0.02 \); (B) proportion in mosquito = 0.075 (±0.24) + 0.85 (±0.42) * proportion in mouse; \( R^2 = 0.37, p=0.05 \). Regression lines in both (A) and (B) do not differ from the linear equation: proportion in mosquito = 0 + 1.0 * proportion in mouse (p>0.05 for both).)
6.4.4. Overall transmission from single and mixed infections

The above analysis showed that both AS and AJ obtained lower transmission from mixed infections than from single infections and that this reduction could not be attributed to a reduction in asexual parasite densities alone: I showed that for a given density AS and AJ still infected fewer mosquitoes from mixed than single infections. This suggests that the overall transmission from mixed infections was lower than that from single infections, and this was indeed the case. On day 7, fewer mosquitoes became infected when feeding on mixed infections than on either AS or AJ single infections (Figure 6.6A, B; p=0.034). This difference did not occur because of lower gametocyte densities in mixed infections (Figure 6.2A, B): more mosquitoes became infected with higher gametocyte densities (p=0.022), but for a given gametocyte density, mixed infections still infected fewer proportions of mosquitoes than single infections (p=0.002). Per gametocyte, the numbers of oocysts per infected mosquito were also lower for mixed infections (p=0.002 for both day 7 and over the whole infection). On day 14 mixed infections infected as many mosquitoes as did AJ infections (p>0.05); in C57 mice these numbers were higher than for AS single infections, whereas in CBA mice they were lower (Figure 6.6C, D; Treatment x Strain interaction: p=0.002). Thus, clone AS suffered great transmission reductions in mixed infections in CBA mice: these mixed infections had much lower gametocyte densities than AS single infections (Figure 6.2B), so that they infected far fewer mosquitoes.

On days 7 and 14 C57 mice infected more mosquitoes than CBA mice. Again this was not due to differences in gametocyte numbers (Figure 6.3A, B): per gametocyte, C57 mice infected more mosquitoes than did CBA mice (p=0.05 for both days 7 and 14). Infected mosquitoes that had fed on CBA mice also had lower numbers of oocysts than those that had fed on C57 mice (p=0.008 and p=0.004 for day 7 and the whole infection respectively).
Figure 6.6. Proportions of mosquitoes that became infected when feeding on mice infected with either AS, AJ or a mixture of AS and AJ. Shown are proportions for C57 mice (left) and CBA mice (right), on days 7 (A and B), 14 (C and D) and 21 (E and F). Within each graph, asterisks denote treatments that are significantly different from the other treatments in the same graph. Proportions are based on the means of 3 mice for each data-point, except for AJ in graph F (2 mice only). Crosses denote proportions of zero. Error bars are 95% confidence limits based on the binomial distribution (Rohlf & Sokal 1981).
This experiment showed strong transmission losses from mixed infections. Both clones AS and AJ obtained much lower transmission from mixed infections than from single infections, both in terms of the numbers of mosquitoes they infected as well as the numbers of parasites per infected mosquito. This transmission reduction was the result of two effects. First, it was shown that the relative proportion of a clone in the asexual parasite population in the mouse was a good predictor for the relative proportion of that same clone in the mosquito. This means that competition led to reduced parasite densities in the mouse and consequently lower transmission. Second, the overall transmission from mixed infections was lower than from single infections, so that for a given parasite density, clones still transmitted less well from mixed than from single infections. Thus, these results suggest that relative between-host fitness is directly related to relative within-host competitiveness in this malaria model, although overall transmission can be much reduced.

Even though these results are based on a relatively small number of mosquitoes, there is at least no suggestion that a reverse relationship between within-host competitiveness and transmission exists, i.e. no evidence of increased relative transmission from mixed infections. We thus have no reason to believe that *P. chabaudi* parasites facultatively increase their investment in transmission stages in response to the presence of competitors as they do in response to sub-curative drug levels (Buckling et al. 1997). This accords well with mixed infections of the *P. chabaudi* clones CR and ER, in which there was also no evidence for such a facultative increase in transmission stage production (Taylor & Read 1998).

It thus seems safe to suggest that these results confirm the oft-held theoretical assumption that within-host parasite competitiveness is a good predictor for parasite transmission. This assumption has been the basis of models asserting that within-host competition is a crucial determinant in the spread of drug resistance (Hastings & D'Alessandro 2000), and that vaccine escape mutants can arise because they are competitively superior and transmit better than strains against which vaccines protect (e.g. Bull 1994). In previous experiments I showed that in *P. chabaudi* there is a direct relationship between virulence and competitiveness (Chapter 3). The current experiment then suggests that there is also a direct relationship between virulence and transmission from mixed infections. Such a relationship has been paramount to a large body of theory predicting that competition in mixed infections leads to selection for increased virulence (Bremermann & Pickering 1983; Sasaki & Iwasa 1991; Van Baalen & Sabelis 1995; Frank 1996; Gandon 1998; Mosquera & Adler 1998). It is important to note that in this experiment I did not find that the avirulent clone AS suffered
more transmission loss than the virulent clone AJ. However, AS was much more virulent in this experiment than in previous experiments (Chapters 3, 4 and 5, De Roode et al. 2004b [Thesis Appendix]); it caused as much weight loss as AJ and consequently suffered less from competition over the whole infection than in previous experiments. This increased virulence is very likely the result of several blood passages of the parasite prior to the experiment, as serial passaging increases virulence in *P. chabaudi* (Mackinnon & Read 1999b; Mackinnon & Read 2004a). Thus, had AS been less virulent, it would probably have suffered more from competition and thus suffered more transmission loss.

Contrary to my expectations (De Roode et al. 2004b [Chapter 4, Thesis Appendix]), AS suffered more transmission loss from mixed infections in CBA mice than in C57 mice. I had expected the reverse, as in CBA mice AS actually benefited from the presence of AJ later on in the infection (after about 16 days), when it produced more parasites in mixed than in single infections. In this, as well as in a previous experiment (De Roode et al. 2004b [Chapter 4, Thesis Appendix]), this facilitation coincided with a large peak of gametocytes around day 21, and it was hypothesised that this could result in transmission of AS parasites at that time. However, these gametocytes resulted in hardly any mosquito infections at all, with only 4 mosquitoes out of a total of 83 infected. Gametocytes have often been found to become less infective over time (Dearsly et al. 1990; Naotunne et al. 1990), and this is probably the result of transmission blocking immunity (Naotunne et al. 1990). These results thus show that the longer persistence of AS in mixed infections in CBA mice did not result in extra transmission: this questions the idea that antigenic variation of parasites is a means to prolong infection in order to obtain more transmission (e.g. Antia & Lipsitch 1997). One other reason that AS suffered extra much transmission loss from CBA mice is that whereas in single infections it produced large numbers of transmissible gametocytes on day 14, in mixed infections these numbers were drastically reduced due to the presence of AJ.

In this study, mixed-clone infections – on a per gametocyte basis - generally infected much lower proportions of mosquitoes than did single-clone infections, and resulted in lower numbers of oocysts in infected mosquitoes. This could be due to immunity in the mosquito host, the mouse host, or both. Between the ingested gametocyte and the oocyst stages in the mosquito, malaria parasites undergo huge mortality (Vaughan et al. 1992): this is at least partly due to the mosquito’s immune system (Sinden 2002; Dimopoulos 2003). Mosquito immune responses include digestive enzymes in the mid-gut and melanotic encapsulation of early oocysts, and some of these responses are believed to be quite specific (Dimopoulos 2003). Possibly, mosquito immunity is more effective against a mixed infection of AS and AJ parasites than against single AS or AJ infections: this could be due to AS and AJ sharing
some antigens or because of them having some immunological properties that make immune responses against them synergistic.

Mixed infections could also be less transmissible due to strain-transcending transmission blocking by the mouse’s immune system. Transmission blocking immunity in malaria reduces infectivity to the mosquito vector by reducing gametocyte survival (Harte et al. 1985), gamete fertilisation (Gwadz 1976) and sporogonic development in the vector (Winger et al. 1988), and in the rodent malaria P. berghei occurs as early as day 6 PI (Fleck et al. 1994). Transmission blocking is often mediated by antibodies killing gametes or zygotes, or preventing fertilisation and ookinete mid-gut penetration (Carter 2001; Kaslow 2002). With so many antigen-targets, it seems likely that there will be considerable cross-immunity between AS and AJ. Indeed, if the transmission blocking antigens of clones AS and AJ are very similar, immunity could work synergistically against these clones. A corollary of this argument is that if two clones were very antigenically different, overall transmission from mixed infections could actually be enhanced, something that has indeed been observed with two different P. chabaudi clones (Taylor et al. 1997b, see Chapter 8 for discussion).

Transmission blocking immunity is an attractive explanation for the results observed here, because it could also explain the differences between C57 and CBA mice. CBA mice suffered more anaemia and weight loss, and this could be due to CBA mice having different immunological responses (as hypothesized in De Roode et al. 2004b [Chapter 4, Thesis Appendix]). C57 and CBA mice show different immune responses when infected with Schistosoma japonicum infections (Hirata et al. 2002), and with P. chabaudi these strains also differ in the levels of cytokines they produce (A.L. Graham et al., unpublished results). Thus, it is well possible that C57 and CBA mice also have different levels of transmission blocking immunity, with CBA mice blocking transmission more effectively.

Despite this attractive explanation of transmission blocking immunity, there are alternative explanations for the reduced infectivity of mixed infections observed here. For example, fertilisation of AS and AJ gametes could result in disrupted oocyst formation; if this was true, I would expect fewer oocysts to be AS/AJ cross-progeny than of the parental types, which is testable by genotyping individual oocysts (Ranford-Cartwright et al. 1991; Taylor et al. 1997a). Another possibility is that AS and AJ clones competed through interference (as observed in bacteria, see for example Riley & Gordon 1999) by excreting some substances that had negative effects on gametocyte infectivity or sporogonic development of the other clone. Finally, mixed infections could have affected mouse blood in such a way that mosquitoes took smaller blood meals or blood meals of lower quality (Ferguson et al. 1994).
effectively reducing the number of gametocytes. This could not have been because mixed infections were more anaemic, because mixed infections did not have lower red blood cell densities than single infections on the mosquito feed days. Higher anaemia also could not explain that CBA mice infected fewer mosquitoes: only on one of the feed days (day 14) did they have fewer red blood cells than C57 mice.

The analysis of this experiment was made difficult by the fact that not many mosquitoes became infected, and that a lot of infected mosquitoes were not PCR positive, let alone had high numbers of sporozoites that could be reliably analysed. Ookinetes (Beier et al. 1992) and oocyst numbers in the field (Pringle 1966; Beier et al. 1987; Billingsley et al. 1994) are usually very low and lower than in the lab (Medley et al. 1993). However, the oocysts that are present generally contain many sporozoites, in the order of thousands (Pringle 1966; Beier et al. 1987). In contrast to this study, in which oocyst positive mosquitoes were often PCR negative, real-time PCR detected \textit{P. falciparum} parasites in \textit{A. stephensi} mosquitoes when oocysts were not visible microscopically (A.S. Bell, unpublished results). It thus seems that the oocysts observed here had either very low sporozoite numbers, were degrading, or had not yet developed properly. Perhaps if I had analysed the oocysts a few days later, more sporozoites would have been present, but it is also possible that the poor transmission observed in the current study is an effect of the serial passaging of the clones used prior to the experiment.

Serial passaging in many parasites results in a reduction of the parasites’ ability to transmit properly and efficiently (Ebert 1998), and certainly occurs in in vitro passaged \textit{P. falciparum} isolates (Day et al. 1993). It also happens in \textit{P. chabaudi} parasites that are blood-passaged from mouse to mouse, circumventing mosquito transmission (A.S. Bell, unpublished results): when a heavily blood passaged and poorly transmitting \textit{P. chabaudi} line was transmitted through mosquitoes only once, it reverted to its original wildtype’s ability to transmit properly and efficiently (A.S. Bell, unpublished results). If this was the case for clones AS and AJ as well, the described experiment could be repeated with mosquito passaged AS and AJ lines, possibly resulting in more infected mosquitoes and higher numbers of sporozoites. Such an experiment could then be used to more robustly confirm the conclusions drawn here. This is certainly necessary: that mixed infections were less infectious than single infections is a finding that is not predicted by any of the existing evolutionary models. As such, this study illustrates the great need of experimentally testing widely held theoretical assumptions.
7. Competitive release of drug resistance following drug treatment of mixed malaria infections


7.1. Abstract

Malaria infections are often genetically diverse, potentially leading to competition between co-infecting strains. Such competition is of key importance in the spread of drug resistance. I studied the effects of drug treatment on within-host competition using the rodent malaria *Plasmodium chabaudi*. Mice were infected simultaneously with a drug-resistant and a drug-sensitive clone and were then either drug-treated or left untreated. In the absence of drugs, the sensitive clone competitively suppressed the resistant clone and reduced its transmission to the mosquito vector. Drug treatment, however, allowed the resistant clone to fill the ecological space emptied by the removal of the sensitive clone, allowing it to transmit as well as it would have done on its own. These results show that under drug pressure, resistant strains can have two advantages: (1) they survive better than sensitive strains and (2) they can exploit the opportunities presented by the removal of their competitors. When mixed infections are common, such effects could increase the spread of drug resistance.

7.2. Introduction

Malaria infections often consist of more than one parasite genotype (e.g. Snewin *et al.* 1991; Daubersies *et al.* 1996; Babiker *et al.* 1999). Humans represent ecological niches for co-infecting malaria parasites, with shared predators (immune responses) and limited resources, so that competition between co-infecting malaria strains is likely to be intense (Read & Taylor 2001). Such competition could strongly affect the relative transmission of drug-resistant strains, and thus the spread of drug resistance (Hastings & D'Alessandro 2000).

Resistant and sensitive strains will co-occur in the same host both when resistance mutations first arise *de novo*, and when hosts acquire resistant and sensitive strains from one mosquito bite simultaneously or from different mosquito bites. In the absence of drug treatment, the transmission success of the resistant strain will depend on its intrinsic fitness and competitive ability. However, if drug treatment does occur, the resistant strain has two potential fitness advantages. First, it will better survive the drug than the sensitive strain. Second, treatment can remove drug-sensitive competitors, thus freeing up ecological space for the resistant
strain to occupy. This second effect, well recognised in theory, has the potential to greatly enhance the rate of spread of drug resistance in a population (Hastings & D'Alessandro 2000). However, there is no direct experimental evidence that removal of competitors by drug treatment does enhance the transmission of drug-resistant parasites. Here I report what I believe to be the first experimental demonstration that competitive release of drug-resistant strains can occur following drug treatment.

I studied mixed infections of drug-sensitive and -resistant malaria clones of the rodent malaria *Plasmodium chabaudi*. This parasite is commonly used as a model for human malaria (Cox 1988), and has been extensively used to study drug resistance (Carlton *et al.* 2001). In the absence of drugs, the drug-resistant strain has been shown to suffer from competition from a -sensitive strain (Chapters 3-6, De Roode *et al.* 2004b [Thesis Appendix]). Here, I compare competition between the two strains in drug-treated and untreated mice.

### 7.3. Material and methods

I used two genetically distinct *Plasmodium chabaudi chabaudi* clones: AS(pyr1A), which is resistant to the antifolate drug pyrimethamine (Walliker *et al.* 1975), and AJ, a sensitive clone. Hosts were eight weeks old CBA/Ca inbred female mice (Ann Walker, University of Edinburgh; Harlan, England). Two experiments were performed. In the first, two groups of five mice were infected with $10^6$ AS parasites, and two groups with $10^6$ AS + $10^6$ AJ parasites, as described in Chapter 4. One group from each of these two infection types was drug-treated within three hours of inoculation and again on days 1, 2 and 3 PT (post-infection), using an oral administration of 8 mg pyrimethamine per kg mouse body weight. The untreated mice are the same ones as described in Chapter 4.

I monitored asexual parasite densities and gametocyte densities - the latter being the transmission stages to the mosquito – as described in Chapter 4. Real-time quantitative PCR was used to distinguish and quantify AS and AJ parasites in mixed infections (Chapter 2, Cheesman *et al.* 2003; De Roode *et al.* 2004b [Thesis Appendix]). This protocol cannot distinguish between asexual parasites and gametocytes, but I used real-time PCR data as estimates of asexual densities: gametocyte densities were 2-3 orders of magnitude lower than asexual densities and thus a negligible component of overall parasite numbers. For each infection, I defined the acute phase as that involving the first wave of parasites, and the chronic phase as beginning when parasite numbers recovered after the collapse of the first wave around day 12. All parasites had disappeared below detectable levels after 50 days.
In the second experiment (the experiment described in Chapter 6), two groups of nine mice were infected as above with either AS parasites or AS+AJ parasites. The subsequent transmission success of AS was assayed by allowing batches of 30 starved *Anopheles stephensi* mosquitoes to feed on 3 mice from each group on each of days 7, 14, and 21 PI, as described previously (e.g. Ferguson et al. 2003b). Eight days after the feeds, mosquitoes were dissected, and DNA was extracted from midguts carrying oocysts. Real-time quantitative PCR was subsequently used to determine the prevalence of AS in these mosquitoes.

![Graphs showing asexual AS parasite densities over time](image)

**Figure 7.1.** Log asexual AS parasite densities over time in untreated (A) and drug-treated (B) mice infected with AS alone or a mixture of AS+AJ, and total numbers of AS parasites produced over acute (C) and chronic (D) phases. All data points (mean ± 1 s.e.m.) are based on 5 replicate mice, except for mixed infections in untreated mice in (A) (4 mice on day 11 and 3 mice from day 12 onwards) and (C) and (D) (3 mice). As the limit of detection was 100 parasites per μl blood, y-axes in (A) and (B) start at 2.
7.4. Results

Two untreated mice infected with AS+AJ died on days 10 and 11 PI respectively, and were excluded from the analysis.

In untreated mice, AS produced far fewer parasites during the acute phase in mixed infections with AJ than it did alone (Figures 7.1A, C). However, in drug-treated mice, where AJ was entirely removed by pyrimethamine (none of the PCR reactions performed were positive for AJ), AS produced as many parasites in mixed infections as it did alone (Figures 7.1B, C; Drug treatment x Alone/Mixed interaction: F_{1,14}=14.4, p=0.002). Thus, AS was competitively suppressed in mixed infections in untreated mice, but this suppression was negated when mice were treated with pyrimethamine, which effectively removed AJ.

During the chronic phase, AS was more numerous in untreated mice in mixed infections than in single-clone infections (due to the parasite peak around day 21; Figures 7.1A, D). Thus, in untreated mice in the chronic phase, AS did not suffer from competition, and actually benefited from the presence of AJ (facilitation). In drug-treated mice, however, AS parasites were similarly numerous in mixed- and single-clone infections (Figures 7.1B, D; Drug treatment x Alone/Mixed interaction: F_{1,14}=13.8, p=0.002).

![Graphs showing gametocyte densities over time](image)

**Figure 7.2.** Log gametocyte densities over time (mean ± 1 s.e.m.) for untreated (A) and drug-treated mice (B) respectively. In (A) gametocyte densities for mixed AS+AJ infections reflect overall AS+AJ gametocytes, as the real-time PCR assay does not distinguish between these (see text); in (B) all gametocytes are produced by AS, as AJ was cleared from mixed infections. All data points are based on 5 replicate mice, except for mixed infections in untreated mice in (A): 4 mice on day 11 and 3 mice from day 12 onwards. As the limit of detection was 100 gametocytes per μl blood, y-axes start at 2.
The large peak of AS parasites in the chronic phase in the untreated mixed infections around day 21 (figure 7.1A) coincided with a large peak of gametocytes, the transmissible stages of the parasite (figure 7.2A). This was in contrast with single AS infections in untreated mice, and infections in drug-treated mice, where gametocytes were mainly produced around day 14 (figures 7.2A, B). Overall, gametocyte numbers were the same for all four infection types (p>0.05 for both Drug treatment and Alone/Mixed). Whether AS really suffered from competitive suppression by AJ in untreated mice thus depends on how many of the gametocytes around day 21 were of the AS genotype, and on how transmissible they were.

The second experiment assayed transmission to mosquitoes on days 7, 14 and 21 Pl. I found that AS infected far fewer mosquitoes from mixed infections than from single infections (Figure 7.3; Alone/Mixed: p=0.002), indicating that gametocytes produced around day 21 had a low transmissibility, probably as a result of transmission-blocking immunity (Naotunne et al. 1990). Thus, the competitive suppression of the resistant clone in untreated infections translated into reduced transmission success.

![Proportion of mosquitoes infected with AS](image)

**Figure 7.3.** Proportions of AS infected mosquitoes that fed either on mice infected with AS alone or mice infected with a mixture of AS+AJ (mean and 95% confidence interval) in the second experiment. Means are based on 9 mice (3 on day 7, 3 on day 14 and 3 on day 21 Pl) from which totals of 205 (AS alone) and 216 (mixed AS+AJ) mosquitoes took a blood meal. Infection with AS was assessed by real-time PCR.
These results show that drug treatment of malaria infections can severely affect ecological interactions between co-infecting strains. The drug-resistant clone was competitively suppressed by the drug-sensitive clone in untreated mice, in terms of both within-host growth and transmission to the mosquito vector. However, drug treatment removed that competitive suppression, and allowed the resistant clone to fill the ecological space emptied, giving it a substantial and additional fitness benefit in addition to the simple survival advantage conferred by resistance. Thus, under drug pressure, resistant strains can have two advantages: they survive better than sensitive strains and they can exploit the opportunities presented by the removal of their competitors. I studied competition between two unrelated clones, and thus not the situation in which a resistant clone arose \emph{de novo} (White & Pongtavornpinyo 2003), but it seems likely that the competitive release following drug therapy would also apply there.

Competitive release following drug treatment will greatly enhance the spread of drug resistance (Hastings & D'Alessandro 2000). Also, with only the resistant strain left in the host, the probability of outbreeding is reduced, thus reducing the chances of meiotic recombination destroying multi-locus resistance (Dye & Williams 1997). In combination, these two processes could enhance the spread of drug resistance, especially in areas with high numbers of strains per infection (Hastings & D'Alessandro 2000).

Of course, this is an argument for judicious use of drugs, not their non-use. Clearance of drug-sensitive strains from mixed infections might enhance the spread of drug resistance, but this has to be offset against the short-term public health benefits, such as reducing overall malaria prevalence. In these experiments, AJ was also the more virulent clone (Chapters 3, 4 and 6, De Roode \textit{et al.} 2004b [Thesis Appendix]), and when it was cleared from mixed infections by drug treatment, mice were less sick (results not shown).

Mice were drug-treated before symptoms occurred, resulting in competitive release. This situation perhaps best mimics the case of prophylactic drug use, or what might occur to new co-infections in high transmission areas where drug use is common. Many more complex experiments will be necessary to determine if competitive release occurs when treatment follows symptoms, and in semi-immune individuals. The facilitation observed in chronic infections (Figures 7.1A, D) suggests the situation might be very complex.

Within-host competition in \textit{P. chabaudi} is now firmly established (e.g. Snounou \textit{et al.} 1992; Taylor \textit{et al.} 1997a; De Roode \textit{et al.} 2004b [Thesis Appendix]). Evidence for competition
between coinfecting genotypes in human malaria infections is necessarily indirect, but consistent with this (Read & Taylor 2001). In older children and adults, for example, parasite densities do not increase with increasing numbers of clones, thus indicating that parasite clones are not regulated independently (Smith et al. 1999a). Given this, and the high frequency of mixed infections in human malaria (e.g. Snewin et al. 1991; Daubersies et al. 1996; Babiker et al. 1999; Felger et al. 1999) often consisting of both resistant and sensitive genotypes (Babiker et al. 1991b), and the fact that genetic diversity can be altered by antimalaria prophylaxis (Beck et al. 1999), it is highly likely that what I have observed in a rodent model also occurs in human malaria. Indeed, a recent study has already implicated release of within-host competition as a key-factor in the spread of drug resistance in Uganda (Talisuna et al. 2003).
8. General discussion

Full discussions of specific findings have been provided with each chapter. Here, I highlight some general themes, conclusions, and practical applications. I also discuss some possible future directions.

8.1. Findings

The thesis started off with a quest to find a method to quantify different malaria genotypes in mixed infections. It was found that quantitative real-time PCR was a good technique to do this, providing accurate estimations of the clonal densities of different *Plasmodium chabaudi* strains in the blood of laboratory mice (Chapter 2, Cheesman *et al.* 2003 [Thesis Appendix]). This method is especially useful, since it relies on sequence polymorphisms between different clones: as such polymorphisms are known for a whole suite of *P. chabaudi* clones, the technique can be transferred to different clone combinations, something that is now well under way (A.S. Bell, unpublished results).

Real-time PCR was then used to ask which biological factors are important determinants of within-host competition between malaria strains. Using three clones with very similar genetic backgrounds, but differing in their virulence, I showed a relationship between virulence and competitiveness (Chapter 3), as generally assumed by theoretical models on the evolution of virulence. Virulence was certainly not the only determinant of within-host competition, however, as shown by subsequent chapters. Chapters 4 (De Roode *et al.* 2004b [Thesis Appendix]) and 6 showed that host genotype is an important factor too: in resistant C57 mice, an avirulent clone was rapidly competitively excluded by a virulent clone, whereas in less resistant CBA mice it persisted and indeed overgrew the virulent clone later on during the infection. In Chapter 5, using two different pairs of clones, I showed that timing of infection is also crucially important. Clones generally did not suffer from competition when infecting mice first; but they did when infecting mice simultaneously with, or later than their competitor. Most strikingly, sequential infection could turn over competitive hierarchies completely, so that clones that were the inferior competitor in simultaneous co-infections, were the dominant competitor if they infected mice first.

I studied the result of within-host competition on between-host transmission (Chapter 6), showing that competitive suppression in the mammalian host translated into a proportional reduced transmission to the mosquito vector. This suggests that the observed competitive
suppression of avirulent *P. chabaudi* clones in Chapters 3-5 would indeed have resulted in reduced transmission to the mosquito vector.

Tentatively, I conclude that these experiments confirm that within-host competition plays an important role in the evolution of virulence, as suggested by a large body of theoretical studies: virulent clones were competitively superior to avirulent parasites (Chapter 3), and competitive superiority most likely translated into transmission to the mosquito vector (Chapter 6). This suggests that where mixed infections are common – as is the case with malaria - virulent parasites should be selected for; the result would be evolution towards increased disease severity. Chapters 4 and 5 would also support this view: even though an avirulent clone persisted in CBA mice, and overgrew a virulent clone later during the infection (Chapters 4, 6), this did not result in increased transmission (Chapter 6). Also, even though the avirulent clone became competitively dominant when infecting mice before the virulent clone (Chapter 5), over all the infections studied it did relatively worse than the virulent clone. These results thus suggest that overall, virulent clones had a competitive advantage.

8.2. Different clones: a different story?

One of the major caveats of this thesis is that it is based on a limited number of parasites, mostly using AS and AJ clones (but see Chapter 5). Now that real-time quantitative PCR has proven to be an accurate technique, useful for the study of mixed malaria infections, it is time to move on to study more mixed infections consisting of different clone.

Further study using different clones is certainly necessary. As became clear in Chapter 5, it is not only the relative virulence of clones that determines competitive dominance, but also how antigenically different they are. It was suggested that competition between clones that are controlled by very similar immune responses should be stronger than competition between clones that are controlled by very different immune responses.

Evidence that antigenic diversity of mixed infections is indeed important comes from a comparison of results obtained with different experiments using different *P. chabaudi* clone combinations. Infections consisting of clones CR and ER, for instance, were more virulent to mice than infections of either CR or ER alone (Taylor *et al.* 1998), and also infected more mosquitoes (Taylor *et al.* 1997b). This is in contrast with mixtures consisting of AS and AJ, as described in this thesis, which were as virulent as the virulent clone AJ (results not shown) and which infected fewer mosquitoes than single-AS or -AJ infections (Chapter 6). These
apparent contradictions could be explained by the fact that CR and ER are antigenically different, whereas AS and AJ are antigenically similar. Experiments have shown that mice immunised with CR are better protected against CR than against ER and that mice immunised with ER are better protected against ER than against CR (Buckling & Read 2001). This was not only true for asexual parasites, but also for transmissibility to the mosquito: thus, CR gametocytes in ER-immunised mice were more transmissible than in CR-immunised mice, and ER gametocytes were more transmissible in CR-immunised mice than in ER-immunised mice. In contrast to clones CR and ER, AS and AJ are probably controlled by more similar immune responses; this assertion is based on the fact that mice immunised with AS cleared AJ almost as effectively as AS, and mice infected with AJ cleared AS almost as effectively as AJ (S.J. Cheesman, pers. comm.). Indeed, I found (Chapter 5) that mice infected with AS first and then with AJ 3 or 11 days later, had lower parasite densities than those with AJ alone, showing a protective effect of AS infection against AJ infection.

The implication is that a mouse infected with CR and ER will have to mount two specific immune responses simultaneously, a process that could be more costly than mounting one response only, thus causing increased virulence (Taylor et al. 1998), or less effective, thus causing mixed infections to become more transmissible (Taylor et al. 1997b). That mice indeed have trouble mounting two effective immune responses simultaneously is supported by the fact that when starting as the minority clone, both CR and ER eventually overgrew their competitor in mixed infections, presumably because the mouse’s immune system focused on the initial majority clone (Taylor et al. 1997a). In contrast with CR and ER, clearing AS and AJ simultaneously is no more costly or less effective than clearing AS or AJ alone: the result would be virulence as high as the most virulent single infection, and no enhanced transmission to mosquitoes, exactly as observed.

Using clones AS and CB, regulated by distinct immune responses, also suggested that antigenic diversity is important. It was shown that mice infected with CB 3 days after AS experienced more parasites than those infected with AS alone (Chapter 5), apparently because the immune system focused on AS, thereby not being able to control CB simultaneously. The fact that the reverse did not happen – AS being able to ‘invade’ mice already infected with CB – shows that the situation may be very complex, and only strengthens the claim that more study on this subject is needed.
8.3. Practical implications: Darwinian medicine

Some scientists – notably those building atomic bombs – argue that science should only be about having fun, about having pleasure in finding things out (Feynman 1999). However, with the current climate of governments cutting down on scientific funding, such a view may be hard to maintain. So does the presented research have any practical implications?

8.3.1. Reducing clone multiplicity

For quite some time now, scientists have argued that understanding how diseases evolve should help us in designing ways to control them (Ewald 1980; Williams & Nesse 1991; Ewald 1994; Nesse & Williams 1994; Stearns 1999; Zimmer 2003). Thus, understanding why malaria is virulent could help in designing ways to make it less virulent, to – as some have put it – tame it (Zimmer 2003), or at least to not make it worse. As many theoreticians assert that within-competition between parasite strains drives the evolution of virulence upwards, it has been suggested that reducing clone multiplicity should have favourable consequences (e.g. Adler & Mosquera Losada 2002; Galvani 2003). This is because a lower incidence of mixed infections should result in less within-competition and therefore less selection for increased virulence. Based on the results in this thesis, suggesting that within-host competition indeed drives evolution upwards, it seems plausible that reducing clone multiplicity could have beneficial effects for global malaria burdens.

This is probably good news, as prophylaxis and drug treatment have been shown to reduce clone multiplicity (Beck et al. 1999; Basco & Ringwald 2001), and so has use of the experimental Spf66 vaccine in Tanzanian and Gambian children (Beck et al. 1997; Haywood et al. 1999); intuitively one would also expect insecticide impregnated bed nets to reduce clone multiplicity, but this is not always the case (Fraser-Hurt et al. 1999). It must be noted, however, that reductions of clone multiplicity could also have negative consequences: it has, for example, been suggested that clone multiplicity is an important factor in building up acquired immunity against malaria, and a reduction of clone multiplicity could interfere with this (e.g. Beck et al. 1997; Snow et al. 1997; Beck et al. 1999; Basco & Ringwald 2001). Moreover, the actual presence of circulating strains could directly interfere with the establishment of others (Smith et al. 1999b), so that reducing clone multiplicity could result in higher disease incidence.
8.3.2. Vaccination

It has been suggested that vaccination could have beneficial effects on the evolution of virulence. This is because vaccination would reduce the number of susceptible people, thereby reducing the occurrence of super-infection, and thus within-host competition, consequently leading to reduced virulence (Nowak & Sigmund 2002). However, this argument is based on the assumption that vaccines are perfect, completely preventing infection with a pathogen.

Future malaria vaccines, however, may well be imperfect, reducing malaria burdens and symptoms, but not preventing infection. Four types of malaria vaccine are currently recognised: infection- and transmission-blocking vaccines, and anti-disease and -parasite vaccines. Of these, anti-disease and -parasite vaccines will most likely result in immunity that suppresses parasite densities or reduces disease, but does not prevent infection. As both suppression of parasite densities and reducing disease symptoms could artificially negate the evolutionary costs of high parasite growth rate (i.e. killing the host before transmission is realised), it has been suggested that these vaccines could select for increased virulence (Gandon et al. 2001; Gandon et al. 2003; Read et al. 2004). Experimental studies have now shown that serial passage of *P. chabaudi* parasites in immune hosts results in more virulent parasites than serial passage in naive hosts (Mackinnon & Read 2004a), supporting this claim. The most likely explanation for this is that virulent parasites are selected for because they are competitively superior to avirulent parasites (Chapter 3): that they have an even greater relative advantage in immune hosts, supports the idea that virulent parasites may have a stronger competitive advantage in resistant hosts, as made in Chapter 4.

Further experiments to test this assertion directly using mixtures of parasites in vaccinated and unvaccinated hosts would be important to predict the consequences of vaccination on the evolution of virulence. Even though an avirulent clone suffered more from competitive suppression in a resistant mouse strain than in a susceptible strain (Chapter 4), it did actually not suffer more transmission loss than from the susceptible strain (Chapter 6), confirming that more study is needed.

8.3.3. Drug resistance

In Chapter 7 (De Roode et al. 2004a [Thesis Appendix]), I showed that competitive interactions can be drastically altered by drug treatment. Thus, in the absence of drugs, a drug-resistant clone was competitively suppressed by a drug-sensitive clone, so that it
obtained far lower transmission than it would have done alone. Drug treatment, however, removed the superior drug-sensitive competitor, leaving the resistant clone to fill up the ecological space thus emptied. It has been suggested that such competitive release could strongly enhance the spread of drug resistance (Hastings & D'Alessandro 2000), especially in areas where mixed malaria infections are common. This suggests that, under certain circumstances, it would be wiser not to use drugs, so as to prevent the enhanced spread of drug resistance. Of course, the benefits of drug use for the individual should be offset against the risks for society.

8.4. Future directions

Research is never really finished, and the results described in this thesis may lead to many new questions, such as the following.

**First**, as already discussed in detail, it would be important to study competition using different combinations of clones, to find out how generally virulent clones are competitively superior to prudent clones. As I am writing this thesis, experiments are already underway to address this question, using 4 different clones (AS, CW, AJ, AT), resulting in 6 different clone combinations (AS+CW, AS+AJ, AS+AT, CW+AJ, CW+AT and AJ+AT; A.S. Bell, J.C. de Roode & A.F. Read, unpublished results).

**Second**, it could be asked how important the immune system is in mediating within-host competition. If immune-mediated competition were indeed important, then we would expect human interventions such as vaccination to have a large impact on within-host competition and the subsequent evolution of virulence. This question is also already being addressed by studies on competition between AS and AJ in mice with or without intact T-cell repertoires (L. Råberg, J.C. de Roode & A.F. Read, unpublished results).

**Third**, within-host competition should be studied in semi-immune mice. So far, I have studied competition in naive mice, but in malaria-endemic areas, many, if not all, people are semi-immune. As different clones will be affected to different extents by pre-existing immunity, this could strongly affect the outcome of within-host competition. Experiments could be done with mice immunised with *P. chabaudi*, followed by drug-treatment (e.g. Mackinnon & Read 2003; 2004a), or indeed with mice immunised with blood-stage vaccines (K. Grech, R. Anders, S.J. Cheesman R. Carter & A.F. Read, unpublished results). Such experiments could also be used to ask whether host resistance results in stronger within-host
competition. Different mouse strains with different levels of resistance could also be used to address this question.

**Fourth**, besides repeating the experiment on within-host competition and between-host fitness (Chapter 6), competition between malaria clones could be studied over the whole course of the malaria life cycle, including all stages in the mosquito vector. This would be important, because parasites with different host species in their life cycle are often constrained in their evolution by traits that might have different optima in different hosts (Gandon 2004). There is, for example, no relationship between *P. chabaudi* parasite virulence in mouse and mosquito (Ferguson *et al.* 2003b), and the same could be true for competitiveness. Competition in the mosquito is probably very strong: it has been claimed that naturally infected *Anopheles gambiae* mosquitoes usually do not carry more than 5 oocysts, and that parasite transmission through the mosquito is so tenuous that as little as five parasites survive (Sinden 2002); many mosquitoes that become infected with *P. falciparum* indeed never obtain salivary gland infections (Beier *et al.* 1990), and mosquito salivary glands have been shown to harbour fewer parasite genotypes than midguts (Arez *et al.* 2003).

**Fifth**, it would be important to design field studies to test whether within-host competition is also a driving force for virulence evolution in human malaria populations; this would also be a necessity to support the practical implications of this thesis as outlined above. One way to do this would be to study several areas, some with a high and some with a low incidence of mixed infections. It could then be asked whether, on average, parasites from areas with many mixed infections are more virulent than those from areas with fewer mixed infections. One problem with such studies would be how to measure parasite virulence ethically. Host factors, such as mutations conferring resistance to malaria infection (Cockburn *et al.* 2004), also affect disease severity; and even groups of people that live geographically close together in areas with different incidences of mixed infections can have dramatic differences in their susceptibility to malaria (Paganotti *et al.* 2004), obscuring parasite virulence effects. One way around this problem would be to isolate malaria strains from people living in different areas and to study *in vitro* correlates of virulence instead, such as multiplication rate (Chotivanich *et al.* 2000) and rosetting (Rowe *et al.* 1995). In due course, when parasite virulence phenotypes can be related to specific genes, frequencies of those genes could also be used to test whether virulence genes are more strongly selected in areas with high rather than low incidences of mixed infections.
Finally, it would be crucial to study within-host competition in different host-parasite systems. This thesis presented the first experiment thus far to test for a relationship between virulence and within-host competitiveness; for a real progress in the field of virulence evolution, however, such a relationship needs to be studied in different host-parasite systems as well. Only if a relationship between virulence and competitiveness generally exists for different parasite species (so that Patricks Nasty play a more general role than in rodent malaria alone), can we conclude that within-host competition is an important factor in the evolution of virulence. Only if that is true, will it make sense to even try to predict the consequences of human interventions on the subsequent evolution of virulence. In my view therefore, a recent publication questioning whether virulence evolution theory could be used to ‘tame our pathogens’ (Ebert & Bull 2003; Zimmer 2003) is dramatically premature, and very likely based on a lack of empirical data.

In conclusion, this thesis has given us some answers, but raised a lot more questions. Not that that is a bad thing; indeed, as Theodosius Dobzhansky once remarked (1973): “Imagine that everything is completely known and that science has nothing more to discover: what a nightmare!”
9. References


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10. Thesis Appendix

Schematic clone history of parasites used

IFA control experiments and correction factors

(An appendix written by A.F. Read and A.M. Anwar, describing the correction factors used to calculate AS and CB parasite densities in Chapter 5)

Scientific publications


(A manuscript based on research carried out during my M.Sc., but written during my PhD)


(Partly based on Chapter 2)


(Based on Chapter 4)


(Based on Chapter 7)

Journalistic publications


Schematic clone history of parasites used

Clones were used as follows.

Chapter 2: AS, AS(pyr1A) (sometimes denoted AS) and AJ

Chapter 3: AS, AS(pyr1A), AS(pyr1B) (denoted AS\text{\textsubscript{moderate}}, AS\text{\textsubscript{avirulent}} and AS\text{\textsubscript{virulent}}), and AJ

Chapter 4: AS(pyr1A) (denoted AS) and AJ

Chapter 5: AS and CB; AS(pyr1A) and AJ

Chapter 6: AS(pyr1A) (denoted AS) and AJ

Chapter 7: AS(pyr1A) (denoted AS) and AJ
IFA control experiments and correction factors

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Background

Immuno-fluorescent antibody assays (IFA) can generate a biased estimate of the ratio of the two clones (Taylor et al. 1997). This may be for a number of reasons. First, the antibodies may vary in their affinities to their respective protein targets. Second, the expression of the target antigen may differ between clones. The timing of schizogony, and presumably therefore the expression of MSP-1, the target antigen, are known to differ between AS and CB (McLean 1986). Finally, there may be some perceptual bias when counting positive parasites fluorescing at different wavelengths and intensities. To test for any biases, we performed a number of control experiments, where we mixed known concentrations of parasites and compared the ratio obtained from IFA with that of the mixture.

Materials and methods

To investigate any biases in the estimates of clonal frequencies obtained by IFA, volumes of blood from mice infected with known concentrations of AS or CB parasites were mixed in vitro to generate samples with a range of known clonal frequencies. We then independently estimated those frequencies by IFA and compared them with the ‘expected’ frequencies. Note that these ‘expected’ values are themselves not without error. Much of this error will be random (e.g. noise in pipetted volumes), but where blood from a single mouse is used to generate a series of mixtures, error in the estimate of the concentration of parasites in that mouse will generate systematic differences for that series between the expected frequencies and those estimated by IFA. To look for bias due to the IFA, we thus need to look for consistent differences between observed and expected frequencies across a number of series of mixtures.

Six separate series – hereafter referred to as trials – were conducted. In trials 1-3, infected blood came from a single mouse for each clone and was used to generate 14 or 15 different
mixtures. A different pair of mice was used in each of the three trials. A further three trials went as follows. Blood was taken from four donor mice for each clone and used to generate 7 or 8 different mixtures, with the blood from any one mouse being used in a maximum of two mixtures. This entire procedure was then repeated one hour and then two hours later on the same eight mice to generate a total of three separate series of mixtures (trials 4-6 below). Thus, the potential problem of introducing consistent bias into the values of the 'expected' clone frequencies though non-independent estimates of parasite concentration varies somewhat between the trials. In what follows, we take the conservative view, and report results for all 67 mixtures and separately for the mixtures within each trial.

In all trials, mice were bled 1130 and 1330 hrs GMT, smears made, blood count samples made, then 0.5ml of blood was mixed with PBS, and mixtures made from that. These mixtures were then rinsed and pipetted onto multispot slides, and clonal frequencies estimated by IFA exactly as described for the experimental samples. Mixtures ranged from 1.5% to 92.4% CB.

Results

The frequency of CB was overestimated by IFA for 88% the mixtures – all of the mixtures in trials 1 and 3, all but one of the mixtures in trials 4-6, and 11 of the 15 mixtures in trial 2 (Figure 1). On average, the frequency of CB was overestimated by 10.7% (95% c.i. 7.9-13%). The magnitude of the overestimate was uncorrelated with the total number of parasites scored by IFA, or the expected proportion CB.

Whatever the cause of this bias, we can correct for it statistically. We seek a method of adjusting the proportion of IFA positive parasites that were scored as CB (pCB\text{IFA}), such that the adjusted proportion (pCB\text{IFA-adj}) and expected proportion (pCB\text{ex}) are on average equal – i.e. are related by a linear OLS regression with a slope of 1 and and intercept of zero. This was done using the relationship

\[ p\text{CB}_{\text{IFA-adj}} = \frac{m}{m + n(2.49 -0.005n)} \]  

where \( m \) is the number of CB positive parasites counted and \( n \) the number of AS positive parasites. The derivation of (1) is outlined in the Appendix. Using the values so obtained, gives the least squares regression
\[ p_{CB_{MIX}} = 1.01^* (p_{CB_{IFA-adj}}) - 0.008, \quad (2) \]

a relationship which is not significantly different from the required \( p_{CB_{IFA}} = p_{CB_{IFA-adj}} \) (95% c.i. intercept: -0.05-0.03; slope: 0.922-1.098). When the IFA data were adjusted using equation (1), there was no evidence any consistent difference between observed and expected \( pCB \), with 33 overestimates and 34 underestimates (mean difference (±s.e.) between \( p_{CB_{IFA-adj}} \) and \( p_{CB_{mix}} \) =0.004 ±0.082, \( t_{66} = .373, p=0.71 \)). Thus, observed clonal proportions adjusted using (1) equal expected clonal proportions, on average (Figure 2).

The data from these control experiments also gives some estimate of the accuracy of the clonal frequencies estimated by IFA and equation 1. Much of the unexplained variance about the 1:1 line in figure 2 is explained by differences between experiments (\( F_{5,60}=10.58, p<0.0001 \); adding experiment as a factor takes the \( r^2 \) of equation 2 from 0.89 to 0.99). Assume that the experiment effect arises from errors in the estimates of parasite concentrations used to calculate clonal frequencies in the mixtures. The within-experiment error then gives an indication of the accuracy of the corrected estimates of \( pCB \), particular in trials 1-3 which contained no within-experiment variation in parasite concentrations. Within those three trials, and using the adjusted values, the mean (± s.d.) absolute deviation between expected and observed was 0.044 (±0.037), with half the estimates being less than 3% off the expected values and 90% being out by less than 8%. In fact, the accuracy maybe better than that: these estimates assume there is no error when making the mixtures themselves (e.g. that blood volumes are measured without error).
Figure 1. Proportion of parasites that were CB as estimated from IFA and the proportion expected, with data for each trial shown separately. Line is equality.

Y=-.008 + 1.01*X;R^2=.89

Figure 2. Filled squares = values corrected using (4); open circles = obs, uncorrected IFA ratios. 67 points, x-axis = corrected ratio, using (4); y=exp ratio from mixtures.
Derivation of Equation 1

We begin by attempting to find regression equations relating the number of parasites IFA-positive parasites of each clone to the number expected, given the frequency of each clone in the mixture and the total number of IFA-positive parasites. These regressions lines should pass through the origin and thus have the general form

\[ y = \beta x \]  

(A1)

where \( y \) is expected count, and \( x \) the observed count. Estimates of the slope \( \beta \) can then be used to estimate the true count.

The relevant data for CB are shown in Fig 3a. Armitage and Berry (1987 p. 272-273) give three methods of estimating \( \beta \) in this sort of situation. The OLS estimate of slope (b) assumes that the residual variance of \( y \) is independent of \( x \), which is clearly inappropriate here. If the residual standard deviation of \( y \) increases in proportion to \( x \), as it does here, the best estimate of \( \beta \) is \( b_2 = \Sigma(y/x)/n \). The average (±s.e.) value of \( b_2 \) across the six trials is 0.784 (±0.036), which in this case is in fact comparable to the OLS slope (0.81 ±0.036). Across all 67 mixtures, \( b_2=0.772 \) and \( b=0.800 \). Thus, for clone CB, we can estimate the correct CB count as

\[ 0.784 \times \text{(IFA count of CB parasites)} \]  

(A2)

The situation with the AS counts is not quite so simple, with a pronounced curvilinear relationship between the number of AS-positive parasites counted and the number expected (Figure 3b). Indeed, the squared term in a least squares second order polynomial forced through the origin are significant across all the data and for five of the six trials. A variety of standard transformations did not satisfactorily linearise the relationship, so we seek the quadratic analogy of A1:

\[ y = \beta x + \gamma x^2 \]

Here we take the least squares coefficients (b', c) as unbiased estimators of \( \beta \) and \( \gamma \). Unlike the CB counts, the residual variance of \( y \) is independent of \( x \) for the AS data. Across all
points, there is no correlation between the observed counts and the absolute value of the residuals from the 2nd order polynomial \((F_{1,65} =0.68, p=0.41)\) or the squared deviations \((F_{1,65} =0.13, p=0.72)\), and nor is there for any of the independent experiments \((p>0.15\) in all cases). The average (±s.e.) values of \(b'\) and \(c\) across the six trials are 1.95 (±1.74) and -0.004 (±0.001) respectively; the analogous values across all 67 mixtures are 1.72 and -0.003. Thus, for clone AS, we can estimate the correct AS count as

\[1.95 \times (\text{IFA count of AS parasites}) - 0.004 \times (\text{IFA count of AS parasites})^2.\]  

(A3)

Putting together (A2) and (A3) gives (1).

A variety of other approaches were also tried. Models assuming the count of one clone was correct and attempting to adjust the other were unable to satisfactorily relate observed and expected frequencies. Another approach is to fit a quadratic forced through 0,0 and 1,1 to the observed and expected clonal frequencies (Taylor et al. 1997). This relationship has the form

\[\text{pCB}_{\text{MIX}} = \text{pCB}_{\text{IFA}} + \alpha \times \text{pCB}_{\text{IFA}} \times (\text{pCB}_{\text{IFA}} - 1)\]  

(A4)

and requires that we estimate the coefficient \(\alpha\). However, values obtained by least squares either across all 67 mixtures (0.578) or by averaging the value obtained for each trial (0.5), or by taking the most extreme trial values (0.223, 0.959), all consistently generated relationships between adjusted pCB and expected pCB with slopes significantly less than 1.
Observed number of IFA positive parasites of each clone against the number expected for all 67 control mixtures, for (a) clone CB and (b) clone AS. In both cases, the plotted lines are respectively, OLS first and second order polynomials forced through the origin.

Figure 3: Observed number of IFA positive parasites of each clone against the number expected for all 67 control mixtures, for (a) clone CB and (b) clone AS. In both cases, the plotted lines are respectively, OLS first and second order polynomials forced through the origin.

References


Rodent malaria parasites suffer from the presence of conspecific clones in three-clone 
*Plasmodium chabaudi* infections

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(Received 21 October 2002; revised 28 March 2003; accepted 31 May 2003)

**SUMMARY**

We studied infection dynamics of *Plasmodium chabaudi* in mice infected with 3 genetically distinct clones - 1 less virulent than the other 2 - either on their own or in mixtures. During the acute phase of infection, total numbers of asexual parasites in mixed-clone infections were equal to those produced by the 3 clones alone, suggesting strong in-host competition among clones. During the chronic phase of the infection, mixed-clone infections produced more asexual parasites than single-clone infections, suggesting lower levels of competition than during the acute phase, and indicating that a genetically diverse infection is harder to control by the host immune system. Transmission potential over the whole course of infection was lower from mixed-clone infections than from the average of the 3 single-clone infections. These results suggest that in-host competition reduces both growth rate and probability of transmission for individual parasite clones.

Key words: malaria, *Plasmodium chabaudi*, virulence, evolution, competition, mixed infections.

**INTRODUCTION**

Human malaria infections often consist of different parasite genotypes occupying the same host (Babiker *et al.* 1991; Conway, Greenwood & McBride, 1991; Arnot, 1998; Babiker, Ranford-Cartwright & Walliker, 1999; Felger *et al.* 1999; Smith *et al.* 1999), but the effect of multiplicity on virulence is unclear (reviewed by Read & Taylor, 2001). Previous experiments conducted in our laboratory with the rodent malaria model *Plasmodium chabaudi* have shown that mixed-clone infections with 2 parasite clones can result in higher virulence (Taylor, Mackinnon & Read, 1998; Timms, 2001), and can leave transmission unchanged or even increased (Taylor, Walliker & Read, 1997; Timms, 2001).

Here we studied experimental mixed infections of 3 genetically distinct clones, 2 of which were virulent, and the third avirulent. We infected laboratory mice with 1 of these clones, or with a mixture of them, to address the following questions: (i) is within-host growth of individual clones reduced by the presence of other clones, (ii) is transmission reduced by the presence of other clones, and (iii) do hosts experience more virulence from mixed infections than from single infections?

**MATERIALS AND METHODS**

**Parasites and hosts**

We used 3 cloned *P. chabaudi* lines, denoted AS, AT and ER (Beale, Carter & Walliker, 1978), originally derived from different isolates from thicket rats. Between isolation from the field and use in this experiment, AS, AT and ER had been blood passaged in mice 8, 7 and an unknown number of times, respectively. These clones were chosen for their different growth rates and virulence, AS having a much slower growth rate and virulence than AT and ER which were similar (Mackinnon & Read, 1999).

Hosts used for the experiment were 6-week-old C57Bl/6 inbred female mice (B&K Universal, England), fed on 41B maintenance diet (Harlan, England) and drinking water supplemented with 0·05% para-amino benzoic acid (PABA) to enhance parasite growth (Jacobs, 1964). Mice were kept in a 12:12 h light-dark cycle.

**Experimental design and inoculation of mice with parasites**

We used 2 main treatments. One treatment was single-clone infections of clone AS, AT or ER, the other one was mixed-clone infections with one of the following clone ratios in the inocula: 8AS:1AT:1ER, 1AS:8AT:1ER, 1AS:1AT:8ER and 1AS:1AT:1ER. We chose highly skewed ratios as well as an evenly distributed ratio, to see if starting...
conditions were important for the outcome of competition. Inoculations consisted of the same total number of parasites (10⁶ parasite-infected red blood cells in 0.1 ml) and were delivered i.p.; they were prepared from donor mice by diluting blood in a calf-serum solution (50% heat-inactivated calf-serum, 50% Ringer’s solution [27 mM KCl, 27 mM CaCl₂, 0.15 mM NaCl], 20 units heparin/ml of mouse blood). The single-clone infection of AS consisted of 4 mice, while all other groups consisted of 5.

Monitoring of parasite densities and gametocyte densities

Thin blood smears were made from tail blood every 2 days from day 4–16, 3 times a week from day 24 to 35 and twice a week from day 39 to 70 post-inoculation (p.i.). After staining with Giemsa, the proportion of red blood cells infected with asexual parasites (parasitaemia) and gametocytes (gametocytaemia) were counted microscopically using 1000× magnification. When asexual parasitaemia was high, about 500 red blood cells were counted in at least 4 microscopic fields, whereas with lower parasitaemias 10 microscopic fields were counted for the number of parasites per field, and the average number of cells per field was calculated. Gametocytaemia was assessed from approximately 50 microscopic fields (corresponding to 25–30 000 red blood cells), using polarized light, which highlights mature gametocytes against a dark background. Red blood cell densities were estimated by flow cytometry (Coulter Electronics) from a 1 : 50 000 dilution of a 2 μl sample of tail blood into Isoton solution on days 0, every 2 days from day 4 to 14, on days 17, 19 and 21 and then weekly from day 28 to 70 p.i. Asexual parasite density and gametocyte density were calculated from the product of red blood cell density and parasitaemia or gametocytaemia. On some days, parasitaemia, but not red blood cell density, was recorded, in which case the average red blood cell density on the previous and ensuing measurements was used for these calculations.

Monitoring of virulence

We used maximum weight loss and maximum red blood cell loss in the mouse as indicators of morbidity, which has been shown to correlate with mortality (Mackinnon, Gaffney & Read, 2002). Red blood cell density was measured as described above, and mouse weights were recorded on days 0, 4, every day from day 6 to 14, days 17, 19, 21, 24 and weekly from day 28 to 70 p.i.

Monitoring of clone dynamics using PCR-detection

Samples of 2 μl of mouse blood from the tail were stored in 200 μl of PBS solution (BDH) until DNA extraction, 1–4 h later, using red blood cell lysis buffer (Boehringer). PCR was performed as described by Taylor, Walliker & Read (1997), with the exception that we used 2 μl for the outer reaction, and 3 μl of 10× diluted outer reaction product for the inner reaction. Products were analysed on 2% metaphor agarose gels (FMC Bioproducts) using ethidium bromide staining. AS showed a band of approximately 300 bp, AT showed a band of approximately 320 bp and ER showed a band of 359 bp. At least 2, and usually 3 PCR reactions were performed on blood samples taken on days 4, 6, 8, 12, 17, 19, 21, 24, 26, 28, 31, 33, 35 and 39.

Statistical analyses

Trait definition. The areas under the asexual parasite and gametocyte density by time curves (Fig. 1A, B) from days 0–12, days 12–39 and from days 39–70 p.i. were calculated as measures of the total number of parasites or gametocytes produced during acute, first and second part of the chronic phase of the infection, respectively, and are referred to as ‘total parasite densities’ and ‘total gametocyte densities’. We chose day 12 as the end of the acute phase as most infections were over their first peak at that point and started to rise again (results for individual mice not shown). Day 39 was chosen as the second break point because all infections had reached low parasitaemias by then. In the analysis, total gametocyte densities over days 12–39 and 39–70 were grouped together; this made the residual errors from the analysis follow a normal distribution, meeting the statistical assumption of normality. Data on asexual parasite density and gametocyte density were log₁₀ transformed prior to analyses to reduce overdispersion in the distribution. As the threshold for detecting parasites by microscopy was around 1/10⁵ red blood cells, when observed values of asexual parasite or gametocyte numbers were zero, they were replaced with 1/10⁴ red blood cells so that zero values fell within the normal distribution range. As indicators of virulence, the minimum weight and red blood cell density for each mouse were subtracted from the average of their values on days 0 and 4 to give ‘maximum weight loss’ and ‘maximum red blood cell loss’. ‘Last day seen’ was defined for each clone as the day on which the last positive PCR result was obtained.

Hypothesis testing

The main aim of the analysis was to determine whether mixed-clone infections could be predicted by the 3 following alternative hypotheses. (1) The ‘Average all’ hypothesis: mixed-clone infections equal the average of the 3 single-clone infections, i.e. there is competition such that each clone in a mixed-clone infection obtains only a share of the
Competition in rodent malaria infections

Fig. 1. Average log_{10} asexual parasite density (A) and log_{10} gametocyte density (B) through time in mice infected with either clone AS, AT or ER of Plasmodium chabaudi or a mixture of these 3 clones. Numbers represent the means of 4 mice (AS), 5 mice (AT, ER) and 19 mice (Mixed).

Because parasite and gametocyte densities of 0 were set to 10^(-10) (see text), the y-axis starts at 4.

The value from each single-clone infection was weighted by one third and summed to give the predicted value of the mixed-clone infections. (2) The 'Average virulent' hypothesis: mixed-clone infections equal the average of AT and ER single-clone infections, i.e. the avirulent clone (AS) is out-competed, and the 2 virulent clones dictate the infection. (3) The 'Sum' hypothesis: mixed-clone infections equal the sum of all single-clone infections, i.e. clones do not compete, and virulence is as high as the sum of the virulence induced by each clone on its own.

To test these hypotheses, the means of mixed-clone infections were compared with predicted means by the method of linear contrasts using the CONTRAST statement in PROC GLM of SAS (SAS/STAT, 1990). This method applies the appropriate weights, as dictated by the hypothesis (e.g., in the case of the 'Average all' hypothesis, 0·33 for each single-clone infection, and −1 for the pooled mixed-clone infections), to the design matrix of the linear equations, and then tests the significance level from the sum of squares of these equations as usual. Note that this method allows for error on the predicted means (which were determined from the single-clone infections) as well as that on the observed means.

We also tested for differences in the rate of decline in the heights of the peaks of parasite densities through time. This was done by fitting a model to all data on parasite density peaks that occurred throughout the infection with fixed effects for treatment (4 levels), day of peak (linear covariate), an interaction between these and a random effect for mouse to account for non-independence between repeated measures on the same mouse (PROC MIXED in SAS).

Before testing our hypotheses, we tested for differences among single-clone infections, and among mixed-clone infections, using analysis of variance in PROC GLM of SAS, with a treatment factor with 3 levels (1 for each clone) or 4 levels (1 for each mixed-clone infection). Treatment and subtreatment means were compared by t-tests based on the residual variance after fitting the model. We also analysed 'last day seen', to determine whether mixed-clone infections were indeed still mixed-clone infections.

RESULTS

During the 70-day period of the experiment, parasite densities exhibited up to 6 peaks (average of 3·8±0·13), which decreased in height and frequency through time (Fig. 1A). Most of the gametocytes were produced during the first and second wave of asexual parasites (Fig. 1B). Afterwards they were detectable only during subsequent peaks in asexual parasite densities. One mouse in the group inoculated with 1AS:1AT:8ER died on day 10 p.i. This mouse had a relatively, but not exceptionally, high parasitaemia (34·6%) on day 8 p.i., but its initial weight was lower than the weights of the other mice in the same group.

Differences among single-clone infections and among mixed-clone infections

Mice infected with AS produced lower parasite densities over days 0–12 and 12–39, produced more gametocytes over the whole course of infection, and lost less weight and red blood cells than AT and ER (P<0·02, P<0·04, P<0·01, P<0·001, P<0·001 respectively) which themselves did not differ. AT and ER only differed in their total number of gametocytes over days 0–12, being higher in AT (P<0·01).
Fig. 2. (A) Total number of asexual parasites produced during the acute phase of infection (days 0–12) for single-clone infections (AS, AT, ER), mixed-clone infections (Mixed) and the predicted numbers based on the ‘Average all’ (Av. all), ‘Average virulent’ (Av. vir.) and ‘Sum’ hypotheses. Bars represent ±1 s.e. Significance levels for testing whether predicted values under the ‘Average all’, ‘Average virulent’ or ‘Sum’ hypotheses differ from ‘Mixed’ are denoted as: *** P<0.001; ** 0.001 < P<0.01; * 0.01 < P<0.05, † 0.05 < P<0.1.

(B) As for (A), but for first part of chronic infection (days 12–39). (C) As for (A), but for second part of chronic phase of infection (days 39–70). Note that no significance symbol is present for the ‘Sum’ hypothesis, because this hypothesis could not be tested due to differences in variance.

Among mixed-clone infections, mice in the 1AS:1AT:1ER treatment lost significantly less weight (P<0.05), had lower total parasite density over days 0–12 (P<0.05) and lower total gametocyte density over days 12–39 (P<0.05) than the other mixed-clone infections, but were similar for other traits. We therefore decided to pool the 4 different mixed treatments together prior to further hypothesis testing.

Survival of individual clones

In both single-clone infections and mixed-clone infections, AS disappeared below the PCR detection level before AT and ER (18.2 days for AS, 28.9 and 31.0 days for AT and ER single-clone infections, P<0.001; 18.4 days for AS, 26.9 and 22.0 days for AT and ER in mixed-clone infections, P<0.001). There was no interaction between treatment and clone effects, showing that clone AS was present for less time than AT and ER in both single-clone and mixed-clone infections. This could be explained by lower parasite densities, since there was a strong relationship between parasite density and PCR-positive rate (data not shown). We have no information on which
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Hypothesis testing

Total asexual parasite densities during the acute phase (days 0-12; Fig. 2A) of mixed-clone infections did not differ from parasite densities predicted under the 'Average all' or 'Average virulent' hypotheses (P>0.10 for both), but were far lower than those under the 'Sum' hypothesis (P<0.001). Between days 12 and 39 the total number of parasites in mixed-clone infections was higher than that under the 'Average all' hypothesis, lower than that under the 'Sum' hypothesis, but the same as that under the 'Average virulent' hypotheses (P<0.001, P<0.001 and P=0.07 respectively). After day 39 parasite densities in mixed-clone infections were much higher than those predicted by the 'Average all' and 'Average virulent' hypotheses (P=0.02, P=0.03 respectively), although whether they were actually the same as those under the 'Sum' hypothesis, could not be tested statistically, as their variances differed greatly (Fig. 2C). The greater number of parasites in mixed-clone infections after day 12 was partly attributable to there being more peaks in mixed-clone infections (4.1±0.16) than single-clone infections (3.4±0.19, P<0.01). It was also partly due to higher individual peaks since the rate of decline in the height of peak densities through time was significantly slower than in single-clone infections (Fig. 3; P<0.001).

Over days 0-12 total numbers of gametocytes in mixed-clone infections did not differ from those under the 'Average all' or 'Average virulent' hypotheses (Fig. 4A; P=0.27, P=0.52 respectively), but were much lower than those under the 'Sum' hypothesis (P<0.001). Between day 12 and day 70 mixed-clone infections produced as many gametocytes as predicted under the 'Average all' and 'Average virulent' hypotheses (Fig. 4B; P=0.06, P=0.59 respectively), but far less than predicted under the 'Sum' hypothesis (P<0.0001). Taken over the whole infection, mixed-clone infections had lower numbers of gametocytes than those predicted under the 'Average all' hypothesis (Fig. 4C; P=0.03), but they were not different from those under the 'Average virulent' hypothesis (P=0.68). They were again lower than those under the 'Sum' hypothesis (P<0.001).

Maximum weight loss (Fig. 5A) was as predicted by the 'Average all' and 'Average virulent' hypotheses. See Fig. 2 (A) for a description of annotations. (B) As for (A), but for chronic phase (days 12-70). (C) As for (A), but for the whole course of infection (days 0-70).
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Observed Expected

B 20 16 12 8 Max. red blood cell loss (x 10^9/ml)

AS AT ER Mixed Av. all Av. vir. Sum

Fig. 5. Maximum weight loss (A) and maximum red blood cell loss (B) in single-clone infections (AS, AT, ER), mixed-clone infections (Mixed) and as predicted under the 'Average all' (Av. all), 'Average virulent' (Av. vir.) and 'Sum' (Sum) hypotheses. See Fig. 2 (A) for a description of annotations.

During the acute phase, parasite densities in mixed-clone infections were as high as the average of all 3 ('Average all' hypothesis) or the 2 virulent single-clone infections ('Average virulent' hypothesis), and much lower than predicted by their sums ('Sum' hypothesis). This indicates that during this phase, when parasite densities are high, clones must compete strongly for ecological space. After the acute phase, especially well into the chronic phase, when densities decrease, parasite densities in mixed-clone infections started to be higher than those predicted by the 'Average all' and 'Average virulent' hypotheses, indicating that competition lessened. However, parasite densities were still not as high as those predicted by the 'Sum' hypothesis, suggesting that some level of competition persisted. Our PCR results showed that, in general, all 3 clones survived the acute phase, suggesting that all 3 clones were affected by competition, though to what degree we cannot tell.

The fact that parasite clones in these mixed-clone infections suffered from competition, is not surprising, as there are several mechanisms by which this could happen. First, mice became very anaemic (probably through a combination of both parasite rupture, as well as phagocytosis of red blood cells and decreased red blood cell production (Cox, 1988; Jakeman et al. 1999; Menendez, Fleming & Alonso, 2000)), possibly resulting in competition between clones for remaining red blood cells. Yap & Stevenson (1994) showed that by artificially preventing anaemia through blood transfusion during and after peak parasitaemia in P. chabaudi, the patenty of infection could be prolonged for up to 5 days after peak parasitaemia. Mice infected with Plasmodium chabaudi are also known to become hypoglycaemic (e.g. Li, Seixas & Langhorne, 2001), so low levels of glucose could also limit parasite growth and enhance competition.

Secondly, parasite clones could adversely affect each other through strain-transcending immunity. During the acute phase, immunity is thought to be largely non-specific (Li et al. 2001; Taylor-Robinson, 1995), although clone-specific immunity also plays a role (Jarra & Brown, 1989; Snounou et al. 1989) and antibodies seem to target parasitized red blood cells for phagocytosis through a very parasite clone-specific process (Mota et al. 1998). This means that if one clone provokes an immune response, it could adversely affect another. This has also been inferred for mixed-species and mixed-clone Plasmodium infections (Bruce et al. 2000). During the chronic phase there also seems to be a Th2 antibody-mediated response (Li et al. 2001; Phillips et al. 1997; Taylor-Robinson, 1995). We noticed that during this phase, mixed-clone infections seemed to be harder to clear by the host immune system than single-clone
infections (they had more and higher parasite peaks), suggesting an inefficient immune clearance due to the fact that the host is faced with antigenic variants of several, rather than one, parasite clones. Several malaria species, e.g. \textit{P. knowlesi} (Brown & Brown, 1965), \textit{P. falciparum} (Biggs et al. 1991; Roberts et al. 1992) and \textit{P. chabaudi} (McLean, Macdougall & Phillips, 1990; McLean, Pearson & Phillips, 1982; Phillips et al. 1997), have been shown to undergo antigenic variation, leaving them to grow relatively unimpeded until new specific antibodies are directed against them. Bruce \textit{et al.} (2000) also invoked antigenic variation, among other mechanisms, to account for sequential episodes of infection in mixed-species and -clone infections in \textit{Plasmodia} infecting humans.

Regardless of the underlying mechanism, it is clear that the 3 clones suffered from being in a mixed-clone infection, and this was also true for gametocytes, the life-stages that are transmitted to mosquitoes. Never did gametocytes reach densities as predicted by the ‘Sum’ hypothesis, thus demonstrating competition in the same way as with asexual parasites. It is important to note that we looked at gametocyte numbers, not infectivity to mosquitoes: therefore we looked at potential transmission rather than actual transmission.

One notable result here was that the avirulent clone (AS) had higher total gametocyte densities than the virulent clones AT and ER. In previous studies, it was found that less virulent clones had less transmission potential than more virulent clones (Mackinnon & Read, 1999). However, the lower gametocyte production in the virulent clones in this experiment was entirely due to there being no gametocytes during the acute phase when red blood cell density was very low (days 10–12). A similar cost to gametocytogenesis of high virulence, possibly mediated through red blood cell supply, was observed by Mackinnon \textit{et al.} (2002).

Our results are in some ways in contrast with previous experiments carried out in \textit{P. chabaudi}. In the current experiment, mice with mixed-clone infections had as much weight and red blood cell loss as predicted under the ‘Average virulent’ hypothesis. This is in contrast to Taylor \textit{et al.} (1998), who found an increased virulence of mixed infections of 2 similarly virulent \textit{P. chabaudi} clones. Timms (2001) also found that virulence increased with some 2-clone infections, but not with other clone pairs.

We also found that the per clone potential transmission in the current experiment was lower in mixed-clone infections than in single-clone infections, whereas Taylor \textit{et al.} (1997) found that the 2 clones in a mixed-clone infection produced more gametocytes and infected more mosquitoes than when on their own. Transmission from mixed-clone infections might truly be variable: Ferguson & Read (2002), for example, showed higher gametocyte production in mixed-clone infections in one replicate, but not in the other replicate of an experiment.

In the field, different relationships between diversity of infections and virulence are also common (Read & Taylor, 2001).

It is, however, equally possible that different combinations of clones and different numbers of clones have different outcomes for clone competition, transmission and virulence. We are currently developing the techniques in our lab to study a whole range of clone combinations to address this question. This is a necessary step if we are ever to understand the impact of mixed-clone infections on virulence in the field.

We thank the staff of the March Animal House, University of Edinburgh, for excellent animal husbandry, and R. Carter, H. Ferguson, A. Graham, T. Lamb, the editor and 2 anonymous referees for helpful comments on an earlier version of this manuscript. This work was supported by the BBSRC, the University of Edinburgh and the Leverhulme Trust. Support for J. C. D.R. was kindly provided by the Stichting Wagenings Universiteit Fonds, the Hendrik Muller’s Vaderlandsh Fonds, and the Fundatie van de Vrijvrouwe van Renswoude te Delft, the Netherlands. The Darwin Trust of Edinburgh funded J. C. D.R. for the final stages of this research.

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Real-time quantitative PCR for analysis of genetically mixed infections of malaria parasites: technique validation and applications

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Received 31 March 2003; received in revised form 25 June 2003; accepted 27 June 2003

Abstract

A technique that can distinguish and quantify genetically different malaria parasite clones in a mixed infection reliably and with speed and accuracy would be very useful for researchers. Many current methods of genotyping and quantification fall down on a number of aspects relating to their ease of use, sensitivity, cost, reproducibility and, not least, accuracy. Here we report the development and validation of a method that offers several advantages in terms of cost, speed and accuracy over conventional PCR or antibody-based methods. Using real-time quantitative PCR (RTQ-PCR) with allele-specific primers, we have accurately quantified the relative proportions of clones present in laboratory prepared ring-stage mixtures of two genetically distinct clones of the rodent malaria parasite Plasmodium chabaudi chabaudi. Accurate and reproducible measurement of the amount of genomic DNA representing each clone in a mixture was achieved over 100-fold range, corresponding to 0.074% parasitised erythrocytes at the lower end. To demonstrate the potential utility of this method, we include an example of the type of application it could be used for. In this case, we studied the growth rate dynamics of mixed-clone infections of P. chabaudi using an avirulent/virulent clone combination (AS (PYR) and AJ) or two clones with similar growth rate profiles (AQ and AJ). The modification of the technique described here should enable researchers to quickly extract accurate and reliable data from in-depth studies covering broad areas of interest, such as analyses of clone-specific responses to drugs, vaccines or other selection pressures in malaria or other parasite species that also contain highly polymorphic DNA sequences.

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Keywords: Plasmodium chabaudi; Real-time quantitative PCR; Parasite clone

1. Introduction

Quantitative information on the genetic composition of the parasites present in malaria infections is important in many situations. For example, in the study of mixed infections containing drug-resistant and drug-sensitive parasites, in studies designed to investigate the competitive fitness of avirulent versus virulent genotypes, in vaccine trials, or in studies aimed at investigating 'strain-specific' immune responses to different parasite clones.

To date, progress on fundamental studies of this nature, has been hampered by the lack of a reliable and robust technique that can rapidly distinguish and accurately quantify genetically distinct parasite clones of the same species present in a mixed infection. Existing methods have significant drawbacks: monoclonal antibody-based methods are able to distinguish different parasites clones by virtue of their variant surface antigens, but, in order to be quantitative, they require labour intensive microscopic analysis using reagents that are often in scarce supply. Standard PCR, although sensitive, cannot reliably quantify mixed genotype infections as it is based on the analysis of end-point amplification products [1].

Real-time quantitative PCR (RTQ-PCR) circumvents the limitations imposed by standard PCR by identifying the position in the PCR reaction where the amount of DNA increases logarithmically from just above background to the plateau phase (called the log-linear phase). Continuous monitoring of PCR samples by fluorescence then allows the small fraction of cycles that fall within the log-linear range to be used to provide reliable information on the initial template concentration.

Many application-specific RTQ-PCR instruments and fluorescence detection systems are now available for research and diagnostics [2]. These packages offer real advantages over standard PCR in terms of their user-friendliness, speed
of operation and sensitivity of measurement. With the ability to quantify accurately over six to seven orders of magnitude, many systems are now capable of quantifying the amount of targeted DNA sequence to less than 1000 copies. This has made accurate quantification of very low parasite densities [3] as well as gene expression studies possible [4].

We report here, an adaptation to RTQ-PCR that has made it possible to measure accurately the relative proportions of parasites carrying different alleles of the same gene in mixed infections of genetically distinct clones of P. chabaudi chabaudi. The experiments described in this paper have two aims: (1) to test the ability of the technique to accurately and reliably quantify the proportions of two genetically distinct clones in a laboratory prepared mixture containing known proportions of each clone, and (2) provide a working example of the type of biological question that could be addressed by this technique. In the latter case, we have analysed the growth rate profiles of a virulent and avirulent clone combination (AJ:AS (PYR)) or a virulent/virulent clone combination (AJ:AQ) in laboratory mice. Although this method was developed for use with the P. chabaudi rodent malaria model for the purpose of studying clone-specific immune or drug-responses, the technology should be readily transferable to human malaria parasites or other parasite species whose genetic material contains a high degree of DNA sequence polymorphism.

For researchers interested in using this technique to determine the relative proportions of genetically distinct malaria clones present in clinical samples, however, an important point should be noted; because quantitation depends on use of allele-specific primers as the defining points of allelic variation, prior knowledge of all the clone genotypes present in a sample would be an essential prerequisite to ensure that unknown or novel parasite variants did not escape detection. Use of a primary PCR-based typing technique prior to quantitation may help circumvent such problems [5,6].

2. Material and methods

2.1. Preparation of blood mixtures from individual mice and DNA extraction

The origins of the cloned lines of P. chabaudi chabaudi “isolates” AS and AJ and the pyrimethamine-resistant clone AS (PYR) used in this study are described in [7,8]. Blood samples were collected from 5- to 8-week-old inbred female CBA mice infected with AS (PYR) or AJ clones. The parasite clones were harvested at the ring stage of the developmental cycle prior to DNA synthesis [9], to try to ensure that each parasite contained a single haploid genome.

The percentage parasitaemia of each clone was 7.4% for AS (PYR) and 18.7% for AJ as determined by microscopic analysis of Giemsa-stained thin blood smears obtained from mouse-tail blood. Red blood cell numbers in each sample were calculated by flow cytometry using a Coulter counter.

Blood samples infected with AS (PYR) or AJ clones were collected by exsanguination of individual mice under general anaesthesia and diluted in citrate saline, pH 7.2. The blood samples were then adjusted using blood taken from an uninfected mouse so that each sample contained the same number of parasites with the same red blood cell density (7.4 x 10^7 parasites in 1 x 10^9 RBC). These two suspensions, containing equal numbers of AS (PYR) or AJ parasite clones were mixed in different proportions in replicate ranging from 0.25% AS (PYR):99.75% AJ through to 99.75% AS (PYR):0.25% AJ. Blood samples were centrifuged briefly to pellet the erythrocytes, and the supernatant removed before the samples were frozen at -70°C prior to DNA extraction. Genomic DNA was extracted using the High Pure PCR Template Preparation Kit (Roche).

2.2. Preparation of blood mixtures from mouse-tail blood and DNA extraction

The percentage parasitaemia of each clone was determined by microscopic analysis of Giemsa-stained thin blood smears obtained from mouse-tail blood at 31% for AS (PYR) and 33.9% for AJ. Thirty microliters of AS (PYR) and 27 μl of AJ-infected mouse-tail blood was diluted to a final volume of 1500 μl in citrate saline. Each solution was used to prepare mixtures of AS (PYR) and AJ clones at ratios of 1:99, 10:90, 50:50, 90:10 and 99:1. Ten-fold and 100-fold dilution of these mixtures were prepared using uninfected blood from a control mouse as diluent to represent parasitaemias of 3 and 0.3%. Blood samples were centrifuged briefly to pellet the erythrocytes, and the supernatant removed before the samples were frozen at -70°C prior to DNA extraction using Instagene Matrix (BioRad).

2.3. Design of allele-specific primers

The primers used in these experiments were designed to amplify a fragment of the Merozoite Surface Protein 1 (MSP-1) of P. chabaudi chabaudi clones AS ([10]; accession no. L22982) AJ or AQ (Cheesman and Carter, unpublished sequence data). The MSP-1 gene, located on chromosome 8 [11] contains regions of high sequence diversity between clones that facilitate the design of allele-specific primers that can act as clone-specific genetic markers. The primer pairs were designed to yield PCR products of similar sizes for AS (PYR), AJ and AQ alleles. Many of the primers used during the course of this work lacked the high degree of specificity required for RTQ-PCR and were discarded. The following primers produced allele-specific amplicons free of primer-dimer or non-specific product contaminants. AS (PYR) forward and reverse primers sequences: 5'-ACAGTAAACACAAAGGAAAC-3' and 5'-GATCAGTGTTAGAGCTGG-3', AJ forward and reverse primer sequences: 5'-ACAGACACACAAAGGAAAC-3' and 5'-TTGGGGTTTCTGTTAGCT-3'. For the AQ and AJ reactions a different set of AJ primers were used:
AJ forward: 5'-ACTGAAGCAACAACACCGAC-3' and AJ reverse: 5'-GTGGTTGATGCACTTGGGGTTTC-3'. The reverse reaction was 5'-TTACCCCAAAACCGTAGTAC-3' and 5'-GTAGTTGGTGCTGTGGAG-3'. Primers were made by Oswell Research Products Ltd. and were HPLC purified. Primer pairs were designed with the aid of PrimerFinder Software (http://eatworms.swmed.edu/~tim/primerfinder/) to minimise the possibility of self-dimerisation or heterodimerisation that could lead to non-specific PCR amplicons.

2.4. Preparation of genomic DNA standards for RTQ-PCR

DNA standards were prepared by the following method to increase the quantity of genomic DNA recovered so that highly concentrated stocks of standard were available for long-term use.

Whole blood samples infected with AS (PYR) or AJ parasite clones were collected from five mice and used to prepare genomic DNA [7]. Briefly, mouse white blood cells were removed by column purification using CF11 (Sigma) and Plasmolipid filters (Euro-Diagnostica). Parasites were isolated from host erythrocytes by saponin lysis and genomic DNA prepared by phenol-chloroform extraction. DNA standards were treated with RNase before re-extracting with phenol-chloroform. The purity and quantity of the DNA was determined by fluorimetry, UV spectrophotometry, and electrophoresis of serially diluted ethidium-bromide stained samples on agarose gels. Standards consisting of 10-fold serial dilutions of genomic DNA were freshly prepared from concentrated stocks for each RTQ-PCR experiment.

Accurate quantification of the clones present in the mixed infection experiments described here necessitated very careful measurement and dilution of each standard (AS (PYR), AJ and AQ) to ensure they were close to equal in their DNA concentration. We found that fluorometric analysis was the most reliable determinant of DNA concentration and UV spectrophotometry the best determinant of DNA quality.

2.5. PCR

PCR was performed using a Roche LightCycler [12]. Ten microlitre reactions were set up using FastStart DNA Master SYBR Green I kit reagents according to the manufacturer's instructions in LightCycler capillaries (Roche).

Prior to the work described here, reaction components and cycling conditions were optimised to give reliable and reproducible results. Magnesium chloride and primer concentration, denaturation, annealing and elongation rates and times were varied to determine the conditions under which only the specific amplicon was produced. The average amplification efficiencies of the reactions were closely matched for each set of standards because small differences can result in large errors in quantification between samples. The average amplification efficiency, E, was determined from the slope of the standard curves produced in each experiment using the equation $E = 10^{-\frac{1}{Q}}$. PCR reactions where the amplicon doubles at every cycle have an optimal efficiency of 2.0 compared to reactions where no amplification occurs and the efficiency is 1.0. Factors that may influence the amplification efficiency include, for example, insufficient optimisation of reaction conditions, poor template quality, poor primer design, or the presence of PCR inhibitors in the reaction.

Reactions were set up with AS- or AJ-specific primer sets containing 0.4 μM forward and reverse primers and 4.5 and 3.5 mM MgCl₂, respectively. All samples were analysed in replicate within each LightCycler run. The amplification program comprised 40 cycles with the following steps: an initial "Hot Start" at 95 °C for 600 s followed by 95 °C with a 0 s hold, cooling at 20 °C/s to 95 °C with a 6 s hold for AS (PYR) or cooling at 20 °C/s to 61 °C with a 6 s hold for AJ, heating at 20 °C/s to 72 °C with a 7 s hold. The fluorescent signal produced from the amplicon was acquired at the end of the polymerisation step at 72 °C. Heating at 20 °C/s to 95 °C with a 0 s hold, cooling at 20 °C/s to 65 °C and heating at 0.2 °C/s to 95 °C in a continuous acquisition mode produced the melting curve data.

Samples of a 10-fold serial dilution of AS (PYR) or AJ gDNA in the range 60–0.006 ng were used as quantification standards for the LightCycler calibration curve. Samples prepared from artificial mixtures of parasite clones were analysed in replicate within the same run. Maximum recovery filter tips (AXYGEN® Scientific) and "No Stick" microtubes (Alpha Laboratories) were used in these experiments. LightCycler data were analysed by the Fit Points method.

Reactions that were set up to measure the proportion of AJ and AQ clones present in a sample used the same reaction conditions as described for the AS (PYR) and AJ clones except 4 mM MgCl₂ was used for AQ and 3.5 mM MgCl₂ for AJ reactions. The annealing temperature used for AJ and AQ reactions was 63 °C with a 6 s hold.

2.6. P. chabaudi mixed-clone infection experiments

Two sets of three CBA female mice were infected with a mixture consisting of $1 \times 10^6$ parasites of clones of AS (PYR) and AJ (Group 1) or $2.5 \times 10^6$ parasites of clones of AQ and AJ (Group 2). Each Group 1 mouse received a total of $2 \times 10^6$ parasites and each Group 2 mouse $5 \times 10^6$ parasites. Parasitaemias were monitored and recorded over the course of the infection by microscopic analysis of Giemsa-stained thin blood smears. Five microliters of tail blood was collected from Group 1 mice and 10 μl of tail blood from Group 2 mice into citrate saline on days 3–8 post-infection. The samples were centrifuged briefly and the RBC pellets stored at −70 °C prior to DNA extraction using the Instagene Matrix (BioRad) kit for AS (PYR) and AQ samples and the High Pure PCR Template Preparation Kit (Roche) for AQ and AJ samples.
3. Results

3.1. Allele-specific melting peaks and cross-reaction tests

A critical assumption underlying these experiments is that the primers used to detect AS (PYR) and AJ MSP-1 alleles are specific for each clone. To test this we conducted a cross-reaction test where AS (PYR) genomic DNA was amplified with AS- and AJ-specific primers, and AJ genomic DNA was amplified with AJ- and AS-specific primers (Fig. 1a and b). These results show the allele-specific melting peak produced by each clone. Only the clone-specific allele was amplified in each case. Use of the ‘wrong’ primer set produced either no signal or melting peaks characteristic of primer-dimer formation. No non-specific PCR products were produced when the correct clone-specific primers were used, as judged by analysis of the melting peaks produced by the LightCycler software. Cross-reaction tests were also conducted on AQ and AT DNA; in each case only the correct allele-specific melting peak was detected (data not shown).

Fig. 1. Fluorescence melting curve data derived from cross-reaction tests performed to confirm the specificity of AS (PYR) and AJ primers for their target sequence. (a) The AS-specific melting peak produced by the pure AS (PYR) clone using AS-specific primers and the signal produced using pure AJ genomic DNA. (b) The AJ-specific melting peak produced by the pure AJ clone using AJ-specific primers and the non-specific signal produced using AJ-specific primers with pure AS (PYR) genomic DNA.
Table 1: The proportions of AS (PYR) and AJ clones quantified by RTQ-PCR from duplicate mixtures (Experiments 1 and 2) containing different proportions of AS (PYR) and AJ blood-stage parasite clones.

<table>
<thead>
<tr>
<th>Expected % AS clone</th>
<th>Measured in Experiment 1</th>
<th>Measured in Experiment 2</th>
<th>Mean of Experiments 1 and 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>% AS clone</td>
<td>% AJ clone</td>
<td>% AS clone</td>
<td>% AJ clone</td>
</tr>
<tr>
<td>0</td>
<td>100</td>
<td>0.00</td>
<td>100.00</td>
</tr>
<tr>
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<td>100.00</td>
</tr>
<tr>
<td>0.50</td>
<td>99.5</td>
<td>0.38</td>
<td>99.62</td>
</tr>
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<td>0.00</td>
<td>100.00</td>
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<td>97.97</td>
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<td>94</td>
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<tr>
<td>99.75</td>
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</tr>
<tr>
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<td>0.00</td>
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</tr>
</tbody>
</table>

3.2. Measurements of MSP-1 AS and AJ alleles in laboratory prepared blood mixtures containing known proportions of each clone

Mouse blood infected with \(7.4 \times 10^7\) ml\(^{-1}\) of parasites representing clones of \(P.\ chabaudi\) \(chabaudi\) AS (PYR) or AJ both at an RBC density of \(1 \times 10^8\) RBC/ml (i.e. 7.4% parasitaemia) was used to make duplicate artificial mixtures containing different proportions of each clone. The proportions of the mixtures tested ranged from 0.25 to 100% AS (PYR) or AJ. In the lower range, for example, mixtures comprised 0.25% AS (PYR) and 99.75% AJ or the reverse, 0.25% AJ and 99.75% AS (PYR).

Table 1 shows the actual proportions obtained by RTQ-PCR for duplicate Experiments 1 and 2, together with the mean value obtained for both experiments. The results show that the mean proportions of AS (PYR) and AJ clones quantified in both experiments correlates well with the proportions expected from the mixtures. Data from the individual experiments show, however, that some of the reactions measuring in the lower ranges occasionally produced no specific PCR product (e.g. in Experiment 1 the value expected for AS (PYR) of 0.25% was quantified at 0%). Given that the accuracy of measurement in the higher ranges (above 2%) is very good, we attribute such discrepancies to the errors involved in measuring and dispensing very small volumes accurately during the actual preparation of the blood mixtures themselves.

All samples analysed in this experiment, with the exception of the standards, contain mouse host nucleated cell DNA. To ensure that both primer sets did not cross-react with the mouse DNA, we also analysed samples containing no parasites. No amplicon was produced by either set of AS (PYR) or AJ primers from such material (Table 1). The standard curves produced for AS (PYR) and AJ DNA in these experiments were linear over the four-log range of DNA concentration from 6 pg to 60 ng that was used (data not shown). The average amplification efficiencies \((E)\) for both sets of standards were checked to ensure that they were comparable in order to reduce the likelihood of quantification bias in the parasite mixtures analysed here. The average amplification efficiencies were calculated at 1.978 for AS (PYR) and 1.980 for AJ.

3.3. Sensitivity of measurement

We investigated whether the technique was sensitive enough to accurately quantify material containing a 50:50 mixture of AS (PYR) and AJ clones over two orders of magnitude below those used in the initial experiments (i.e. corresponding to 7.4, 0.74 and 0.074% parasitaemia or 74,000, 7400 and 740 parasites in the fraction of the sample analysed). The results, shown in Table 2, demonstrate that even at the lowest level tested (740 parasites), the deviation from the expected value was less than 11%. We also tested whether using DNA samples prepared by a less expensive, quicker method (Instagenie Matrix (BioRad)) using small quantities of mouse-tail blood would also produce accurate results on the LightCycler (Roche) system. For this experiment, artificial blood mixtures were prepared from mouse-tail blood in the proportions AS:AJ; 1:99, 10:90, 90:10, 99:1 and 50:50. These suspensions were used to provide a 10- and 100-fold dilution series to represent parasitaemias of 30, 3 and 0.3%, respectively. DNA was extracted from these samples using Instagenie Matrix (BioRad). Each sample was quantified twice and the proportion...
Table 2

<table>
<thead>
<tr>
<th>Calculated total number of AS or AJ parasites in fraction of sample analysed</th>
<th>Mean concentration of AS clone detected (ng/μl)</th>
<th>Mean concentration of AJ clone detected (ng/μl)</th>
<th>Expected % of AS or AJ detected</th>
<th>Mean % AS detected</th>
<th>Mean % AJ detected</th>
</tr>
</thead>
<tbody>
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<td>74000</td>
<td>0.5135</td>
<td>0.598</td>
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<td>46.2</td>
<td>53.8</td>
</tr>
<tr>
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<td>0.03165</td>
<td>50</td>
<td>50.3</td>
<td>49.7</td>
</tr>
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<td>0.007315</td>
<td>0.00594</td>
<td>50</td>
<td>55.2</td>
<td>44.8</td>
</tr>
</tbody>
</table>

of AS (PYR) measured was based on the mean of these experiments. The observed proportion of AS (PYR) was then plotted against the expected proportion of AS (PYR), as shown in Fig. 2. The results show that DNA extracted from mouse-tail blood using Instagene Matrix gives accurate estimates of the proportions of AS (PYR) and AJ clones present in the artificial mixtures, over a range of 30, 3 and 0.3% parasitaemia. All data points fit almost on a straight line of expected values.

3.4. Analysis of between run variation

In the experiments described so far we have shown that RTQ-PCR using allele-specific primers is both accurate and sensitive. We also investigated how robust the method was in terms of how well random errors within the system appear to be controlled between runs. Ten-fold serial dilutions (10- to 100,000-fold) of P. chabaudi Pd and AQ DNA were made on five independent occasions. Each dilution series was amplified with AQ- or Pd-specific MSP-1 primers on the LightCycler (Roche) in five independent runs. The Crossing Points, defined as the position in the reaction where the fluorescence of the PCR products become greater than the background signal from the SYBR Green 1 fluorescent dye, for each series were calculated using the LightCycler Software. Fig. 3a and b shows the mean values and standard deviation of each dilution series of AQ and AJ DNA. Both curves are linear over a five-log range. The percentage relative standard deviation (% rsd = (standard deviation/mean) x 100), a measure of the overall precision of the method was calculated at <3.6% for AQ and <2.3% for AJ DNA across the sample dilution range, indicating that the method is extremely robust across a wide range of dilutions for both clones.

3.5. Real-time quantitative PCR analysis of mixed-clone infections of AS and AJ or AQ and AJ in laboratory mice

Mixed infections of AS (PYR) and AJ (Fig. 4a) and AQ and AJ clones (Fig. 4b) were analysed using RTQ-PCR in three individual mice per group. The initial inoculum contained a 50:50 mixture of each clone, i.e. AS and AJ (Fig. 4a) or AQ and AJ (Fig. 4b). For the Group 1 mice, AJ rapidly dominated the infection over AS (PYR), but the proportion remained stable between the 3rd and 8th day of infection in all three mice. In the Pd + AQ mixture, AJ also dominated the infection, but less than in the AS + AJ mixture, and the proportions also remained stable between days 3 and 8 post-infection.

In both examples of mixed-clone infections, viz. AS + AJ and AJ + AQ, the proportions of each clone remained remarkably constant during the course of infection and between replicate infections in different mice. This is particularly striking, given the variation observed in terms of the day and peak level of parasitaemia, as was especially noticeable for the AJ + AQ combination (Fig. 4b). The observation suggests a rather tight biological and genetic control over the dynamic behaviour of the parasite clones in a malaria infection. Use of isogenic lines of laboratory mouse with cloned 'strains' of rodent malaria parasite has revealed biological phenomena that could not have been detected in natural human malaria infections.

4. Discussion

The aim of these experiments was to establish if we could adapt RTQ-PCR to accurately identify and measure the proportions of two genetically distinct clones of the rodent malaria parasite P. chabaudi chabaudi in a laboratory-prepared blood-stage infection. This approach has not, to our
Fig. 3. Reproducibility of the RTQ-PCR using MSP-1 AJ or AQ allele-specific primers. Five 10-fold serial dilutions of AJ or AQ DNA templates were prepared and amplified on five independent occasions. The means of the five Crossing Points are plotted for each concentration of DNA used. Error bars represent the standard deviation of each dilution within the series. The precision of the measurements, expressed as the percentage relative standard deviation (% rsd) was calculated at (AQ): undiluted DNA sample, 1.46%; 1/10 dilution, 3.62%; 1/100 dilution, 2.65%; 1/1000 dilution, 2.06%; 1/10,000 dilution, 1.82%; 1/100,000 dilution, 1.43%. (AJ): Undiluted DNA sample, 2.10%; 1/10 dilution, 1.13%; 1/100 dilution, 0.54%; 1/1000 dilution, 1.39%; 1/10,000 dilution, 1.50%; and 1/100,000 dilution, 2.35%.

knowledge, been attempted before in this context, and was made possible by the use of primers designed against MSP-1 gene sequences containing large regions of variability between the clones used in this study, AS (PYR), AJ and AQ.

Artificial blood mixtures containing a calculated number of parasite clones demonstrated that the technique is accurate and sensitive enough to measure over a wide range of different proportions ranging from 0.25% AS (PYR) and 99.75% AJ or the reverse, 0.25% AJ and 99.75% AS (PYR).

We used two different methods to prepare DNA for these experiments to determine whether the real-time method required highly purified template DNA such as was made using the High Pure PCR Template Preparation Kit (Roche) for accuracy or would give comparable results with templates prepared by less rigorous methods using smaller blood volumes (Instagene Matrix (BioRad)).

LightCycler data derived from DNA samples prepared by both methods gave results that were accurate over two orders of magnitude below the starting concentration.

The precision of the technique was tested over a five orders of magnitude dilution series with AQ and AJ genomic DNA diluted and amplified on five separate occasions. The percent relative standard deviation, even at the greatest dilution (1/100,000) was only 1.43% for AQ and 2.35% for AJ, indicating that the technique is repeatable over a wide range of DNA concentration. The precision across the whole dilution series was calculated to be less than 4% rsd for AQ primers and less than 2.5% rsd for AJ primers.

To demonstrate an application of the technique, we investigated the dynamics of mixed infections of *P. chabaudi* clones AJ with AQ or AJ with AS (PYR) in inbred CBA female mice. The starting inocula consisted of a 50:50 mixture of the parasites of each clone. In the AJ and AQ mixture, there was a moderate predominance of AJ over AQ, leading to about 70% AJ by day 8 of infection. In the AJ and AS (PYR) mixture, the predominance of AJ over AS (PYR) was much stronger leading to 90–95% AJ by day 8 of the infection. In each combination, there was remarkable consistency in the behaviour of the mixed clones between
Fig. 4. The mixed infection dynamics of *P. chabaudi* clones AS (PYR) and AJ ((a); Group 1 mice) and AQ and AJ ((b); Group 2 mice) measured using RTQ-PCR. Individual mice were inoculated with a mixture of $1 \times 10^6$ parasites of clones AS (PYR) and AJ (Group 1) or $2.5 \times 10^6$ parasites of clones of AQ and AJ (Group 2) representing a total of $2 \times 10^6$ or $5 \times 10^6$ clones, respectively. (a) shows the proportion of parasite clones quantified for AS (PYR) and AJ, and (b) AQ and AJ clones at days 3–8 post-infection together with the corresponding parasitaemia for the course of each infection.
individual infections. The relationships between the growth of AS (PYR) and AJ in the mixtures reflects the difference in growth rate we have observed between these two clones when examined in single-clone infection situations, with AJ consistently outgrowing AS (data not shown). Our data shows that AS (PYR) grows even more slowly than expected when in competition with AJ. The method is thus shown to be able to explore behaviour of the parasites not previously available to observation.

Having verified in these pilot experiments, the utility of the RTQ-PCR adaptation using AS (PYR), AJ and AQ clones of P. chabaudi, we have also confirmed the general applicability of the technique to other clone combinations (e.g. AS (PYR) and CB and AJ and CB clones of P. chabaudi, (results not shown). It is now our intention to use this technique to genotype and quantify the relative proportions of parasites present in mixed-clone infections of P. chabaudi within the context of 'strain-specific' immunity and drug-resistance. Our adaptation of the standard RTQ-PCR technique using allele-specific primers, should also provide scope for use with other malaria parasite species in more diverse situations.

Acknowledgements

We would like to thank Les Steven and Richard Culleton for their assistance in working with P. chabaudi infections in mice, Judi Allen and colleagues for use of the LightCycler instrument, Lisa Ranford-Cartwright and Andy Bell for their invaluable help and advise on LightCycler issues and Richard Culleton for critical reading of the manuscript. This work was supported by grants from the Wellcome Trust and BBSRC. J.C. de Roode was supported by the Darwin Trust of Edinburgh.

References

Host heterogeneity is a determinant of competitive exclusion or coexistence in genetically diverse malaria infections

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During an infection, malaria parasites compete for limited amounts of food and enemy-free space. Competition affects parasite growth rate, transmission and virulence, and is thus important for parasite evolution. Much evolutionary theory assumes that virulent clones outgrow avirulent ones, favouring the evolution of higher virulence. We infected laboratory mice with a mixture of two Plasmodium chabaudi clones: one virulent, the other avirulent. Using real-time quantitative PCR to track the two parasite clones over the course of the infection, we found that the virulent clone overgrew the avirulent clone. However, host genotype had a major effect on the outcome of competition. In a relatively resistant mouse genotype (C57Bl/6J), the avirulent clone was suppressed below detectable levels after 10 days, and apparently lost from the infection. By contrast, in more susceptible mice (CBA/Ca), the avirulent clone was initially suppressed, but it persisted, and during the chronic phase of infection it did better than it did in single infections. Thus, the qualitative outcome of competition depended on host genotype. We suggest that these differences may be explained by different immune responses in the two mouse strains. Host genotype and resistance could therefore play a key role in the outcome of within-host competition between parasite clones and in the evolution of parasite virulence.

Keywords: Plasmodium chabaudi; malaria; virulence; within-host competition; evolution; host genotype

1. INTRODUCTION

According to a large body of theory, competition within hosts generates selection for pathogens that do more damage to their host (i.e. are more virulent; Levin & Pimentel 1981; Bremermann & Pickering 1983; Frank 1992, 1996; Van Baalen & Sabelis 1995; Gandon 1998; Mosquera & Adler 1998; Ebert 1999; Read et al. 2002). Parasite fitness in singly infected hosts is assumed to be maximized when the benefits of host exploitation (increased transmission rate) balance the costs (increased host mortality) (Levin & Pimentel 1981; Anderson & May 1982; Bremermann & Pickering 1983; May & Anderson 1983; Sasaki & Iwasa 1991; Frank 1992, 1996; Antia et al. 1994; Van Baalen & Sabelis 1995; Antia & Lipsitch 1997). But, in genetically diverse infections, parasites that slowly exploit hosts will be outcompeted by those that exploit hosts more rapidly. Even if host life expectancy is reduced so that all parasites do worse, prudent parasites do disproportionately worse and are thus eliminated by natural selection.

The majority of mixed-infection models of virulence assume that more virulent strains have a competitive advantage. Logical as this may seem, there is hardly any experimental evidence to suggest that this is indeed the case, and some evidence that the opposite is true (Read & Taylor 2001). Within-host competition is certainly widespread and is mediated by limited resources, strain-transcending immune responses (apparent competition) or direct interference between competing genotypes (Read & Taylor 2001). In rodent malaria, for example, both resource and apparent competition probably play a role (Snounou et al. 1989, 1992; Hellriegel 1992; Taylor et al. 1997a; Taylor & Read 1998; Read & Taylor 2001; De Roode et al. 2003). But, while strains with a higher growth rate probably will do better in resource competition, they could also become a preferred target of strain-specific immune responses, giving less-abundant genotypes an advantage (Bruce et al. 2000; Read et al. 2002).

If there is a lack of experimental evidence to suggest that virulent clones are competitively superior to avirulent clones, there is even less evidence that any superiority is consistent across genetically different hosts. Numerous studies have shown that disease virulence varies with host genotype, with some host types being more susceptible to severe disease than others (e.g. Stevenson et al. 1982; Ebert & Hamilton 1996; Ebert et al. 1998; Imhof & Schmid-Hempel 1998; Carius et al. 2001; Mackinnon et al. 2002). This variation in resistance is likely to be reflected in variation in the strengths of resource- or immune-mediated competition between parasites within hosts. Indeed, one study has now shown that this is the case: an experiment on the endophyte Epichloë bromicola parasitic on the grass Bromus erectus showed that parasite strains that competitively excluded some strains on one host genotype were excluded themselves on another (Wille et al. 2002).

Here, we ask whether host genotype is also an important determinant of competitive outcome in a model of human disease. We studied mixed infections of virulent and avirulent Plasmodium chabaudi in singly infected mice. We then repeated this experiment in mice of different genotypes.
avirulent clones of the rodent malaria parasite *Plasmodium chabaudi* in two different strains of laboratory mice. Based on the theory outlined above, we predicted that the virulent parasite clone would rapidly outgrow the avirulent clone in both mouse strains. This did occur in one mouse strain, but in the other competitive suppression was transient, with the avirulent clone persisting to do better in the chronic phase of the infection than it would have done on its own.

2. MATERIAL AND METHODS

(a) Parasites and hosts

We used two genetically distinct *P. chabaudi* chabaudi clones, denoted AS(pyrlA) and AJ. AS(pyrlA) was derived through pyrimethamine selection from clone AS (Walliker et al. 1975). Both AS and AJ were originally isolated from thicket rats (Beale et al. 1978). For simplicity AS(pyrlA) will be referred to as AS from here on. We chose AS and AJ clones because they differ in their growth rates and virulences (Mackinnon & Read 1999), with AS producing fewer parasites and causing less virulence than AJ.

Hosts were eight-week-old C57Bl/6J and CBA/Ca inbred female mice (Ann Walker, University of Edinburgh). They were fed on 41B maintenance diet (Harlan, UK) and their drinking water was supplemented with 0.05% para- amino benzoic acid to enhance parasite growth (Jacobs 1964). They were kept in a 12 L:12 D cycle. We will refer to these mouse strains as C57 and CBA from here on.

(b) Experimental design and inoculation of mice with parasites

The experiment consisted of three treatments for both C57 and CBA mice: infections with AS alone infections with AJ alone and mixed AS+AJ infections. Each treatment group had five mice, resulting in 30 mice in total. Mice infected with just AS or AJ received 10^6 parasites, whereas mice infected with both clones received 2 x 10^6 parasites, made up of 10^6 AS and 10^6 AJ parasites. The latter quantity was chosen because in our analysis we wanted to compare the performance of a clone on its own with its performance in a mixed infection, requiring equal numbers of each parasite clone at inoculation. Although mice that were infected with both clones received a double dose of parasites, we know from previous work that a twofold difference in parasite numbers has a negligible effect on parasite dynamics and virulence (Timms et al. 2001).

We prepared inoculations from donor mice by diluting blood in 0.1 ml of calf serum solution (50% heat-inactivated calf serum, 50% Ringer's solution (27 mM of KCl, 27 mM of CaCl_2 and 0.15 M of NaCl) and 20 units of heparin ml^-1 mouse blood), and injected them via the intraperitoneum. All procedures were carried out under Home Office guidelines.

(c) Monitoring of virulence and infections

To monitor virulence, we recorded mouse live weights and densities of red blood cells (using flow cytometry; Beckman Coulter). Both body mass and red blood cell density decrease dramatically during infection, and these decreases correlate with host mortality and thus virulence (Mackinnon et al. 2002).

To monitor production of asexual parasites and gametocytes (the sexual transmission stages) we took thin blood smears from tail blood. These were fixed with methanol and stained with Giemsa to determine levels of asexual parasitaemia and gametocytaemia using 1000x microscopy. When asexual parasitaemia was high, we counted 500 red blood cells in at least four microscopic fields. With lower parasitaemias and gametocytaemias we counted at least 20 microscopic fields (corresponding to at least 10 000 red blood cells), and calculated the average number of cells per field. Gametocyte numbers were counted using polarized light. We calculated parasite densities and gametocyte densities as the products of parasitaemias and gametocytaemias, respectively, and red blood cell densities taken on the same day.

We took measurements every day from day 0 to day 23 post-infection (PI), three times a week from day 23 to day 48 PI, once a week from day 48 to day 69 PI and on day 83 PI, when the experiment was terminated. Measurements were taken between 09.00 and 10.30, when peripheral blood almost exclusively harboured hamophil ring stages of the parasites.

(d) Monitoring of individual clones in mixed infections

We collected 5 μl samples of tail blood from mice harbouring mixed infections in citrate saline. At 1 000 r.p.m., we removed the supernatant and stored the pelleted blood at –70 °C for subsequent DNA extraction using Instagene Matrix (BioRad). We used real-time quantitative PCR to measure the DNA concentrations of both AS and AJ in these samples (Cheesman et al. 2003) and calculated the proportions of AS and AJ in the total parasite population. Absolute numbers of AS and AJ were then calculated by multiplying these proportions by the overall parasite density on the same day.

We took blood samples on the same days that we took our other measurements, but performed PCRs only when parasitaemias were higher than 0.1%, which is the lowest level at which we can accurately estimate AS and AJ proportions (Cheesman et al. 2003). Our real-time quantitative PCR protocols cannot distinguish between asexual parasites and gametocytes, and so they estimate the densities of all parasites. In our data analyses, we treated these as estimates of asexual density. Gametocyte densities were two to three orders of magnitude lower than asexual-parasite densities, and gametocytes are thus a negligible component of overall parasite numbers.

(e) Trait definition

Prior to statistical analysis we defined and constructed the following traits that described part of or all of the infection. For measures of virulence we determined the 'minimum weight' and 'minimum density of red blood cells' that mice reached. Mice that died were included in our analysis of minimum weights and red blood cell densities, using weight and red blood cell density on the day of death as the minima they reached. For all other traits, these mice were excluded from the analyses.

Parasite levels in mice rose and fell several times, with the first peak being substantially higher and lasting for longer than the other peaks. For each mouse, we calculated the period until the end of the first wave, and defined this as the acute phase of the infection. The subsequent period, the chronic phase, was defined as starting on the day that parasite numbers began to recover after the collapse of the first wave (day 15.6 ± 0.55 PI, mean ± 1 s.e.). To calculate the numbers of parasites present during the whole infection and during the acute and chronic phases, we calculated the areas under the relevant parasite density by day PI curves. As our limit of detecting parasites was ca. 100 μl^-1, we treated observations of zero parasites as being 100 parasites μl^-1. We could accurately quantify AS and AJ.
proportions of 0.01 at levels of 0.1% parasitaemia or higher (Cheesman et al. 2003); below that we set the clone densities at our limit of detection (100 μl⁻¹). Parasite densities in single infections were set to the same level when parasitaemias were lower than 0.001%.

For gametocytes, we chose slightly different ways of calculating densities, as they showed different dynamics from asexual parasites, having a first wave before day 9 and one or several thereafter. We therefore calculated the total gametocyte densities over days 0–9 and days 10–83 PI, as well as the total density over the whole infection. We were able to analyse overall numbers of gametocytes only, as our real-time quantitative PCR protocol does not distinguish between AS and AJ gametocytes (see §2d).

(f) Statistical analysis

We analysed all traits mentioned in §2e using ANOVAs and ANCOVAs in MINITAB (v. 13.30, Minitab Inc.). The explanatory variables used were mouse 'strain' and infection 'treatment'. Strain had two factor levels (C57 and CBA); treatment had up to three factor levels (AS, AJ and AS+AJ), depending on the analysis. For all our models we first fitted the maximal model including covariate (when relevant), treatment, strain and an interaction between treatment and strain. We then minimized the models by removing non-significant terms (p > 0.05), beginning with the interaction.

We log-transformed initial and minimum densities of red blood cells as well as all parasite and gametocyte densities prior to analysis, to meet the necessary normality and homogeneity-of-variance assumptions.

(g) Follow-up experiment

To confirm the most important findings of this study, we infected groups of five C57 and five CBA mice with AS+AJ, exactly as described in §2b, and took blood samples from them on days 6 and 13 PI. We then extracted DNA and analysed these DNA samples with real-time quantitative PCR, to determine whether both clones were present, and in what proportions.

3. RESULTS

One C57 mouse infected with AJ died on day 12 and one infected with AS+AJ died on day 7; two CBA mice infected with AS+AJ died on days 10 and 11 PI. AJ infections induced greater weight loss and lower minimum red blood cell densities than AS infections (treatment: F1,17 = 25.4, p < 0.001; and treatment: F1,18 = 25.3, p < 0.001, respectively), regardless of mouse genotype (strain and treatment × strain n.s.). Thus, as found previously, AS was the less virulent clone.

(a) AS and AJ parasite densities

In mixed infections in both C57 and CBA mice, there were substantially more AJ than AS parasites during the first 10–14 days (figure 1a,b). After day 10, AS disappeared below detectable levels in C57 mice and never reappeared. It persisted, however, in the three CBA mice that survived the first two weeks. One of these experienced separate AS and AJ parasite waves around days 35 and 55 PI (figure 2a). In the other two mice, AS started to overgrow AJ around days 22 and 18, respectively (figure 2b,c). Later in the infection AJ overgrew AS again in one of these (figure 2c).

Formal analysis confirmed this picture. During the acute phase in both mouse strains, AS was competitively suppressed by AJ, producing far fewer parasites in mixed infections than it did alone (figures 1c,d and 3a; treatment: F1,13 = 155, p < 0.001). This suppression was greater in CBA than in C57 mice (treatment × strain: F1,13 = 9.1, p = 0.01). In C57 mice, AS disappeared below detectable levels before the end of the acute phase, showing competitive exclusion.

In CBA mice, AS was also competitively suppressed during the acute phase, but it was not excluded from the infection. During the chronic phase, it produced more parasites than it would have done on its own (figures 1d and 3b; treatment: F1,13 = 6.8, p = 0.022; treatment × strain: F1,13 = 34, p < 0.001), thus showing that, after the chronic phase, AS actually benefited from the presence of AJ (that is, facilitation, not competition). During both the acute and chronic phases, AS produced more parasites in CBA mice than in C57 mice, whether it was alone or in a mixture (figure 3a,b; strain: F1,13 = 125, p < 0.001; F1,13 = 52, p < 0.001, respectively).

During the acute phase, AJ produced roughly the same numbers of parasites in mixed and single infections, in both C57 and CBA mice (figures 1e,f and 3c; treatment: F1,12 = 1.42, p = 0.25), thus showing that, unlike AS, AJ did not suffer from competition. Like AS, it produced more parasites in CBA than in C57 mice (strain: F1,12 = 27, p < 0.001). During the chronic phase, AJ produced slightly fewer parasites in C57 mice, but more in CBA mice, than it did on its own (figure 3d; treatment × strain: F1,12 = 8.1, p = 0.015). When analysing these numbers for CBA mice only, however, there was no difference between single and mixed infections (treatment: F1,8 = 2.7, p = 0.15), thus showing that, unlike AS, AJ did not experience facilitation during this phase.

(b) Follow-up experiment

In the follow-up experiment, we infected another five C57 and five CBA mice with AS+AJ, and sampled these on day 6 and day 13 PI. In both mouse strains AS was present on day 6. By day 13, however, AS had disappeared below detectable levels in the peripheral blood of C57 mice (figure 4a), but was still present in CBA mice (figure 4b), actually overgrowing AJ at this time. These results thus confirmed the qualitatively different dynamics of mixed infections in C57 and CBA mice observed in the main experiment.

(c) Overall parasite densities

During the acute phase, infections in C57 mice consisted of fewer parasites than those in CBA mice (figure 3e; strain: F1,20 = 110, p < 0.001). In both mouse strains, AJ and AS+AJ infections produced more parasites during the acute phase than did AS infections (treatment: F2,20 = 74, p < 0.001).

During the chronic phase, AJ and AS+AJ infections produced more parasites than did AS infections (figure 3f; treatment: F2,20 = 28, p < 0.001). AJ and AS+AJ infections also differed from each other, but differently in the two mouse genotypes: in C57 mice AS+AJ infections produced slightly fewer parasites than AJ infections, whereas...
in CBA mice they produced more (figure 3f; treatment × strain: $F_{2,20} = 11$, $p = 0.001$). Owing to these higher numbers in CBA mice during the chronic phase, AS+AJ infections produced more parasites in CBA mice over the whole of the infection than did AJ infections (treatment × strain: $F_{2,24} = 26$, $p < 0.001$).

**d) Gametocyte densities**

In C57 mice, most gametocytes were produced during the first 9 days of the infection, with AS having lower densities than AJ and AS+AJ (figure 5a; treatment: $F_{2,27} = 11.71$, $p < 0.001$), whereas in CBA mice most gametocytes were produced after day 9 PI, when AJ produced fewer gametocytes than AS and AS+AJ (figure 5b; treatment: $F_{2,20} = 5.0$, $p = 0.017$; strain: $F_{1,20} = 6.04$, $p = 0.023$; treatment × strain: $F_{2,20} = 6.1$, $p = 0.009$). In C57 mice, gametocyte peaks in mixed infections mostly resembled those in AJ infections, the numerically dominant clone (figures 5a and 1a). In CBA mice, mixed infections produced gametocyte dynamics that did not resemble gametocyte production in either AS or AJ single infections, but which peaked during a period when both clones were present in high numbers (figures 5b and 1b). Summarized over the whole infection, AJ produced most gametocytes in C57 mice, but fewest in CBA mice, in which AS produced most (strain: $F_{1,20} = 8.6$, $p = 0.008$; treatment × strain: $F_{2,20} = 8.3$, $p = 0.002$).

**4. DISCUSSION**

Our results show that host genotype affects the outcome of within-host competition between pathogen strains. In C57 mice, the avirulent clone disappeared below detectable levels from the peripheral blood after 10 days of the infection and produced far fewer parasites than it would have done on its own. This competitive suppression almost certainly reduced the clone's transmission potential: the period of greatest gametocyte production in single infections occurred after day 10 PI (figure 5a). As the avirulent clone never reappeared during the course of our study, it seems that it was competitively excluded, something we have not observed before (Taylor et al. 1997b; Taylor & Read 1998; Read et al. 2002; A. F. Read and M. A. Anwar, unpublished data). By contrast, the virulent clone did not suffer at all from competition in mixed infections.

In CBA mice, the avirulent clone was also competitively suppressed during the acute phase, but it persisted and went on to produce more parasites during the chronic phase than it would have done on its own. Thus, in CBA
mice, competitive suppression gave way to facilitation. This may have resulted in enhanced transmission: the density of the avirulent clone peaked at around day 20 PI and coincided with a large gametocyte peak (figures 1d and 5b). Whether the presence of the more virulent clone actually enhanced the overall fitness of the avirulent clone would depend on the host mortality rate induced by the virulent clone and on how many of the gametocytes produced around this time were of the virulent clone.

Our experiment, with just two parasite clones and two mouse strains, generated the whole spectrum of outcomes currently captured in a range of different mixed-strain models of virulence evolution. In extreme coinfection models, clone dynamics are unaffected by the presence of other clones (e.g. May & Nowak 1995; Leung & Forbes 1998); in our experiments, this was so for clone AJ in both mouse strains. Coexistence with competitive suppression of at least one clone, as envisaged in other models (e.g. Sasaki & Iwasa 1991; Frank 1992, 1996; Herre 1995; Van Baalen & Sabelis 1995), occurred within CBA mice. Finally, the competitive exclusion of AS from C57 mouse before much transmission-stage production occurred bears substantial resemblance to superinfection models (which are perhaps better called superseding infection models; Van Baalen & Sabelis 1995). These models allow no coexistence and postulate that competitive suppression instantly reduces transmission to zero (e.g. Levin & Pimentel 1981; Bremermann & Pickering 1983; Bremermann & Thirne 1989; Knolle 1989; Nowak & May 1994; Leung & Forbes 1998; Gandon et al. 2002). Thus, it may prove difficult to capture the real-world complexities of a disease such as malaria (and others; Hood 2003) in models that assume that the outcome of competition is independent of environmental conditions, such as host genotype.

A general conclusion of the above models is that mixed-clone infections will generate selection for increased virulence. However, the precise details of competition will affect the transient evolutionary dynamics and the magnitude of standing virulence in a population, which is what is relevant to animal and human health. Competitive exclusion of avirulent clones, for instance, will affect potential evolutionary trajectories, rates of evolution and average levels of virulence in a population.

Several recent studies have shown that parasite virulence depends on host genotype (e.g. Ebert & Hamilton 1996; Ebert et al. 1998; Imhof & Schmid-Hempel 1998; Carius et al. 2001; Mackinnon et al. 2002), and theory has shown that such host-genotype dependence of virulence can explain why polymorphisms in growth rate and virulence are maintained (Regoes et al. 2000). If the outcome of competition is as dependent on host genotype as is the expression of virulence itself, this could also contribute to the maintenance of growth-rate and virulence polymorphisms in the parasite population. Wille et al. (2002) also suggested this when they found that some strains of the endophyte E. bromicola competitively excluded other strains on one genotype of its host B. erectus, but not on another.

Clone AS was competitively suppressed by AJ, except during the chronic phases of infections in CBA mice, where AS did better than it did on its own. This could be the result of a combination of strain-specific immunity and antigenic variation. In two of the surviving CBA mice we saw AS overgrow AJ after it had been suppressed (figure 2b,c). This might suggest that the immune system had been focusing on the clone that had produced the highest number of parasites up until then (AJ), giving AS some advantage. Strain-specific immunity is certainly well known in P. chabaudi (e.g. Jarra & Brown 1985; Buckling & Read 2001; R. Carter, unpublished results), and facilitation of one pathogen as a result of the immune response focusing on another has also been suggested for fungal species infecting leaf-cutter ants (Hughes & Boomsma 2004). Plasmodium chabaudi is also known to produce antigenic variants at high rates (McLean et al. 1982, 1990; Brannan et al. 1994; Phillips et al. 1997). Novel antigenic variants escape variant-specific host responses, and when there are more clones in an infection, each generating variants, it seems likely that the immune response may be less efficient at controlling the infection. A combination of strain-specific immunity and antigenic variation has also been suggested to play a role in mixed-species and mixed-strain infections of plasmodia in humans (Bruce et al. 2000).

Figure 2. Log AS (solid lines) and AJ (dashed lines) parasite densities over time in mixed AS+AJ infections for the three CBA mice that survived the acute phase. As the limit of detection was 100 parasites $\mu l^{-1}$ blood, y-axes start at 2.
Figure 3. Numbers of parasites produced during (a,c,e) the acute phase and (b,d,f) the chronic phase of the infection for C57 (solid lines) and CBA (dashed lines) mice (mean ± 1 s.e.). Plotted points are: (a) and (b) AS parasite densities in single AS and mixed AS+AJ infections; (c) and (d) AJ parasite densities in single AJ and mixed AS+AJ infections; and (e) and (f) overall parasite numbers in single AS, single AJ and mixed AS+AJ infections. All data points are based on five replicate mice, except for single AJ infections in C57 mice (four mice), mixed AS+AJ infections in C57 mice (four mice) and mixed AS+AJ infections in CBA mice (three mice). The asterisk denotes a parasite density below detection, as AS disappeared from mixed AS+AJ infections in C57 mice.

Figure 4. Proportions of AS (open bars) and AJ (filled bars) (mean ± 1 s.e.) in mixed AS+AJ infections in (a) C57 and (b) CBA mice on days 6 and 13 PI. Data are based on five replicate mice in a follow-up experiment. The asterisk denotes a proportion of 0, as AS disappeared below detection levels from these mixed AS+AJ infections in C57 mice.

Why did infections in C57 and CBA mice show such different dynamics and outcomes of competition? One important possibility is the strain difference in the efficacy of immune control. Mouse strains differ considerably in their resistance to *P. chabaudi* infections (Stevenson et al. 1982). Resistance is associated with reduced parasite densities, and appears to be a complex genetic trait, involving many genes affecting immunity and the production and characteristics of red blood cells (Stevenson et al. 1982; Yap et al. 1994; Fortin et al. 2002). Parasite densities were higher in CBA mice (figure 3e), and such higher densities could reduce the chance of stochastic loss and increase the chance of producing antigenic escape variants.

If differences in overall immune control do explain the strain differences in the outcome of competition, we would expect to find coexistence of the two parasite clones in C57 mice that were made more susceptible, for example by administration of anti-interleukin 12 monoclonal antibodies (Yap et al. 1994). Conversely, we would expect clone AS to disappear in CBA mice that were made more resistant, for example by artificially increasing their interleukin-12 levels (Yap et al. 1994).

If overall resistance does affect the outcome of within-host competition, then we would expect to find differences not only between host genotypes, but also between individuals that differ in their sex, nutritional or health status, vaccination status, or any other factor that influences resistance. Semi-immune mice are much more resistant to
**Figure 5.** Log gametocyte densities over time (mean ± 1 s.e.) for (a) C57 and (b) CBA mice infected with single AS (thick solid lines), single AJ (dashed lines) or mixed AS+AJ (thin solid lines) infections. Shown are overall (AS+AJ) numbers of gametocytes only, as our real-time quantitative PCR cannot be used to distinguish between AS and AJ gametocytes. All data points are based on five replicate mice, except for single AJ infections in C57 mice (four mice after day 12), mixed AS+AJ infections in C57 mice (four mice after day 7) and mixed AS+AJ infections in CBA mice (four mice on day 11 and three mice from day 12 onwards). As the limit of detection was 100 gametocytes μl⁻¹ blood, y-axes start at 2.

*P. chabaudi* infection than are naïve mice (Buckling & Read 2001; Mackinnon & Read 2003). Extrapolating from our results, we would expect that only the virulent clone would survive in such vaccinated hosts. It is important to test this, because this implies that vaccination could increase the frequency of virulent clones in a population, thus leading to unforeseen consequences of vaccination (Gandon et al. 2001, 2003; Read et al. 2004).

We thank D. Walliker for providing us with clone AS(pyr1A), B. H. K. Chan for technical assistance, the March animal house personnel for excellent husbandry, and M. A. Guinnee and M. J. Mackinnon for critical reading of an earlier version of this manuscript. The work was supported by the Wellcome Trust, and J.C.d.R. was supported by the Darwin Trust.

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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.
Competitive release of drug resistance following drug treatment of mixed *Plasmodium chabaudi* infections
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Published: 14 September 2004
Received: 15 June 2004

Accepted: 14 September 2004

This article is available from: http://www.malariajournal.com/content/3/f/33

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Abstract

**Background:** Malaria infections are often genetically diverse, potentially leading to competition between co-infecting strains. Such competition is of key importance in the spread of drug resistance.

**Methods:** The effects of drug treatment on within-host competition were studied using the rodent malaria *Plasmodium chabaudi*. Mice were infected simultaneously with a drug-resistant and a drug-sensitive clone and were then either drug-treated or left untreated. Transmission was assessed by feeding mice to *Anopheles stephensi* mosquitoes.

**Results:** In the absence of drugs, the sensitive clone competitively suppressed the resistant clone; this resulted in lower asexual parasite densities and also reduced transmission to the mosquito vector. Drug treatment, however, allowed the resistant clone to fill the ecological space emptied by the removal of the sensitive clone, allowing it to transmit as well as it would have done in the absence of competition.

**Conclusion:** These results show that under drug pressure, resistant strains can have two advantages: (1) they survive better than sensitive strains and (2) they can exploit the opportunities presented by the removal of their competitors. When mixed infections are common, such effects could increase the spread of drug resistance.

**Background**
Malaria infections often consist of more than one parasite genotype [1-3]. Humans represent ecological niches for co-infecting malaria parasites, with shared predators (immune responses) and limited resources, so that competition between co-infecting malaria strains is likely to be intense [4]. Such competition could strongly affect the relative transmission of newly arisen drug-resistant strains, and thus the spread of drug resistance [5].

Resistant and sensitive strains will co-occur in the same host both when *de novo* mutations arise, and when hosts acquire resistant and sensitive strains from one mosquito bite simultaneously or from different mosquito bites. In the absence of drug treatment, the transmission success of the resistant strain will depend on its intrinsic fitness and competitive ability. However, if drug treatment does occur, the resistant strain has two potential fitness advantages. First, it will better survive the drug than the sensitive strain. Second, treatment can remove drug-sensitive
competitors, thus freeing up ecological space for the resistant strains to occupy; this would increase the relative transmission of the drug-resistant strain. This second effect, well recognized in theory, has the potential to greatly enhance the rate of spread of drug resistance in a population [5]. However, there is no direct experimental evidence that removal of competitors by drug treatment does enhance the transmission of drug-resistant parasites. This paper reports the first direct experimental demonstration that competitive release of drug-resistant strains can occur following drug treatment.

Competition between drug-sensitive and resistant malaria clones was studied using the rodent malaria P. chabaudi. This parasite is commonly used as a model for human malaria [6], and has been extensively used to study drug resistance [7]. In the absence of drugs, the drug-resistant clone is competitively suppressed by a drug-sensitive clone [8]. Here, competition between the two strains in drug-treated and untreated mice is compared.

Methods
Two genetically distinct P. chabaudi clones were used: an AS clone resistant to the antifolate drug pyrimethamine [9], and A1, a sensitive clone. These clones will be referred to as R (for resistant) and S (for sensitive) from hereon. Hosts were eight weeks old CBA/Ca inbred female mice (Ann Walker, University of Edinburgh; Harlan, England). Two experiments were performed. In the first, two groups of five mice were infected with 10^6 R parasites, and two groups with 10^6 R + 10^6 S parasites, as described elsewhere [8]. One group from each of these two infection types was drug-treated within three hours of inoculation and again on days 1, 2 and 3 PI (post-infection), using an oral administration of 8 mg pyrimethamine per kg mouse body weight.

Asexual parasite densities and gametocyte densities – the latter being the transmission stages to the mosquito – were monitored using microscopic examination of thin blood smears and determination of red blood cell densities using flow cytometry (Beckman Coulter), as described elsewhere [8]. Real-time quantitative PCR was used to distinguish and quantify R and S parasites in mixed infections [8,10]. This protocol cannot distinguish between asexual parasites and gametocytes, but real-time PCR data were used as estimates of asexual densities, because gametocyte densities were 2–3 orders of magnitude lower than asexual densities and thus a negligible component of overall parasite numbers. For each infection, two phases were distinguished: the acute phase, involving the first wave of parasites, and the chronic phase, beginning when parasite numbers began to recover after the collapse of that first wave around day 12. All parasites had disappeared below detectable levels after 50 days.

In the second experiment, two groups of nine mice were infected as above with either R parasites or R+S parasites. The subsequent transmission success of clone R was assayed by allowing batches of 30 starved Anopheles stephensi mosquitoes to feed on 3 mice from each group on each of days 7, 14, and 21 PI, as described elsewhere [e.g. [11]]. Eight days after the feeds, mosquitoes were dissected, and DNA extracted from midguts carrying oocysts. Real-time quantitative PCR was subsequently used to determine the prevalence of clone R in these mosquitoes.

All procedures were regulated and carried out under the British Home Office Animals (Scientific Procedures) Act 1986.

Results
Two untreated mice infected with R+S parasites died on days 10 and 11 PI respectively, and were excluded from the analysis.

In untreated mice, there were far fewer R parasites during the acute phase in mixed infections with clone S than in R-only infections (Figures 1a,1c). However, in drug-treated mice, where S parasites were entirely removed by pyrimethamine (none of the PCR reactions performed were positive for clone S), there were as many R parasites in mixed infections as there were in R-only infections (Figures 1b,1c; Drug treatment x Alone/Mixed interaction: F_{1,14} = 1.44, p = 0.002). Thus, R parasites were competitively suppressed in mixed infections in untreated mice, but this suppression was negated when mice were treated with pyrimethamine, which effectively removed S parasites.

During the chronic phase, clone R was more numerous in untreated mice in mixed infections than in single-clone infections (due to the parasite peak around day 21; Figures 1a,1d). Thus, in untreated mice in the chronic phase, clone R did not suffer from competition, and actually benefited from the presence of clone S (facilitation). In drug-treated mice, however, R parasites were similarly numerous in mixed- and single-clone infections (Figures 1b,1d; Drug treatment x Alone/Mixed interaction: F_{1,14} = 13.8, p = 0.002).

The large peak of R parasites in the chronic phase in the untreated mixed infections around day 21 (Figure 1a) coincided with a large peak of gametocytes, the transmissible stages of the parasite (Figure 2a). This was in contrast with single-clone infections of R in untreated mice, and infections in drug-treated mice, where gametocytes were mainly produced around day 14 (Figures 2a,2b). Overall,
Figure 1
Log asexual parasite densities of the resistant clone R over time in untreated (a) and drug-treated (b) mice infected with R alone or a mixture of R+S clones, and total numbers of R parasites produced over the acute (c) and chronic phases (d). All data points (mean ± 1 s.e.m.) are based on 5 replicate mice, except for mixed infections in untreated mice in (a) (4 mice on day 11 and 3 mice from day 12 onwards) and (c) and (d) (3 mice). As the limit of detection was 100 parasites per μl blood, y-axes in (a) and (b) start at 2.

Discussion
These results show that drug treatment of malaria infections can severely affect ecological interactions between co-infecting strains. The drug-resistant clone was competitively suppressed by the drug-sensitive clone in untreated mice, in terms of both within-host growth and transmission to the mosquito vector. However, drug treatment removed that competitive suppression, and allowed the resistant clone to fill the ecological space emptied, giving it a substantial and additional fitness benefit in addition to the competitive suppression of the resistant clone in untreated infections translated into reduced transmission success.
In untreated (a) and drug-treated (b) mice. In (a) gametocyte densities for mixed R+S infections reflect overall R+S gametocytes, as the PCR assay could not distinguish between these (see text); in (b) all gametocytes are produced by clone R, as clone S was cleared from mixed infections. All data points are based on 5 replicate mice, except for mixed infections in untreated mice in (a): 4 mice on day 11 and 3 mice from day 12 onwards. As the limit of detection was 100 gametocytes per μl blood, y-axes start at 2.

Figure 2
Log gametocyte densities over time (mean ± 1 s.e.m.) for untreated (a) and drug-treated (b) mice. In (a) gametocyte densities for mixed R+S infections reflect overall R+S gametocytes, as the PCR assay could not distinguish between these (see text); in (b) all gametocytes are produced by clone R, as clone S was cleared from mixed infections. All data points are based on 5 replicate mice, except for mixed infections in untreated mice in (a): 4 mice on day 11 and 3 mice from day 12 onwards. As the limit of detection was 100 gametocytes per μl blood, y-axes start at 2.

Figure 3
Proportions of mosquitoes infected with the resistant clone R (mean and 95% confidence interval); mosquitoes fed either on mice infected with clone R alone or mice infected with a mixture of clones R and S. Means are based on 9 mice (3 on day 7, 3 on day 14 and 3 on day 21 PI) from which totals of 205 (R alone) and 216 (mixed R+S) mosquitoes took a blood meal. Infection with clone R was assessed by real-time PCR.

to the simple survival advantage conferred by resistance. Thus, under drug pressure, resistant strains can have two advantages: they survive better than sensitive strains and they can exploit the opportunities presented by the removal of their competitors, thereby increasing their relative transmission. Competition was studied between two unrelated clones, and thus did not reflect the situation in which a resistant clone arose de novo [13], but it seems likely that the competitive release following drug therapy would also apply there.

Competitive release following drug treatment will greatly enhance the spread of drug resistance [5]. Also, with only the resistant strain left in the host, the probability of outbreeding is reduced, thus reducing the chances of meiotic recombination destroying multi-locus resistance [14]. In combination, these two processes could enhance the spread of drug resistance, especially in areas with high numbers of strains per infection [5].

Of course, this is an argument for judicious use of drugs, not their non-use. Clearance of drug-sensitive strains from mixed infections might enhance the spread of drug resistance, but this has to be offset against the short-term public health benefits, such as reducing overall malaria prevalence. In these experiments, the drug-sensitive clone was
also the more virulent clone [8], and when it was cleared from mixed infections by drug treatment, mice were less sick, in that they lost less weight and became less anaemic (results not shown).

In this experiment, mice were drug-treated before symptoms occurred, resulting in competitive release. This situation perhaps best mimics the case of prophylactic drug use, or what might occur to new co-infections in high transmission areas where drug use is common. A battery of more complex experiments will be necessary to determine if competitive release occurs when treatment follows symptoms, and when drugs are used to treat semi-immune individuals. The facilitation observed in chronic infections (Figures 1a,1d) suggests the situation might be very complex.

Within-host competition in *P. chabaudi* is now firmly established [8,15,16]. Evidence for competition between co-infecting genotypes in human malaria infections is necessarily indirect, but consistent with this [4]. In older children and adults, for example, parasite densities do not increase with increasing numbers of clones, thus indicating that parasite clones are not regulated independently [17]. Given this, and the high frequency of mixed infections in human malaria [1-3,18] often consisting of both resistant and sensitive genotypes [19], and the fact that genetic diversity can be altered by antimalarial prophylaxis [20], it is highly likely that competitive release of drug resistance also occurs in human malaria. Indeed, a recent study has already implicated release of within-host competition as a key-factor in the spread of drug resistance in Uganda [21].

**Authors' contributions**

JCdR and RC designed and performed the first experiment, while JCdR and ASB performed the second experiment. JCdR analysed the results and drafted the manuscript. ASB developed the real-time PCR assays for analysis of parasite populations inside mosquitoes. AFR assisted in designing both experiments and writing the manuscript. All authors read and approved of the final version of the manuscript.

**Competing interests**

None declared.

**Acknowledgements**

D. Walliker is thanked for assistance with drug-treating mice, B. Chan for technical assistance, R. Carter and S. Cheesman for fruitful discussion and development of real-time PCR assays, M. Guineau for comments on an earlier manuscript, and the staff of the March animal house for excellent husbandry. The study was funded by the Wellcome Trust and the BBSRC, and JCdR was supported by the Darwin Trust of Edinburgh.

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Evolution and ecology, after the malaria genomes

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The first of the three genomes responsible for malaria was published last year; with the recent and much hyped publication of parasite and vector genomes in Nature and Science respectively, the life-cycle triad is now complete. The genomes and associated papers demonstrate that evolutionary biologists and ecologists have much to contribute to malaria control — and to the unravelling of some very interesting biology.

The recently announced sequencing of the genomes of the most virulent human malaria parasite Plasmodium falciparum [1], its most important vector Anopheles gambiae [2], and that of the rodent malaria Plasmodium yoelii yoelii [3], has rightly engendered much enthusiasm among malaria researchers. It is now possible, for instance, to search the full genomes for new drug and vaccine targets, and hundreds of candidates are likely to be found. Quite when this will impact on malaria control is, however, an open question. Novel drugs are certainly required. But testing the candidate vaccines that we already have is currently rate-limiting and, even if the candidates now under trial were actually to work, regulatory bureaucracy means it would be 10–15 years before they could be deployed (by which time another ten million people will have died). The substantial human [4] and economic [5] costs of malaria will be with us for some time.

Malaria research has been dominated by biomedical science, but the failure of that science to control malaria is largely because of evolution and ecology. Both parasite and vector have evolved around drugs; vaccines are not in use due to the vector have evolved around drugs; vaccines are not in use because the parasite possesses adaptations to promote somatic evolution; and the robust ecology of the vector and parasite challenge standard public health approaches. The time is ripe for ecologists and evolutionists to join biomedical researchers in the post-genomic malaria world. Not only will this solve many emerging evolutionary puzzles, but it will also be an absolute necessity to solve the ecological questions that, for example, genetically modified (GM) mosquitoes will pose. Here, we summarize some of the opportunities that emerge from the Nature and Science issues that contained the genomes.

Evolution of antigenic variation

One puzzle is species divergence in antigenic variation genes. Comparison of the P. falciparum and P. y. yoelii genomes reveals that the two species use nonhomologous gene families to change their antigenic properties and thus escape immune recognition [6]. The P. falciparum genome contains 59 var, 149 rif and 28 stevor genes [1]. Rif and stevor genes are probably involved in antigenic variation, but var genes definitely are. They encode proteins on the red blood cell membrane, where they are involved in adhesion to host endothelial receptors [1], thus making them a direct target for the immune system. An individual parasite only transports one var protein to its cell surface, but individuals in a clonal lineage can express different proteins and hence evade the immune system.

The rodent malaria P. y. yoelii does not possess homologues of var, rif or stevor genes [3], in spite of great overall gene synteny between the two species: of ~5300 P. falciparum genes, >3300 P. y. yoelii orthologues were found. A probable candidate for P. y. yoelii antigenic variation genes is the vir gene family, containing no less than 838 genes. They are homologous with the vir gene family in the human malaria parasite P. vivax, encoding proteins that are immunovariant in natural infections and that are likely to be involved in antigenic variation and immune evasion [3].

Differences in the gene families involved could mirror differences in the strategies adopted. Even so, why would these species differ in the genetic basis of antigenic variation? Presumably they share a common ancestor that also had to deal with acquired immunity. The differences cannot be caused simply by adaptations to different hosts, because P. y. yoelii and P. vivax have homologous immune evasion genes. Searches for homologous gene families in other rodent and primate malarias, as well as those in birds and lizards, will be very interesting, especially given that more rigorous Plasmodium phylogenies are beginning to emerge [7]. The nature of selection on diversity-generating mechanisms has long been the subject of conventional evolutionary biology. With the genomes in our hands, it is now time to apply this expertise to infectious disease [6].

Evolution of multigene families

Antigenic variation genes are not the only genes to occur in gene families. In fact, both the Plasmodium and Anopheles genome show many expansions of genes and gene families, possibly providing parasite and vector with a great adaptive ability.

The Anopheles genome contains many gene families, and these are often highly divergent from those in Drosophila melanogaster. For instance, the 242 Anopheles genes from 18 families implicated in innate immunity show marked diversification from Drosophila and a deficit of orthologues [8]. Of the 79 Anopheles G protein-coupled receptors identified as candidate odorant receptors, 27 do not have close Drosophila relatives [9]. It is tempting to attribute these genome-wide differences to selection
pressures from different food sources. But why should detecting rotting fruit or humans require radically different genetic control? And why should the fruit- and blood-borne pathogens require such different immune control? Has gene proliferation been a cause or a consequence of the adaptive plasticity that has the malaria parasite and its vector resilient in the face of 100 years of medical onslaught?

**Vector behaviour and physiology**

Malaria parasites decrease vector fecundity [10] and increase biting rate and blood-meal size [11]. These alterations could be adaptive for the parasite (e.g., reallocation from reproduction to parasite nutrition) or host (reallocation from reproduction to immune function), or adaptive for neither. Differentiating between these alternatives can be done by measuring the fitness consequences of the altered phenotypes for the players involved, but it is not always easy to manipulate these experimentally. A different approach is to determine which genes are running the show.

Holt et al. [2] present a study of blood-meal-fed and non-blood-meal-fed mosquitoes, and show that genes involved in egg melanisation and yolk and oocyte production were upregulated in blood-fed mosquitoes. A next step is to compare gene expression patterns of mosquitoes fed infected or uninfected blood. Alternatively, are there *Anopheles*-like genes in the malaria genome? These might be good candidates as host-manipulation genes.

One candidate gene involved in the decreased fecundity is one of 35 putative regulatory genes [12]. This gene is closely related to an *Aedes aegypti* homologue that is expressed in ovaries only during reproductive arrest and the first 24 hr of a reproductive cycle when ecdysteroids are being synthesized. This receptor is possibly involved in hormonal regulation and physiological pathways that, in *D. melanogaster* and *Caenorhabditis elegans*, are involved in the link between longevity and fecundity. If this is the key regulatory switch between arrest/longevity and reproduction in *An. gambiae*, it could be the target mechanism of any adaptive manipulation of the reproductive physiology of the mosquito by the parasite.

**Ecological challenges of GM mosquitoes**

In the past, malaria control has been achieved mainly by mosquito control [13]. With the increasing problems of insecticide resistance, many malaria researchers now focus their hopes on changing the genetic makeup of the mosquito to block transmission or to decrease mosquito fitness. The genomes will provide many candidate genes to accomplish this. Rapidly advancing technology has already made it possible to create transgenic mosquitoes that do not transmit malaria [14]. Indeed, we are now at a stage at which the technological challenges of manipulating genes in *vivo* are trivial compared to the ecological challenges of using this technology to control malaria [15].

There is room for a lot more thinking about which genes should be used and then how they should be driven into wild populations [15]. This is an area that has already seen significant contributions from evolutionary biologists [16]. But much more is needed, particularly to fill out our incomplete understanding of parasite transmission ecology [17]. For example, we need to know more about gene flow within and between mosquito populations. What are the fitness consequences of genetic modification of mosquitoes? And what counter evolution by the parasite will GM mosquitoes prompt? Scott et al. [17] argue that continued evaluation of GM technology will require release of GM mosquitoes on isolated oceanic or ecological islands that have been thoroughly characterized with respect to the genetic and ecological makeup of local mosquito vector populations and site-specific patterns of pathogen transmission and disease. That is a lot of untrendy 'muddy boots' ecology.

**Outlook**

Clearly, the molecular biologists who make GM mosquitoes will need ecologists to tackle these complicated ecological problems. In a time of increasingly big science, even the early findings from the post-genomic era show that readers of TREE will be as needed in malaria research as are readers of Trends in Genetics, Parasitology or Microbiology. Indeed, in an academic world where we know an enormous amount about banana-dwelling flies, one could argue that a focus by evolutionary biologists and ecologists on an organism that is at least as interesting as a fruit fly is long overdue. Research funding opportunities are arguably the only benefit of the catastrophic death toll from malaria.

**References**

Malaria in the frame

The publication last year of the genome sequence of the malaria parasite *P. falciparum* offers new hope for the discovery of a vaccine, says Jaap de Roode.

- Genome sequence of malaria parasite, *P. falciparum*, reveals 23 million base pairs and a predicted 5279 proteins.
- Search for a single protein vaccine yields 50–100 new candidates.
- ‘Priming’ and ‘boosting’ methods raise the immune response.
- Trials of RTS,S vaccine demonstrate 34–47 per cent protection among African males.
- Estimated seven to 23 years before first African child vaccinated.
With many diseases, for example, polio and whooping cough, vaccines consist of dead or inactivated forms of the bacterium or virus that causes it. Indeed such an approach works well enough with malaria: 90 per cent of volunteers infected with irradiated malaria sporozoites are well protected against malaria. But producing them in large enough numbers has proven impossible: malaria parasites are not so easily produced in flasks or Petri dishes as bacteria or virus particles.

That is why there still is no malaria vaccine, and why scientists have been searching for single malaria proteins that on their own trigger a strong enough immune response to give protection against the disease. Given the parasite’s lifecycle, there seems ample choice: any protein that triggers the immune response, no one has yet found a protein that on its own can mimic infection with the full parasite, consisting of thousands of proteins. With the genome in our hands, however, some researchers now hope that we can identify which of the predicted 5279 proteins could be used in such a way.

Henk Stunnenberg at the University of Nijmegen in The Netherlands, has for some time now worked on a potential vaccine that he hopes will disrupt parasite fertilisation in the mosquito, and block parasite transmission to new patients. With the genome available, he is optimistic that such a vaccine will be within reach. He recently set out to determine which proteins are produced during different stages.

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GTTAAATATAACCTTTT... One click of the mouse on any computer connected to the World Wide Web, and thousands of As, Cs, Gs andTs slide over the computer screen. We are at http://PlasmoDB.org, the website that hosts the database containing the full genome and predicted protein makeup of the malaria parasite *Plasmodium falciparum*. Gone live in 2000, and completed last October, the website now receives thousands of hits every day from researchers all over the world who are looking for things with fancy names such as TRAP, GLURP and SALSA.

Behind these seemingly trivial four letters of the alphabet lies a human tragedy: the As, Cs, Gs and Ts stand for the four building blocks that make up the genetic backbone of a parasite that kills almost one person every 30 seconds. Mounting to over a million deaths a year, most of these are children in sub-Saharan Africa. Another 500 million people suffer from anaemia and immune suppression that leaves them vulnerable to other fatal diseases, exacerbated by their poverty and limited access to cure and treatment. More people suffer from malaria today than ever before in human history; and with the malaria parasite becoming increasingly resistant to available drugs, it seems clear that the world is desperately in need of a vaccine.

More than six years ago, a consortium of labs from around the world decided to take up the huge task of sequencing the full genome of the malaria parasite and so gain more insight in its genetic and protein make-up. The publication of this genome sequence in *Nature* last October revealed 23 million base pairs, predicted 5279 proteins, and was unsurprisingly heralded as the great breakthrough in malaria. Now that we can start to unveil the biology of *P. falciparum*, we can look for its Achilles’ heels that we could use to fight it, and this will undoubtedly lead to the discovery of many new drug targets. Indeed, it will also lead to the discovery of genes, proteins or portions of the malaria parasite that could be used as a vaccine. But most researchers now realise that it will still be many years, and many millions of malaria sporozoites, before all the problems are solved to make a vaccine candidate into an effective and life-saving entity.

Mosquito bite

Malarial disease starts with the bite of a mosquito that injects tiny parasites called sporozoites into the human bloodstream. These sporozoites migrate to the liver, where they invade liver cells, replicate, and burst out of the cells. One sporozoite can give rise to no less than 30000 new parasites that travel to the travel to the blood where they start causing disease. The parasites invade red blood cells, replicate within them and rupture the cells, before invading more red blood cells. This process repeats itself every 48 hours, and results in the fevers so characteristic of malaria. Some parasites make the red blood cells stick to blood vessels (thereby in some cases killing the patient), while others escape this ongoing cycle by turning into gametocytes — precursors of sex cells — of which male and female forms exist. Gametocytes, when taken up by a new blood-feeding mosquito, mature and fertilise each other to give rise to a new generation of malaria parasites, ready to infect a new patient.

**SEM of red blood cells and *P. falciparum* protozoa (yellow)**

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**The lifecycle of *Plasmodium falciparum***

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**CHEMISTRY IN BRITAIN SEPTEMBER 2003**
New opportunities for drug design

The malaria genome will not only come in handy for developing vaccines; many malaria researchers believe it will also be a great tool with which to design new drugs against the parasite.

For decades doctors in developing countries have depended on relatively cheap drugs such as chloroquine and Fansidar. But the malaria parasite has quickly grown resistant to these drugs; it took less than 15 years for resistance to spread through the whole of Africa. Today these drugs are useless in many areas. And because many of the drugs that are used these days are chemically related, resistance against one drug can facilitate resistance against another. The world is thus desperately in need of new classes of drugs, unrelated to the ones now in use.

There is good hope that the Plasmodium falciparum genome will help in identifying these new drugs. Perhaps the most interesting target for drug development is the apicoplast, an organelle located inside the malaria parasite. Related to chloroplasts in plants, it is the only place in the parasite where lipid biosynthesis occurs, and is therefore vital for the parasite's survival. Because this organelle has no human counterpart, it is an ideal drug target: drugs will target the parasite, but leave the malaria patient unharmed.

The sequencing project revealed that about 10 per cent of all the genes in the malaria genome code for proteins that head for the apicoplast. Therefore it should offer no less than 500 new drug targets.

Even before completion of the malaria genome, researchers already identified genes involved in the apicoplast's lipid biosynthesis. They could do this, because during the years that it took to sequence the genome, the database containing the sequence was regularly updated. A group of German researchers identified enzymes in the isoprenoid pathway, and used two existing drugs to target these. Doing this, they could inhibit growth of Plasmodium falciparum parasites in culture, even though these parasites were resistant to other drugs.

Interestingly, one of these drugs, fosmidomycin, had been developed for treating urinary infections. It is now in clinical trials as an antimalarial drug.

Other researchers – from both the US and Great Britain – identified the so-called shikimate pathway in P. falciparum. This is a pathway that in plants is located in chloroplasts, and is involved in the production of, among other compounds, folicate and aromatic amino acids. Treatment with glyphosate, a pesticide commonly used to kill weeds, inhibited growth of Plasmodium falciparum parasites.

The apicoplast is not the only opportunity to create new drug treatments, however. Researchers at St George's Hospital Medical School in London, for example, have recently found a way to stop the malaria parasite's sugar transport from working. By blocking the transporter that absorbs the glucose into the parasite, they were able to kill malaria parasites, because sugar is vital for the parasite's growth in red blood cells.

The next few years will see malarialogists unravelling the molecular biology and biochemistry of the parasite. We can therefore expect many more and new antimalaria drugs.

of the malaria lifecycle. To do this, his team and colleagues at the University of Southern Denmark extracted proteins from blood-stage parasites, gametocytes and gametes. They broke these proteins into smaller pieces (peptides) by using trypsin, an enzyme that cleaves proteins at every lysine or arginine residue it encounters. Stunnenberg then ran these peptides through a mass spectrometer to determine accurately the mass of each of the peptides. Because each of the peptides has its own specific mass (based on which and how many amino acids it consists of), this results in peptide 'fingerprints'. And because computer algorithms are able to predict amino acid sequences and therefore peptide fingerprints on the basis of DNA sequences, Stunnenberg was able to compare his fingerprints with those in the malaria database. Doing this he found about 1300 proteins: 23 per cent of all the predicted malaria proteins.

Most of these are not interesting as vaccine candidates, Stunnenberg points out, because they don't occur on the outside of the parasites, are not seen by the immune system, and will therefore not trigger a useful immune response. The most interesting proteins are those that occur on the outside of parasites, and the researchers set out to determine which of the 1300 proteins were likely to be found here.

They did this by searching for signal peptides – regions of proteins that indicate they occur on the outside of cell membranes – and transmembrane domains – regions that suggest the proteins are attached to a membrane. Doing this they found roughly 150-200 potential transmembrane proteins. 'Half of those occur on gametocytes and gametes,' says Stunnenberg, 'so we have some 50-100 new candidates [for transmission blocking vaccines] that we need to investigate.'

Stunnenberg now hopes to obtain blood serum from malaria patients, and see if this contains antibodies that bind to these proteins. If so, then they are apparently visible to the immune system, and might be good vaccine candidates. It is also possible to find homologous genes in the rodent malaria parasite Plasmodium berghei. In the laboratory, this parasite infects mice, and it is easily manipulated. By disrupting the particular genes that code for these proteins, you could find out if indeed the parasite's reproduction is disrupted.

In a similar way, Stunnenberg, along with colleagues from the University of Leiden, has already identified the so-called Pf48/45 protein, present on the surface of gametocytes. When disrupting the gene in P. berghei, male gametocytes turn out to be infertile, something that also happens with antibodies that blocked the protein. It would therefore be a great vaccine: the human body would produce antibodies against it, blocking fertilisation in the mosquito and preventing malaria transmission. 'It would be an excellent transmission blocking vaccine', Stunnenberg says, 'if it was not so difficult to fold it correctly.' For only in its correct conformation does the protein trigger antibodies in the human body. Mimicking that conformation in the lab has so far proven impossible, and large-scale production of it for use as a vaccine seems therefore out of reach. But within the 50-100 new candidates, Stunnenberg hopes there will be proteins that have similar effects, but which can more easily be produced in the lab.

Priming and boosting

While Stunnenberg focuses his hope on an anti-transmission vaccine, many other researchers have stepped into the 'post-genomic malarial era' to identify vaccines that will prevent parasite invasion of liver cells or red blood cells, and so prevent infection or alleviate disease. Ultimately, they hope, this will result in many proteins that trigger immunity against different stages of the parasite. Combined within one vaccine, these might form the ultimate preventative: a parasite escaping an 'anti-liver' vaccine would still be killed by a vaccine that blocks red blood cell invasion.

With the genome in our hands, it seems likely that tens, maybe even hundreds of such proteins will be found over the coming few years. But Adrian Hill of the University of Oxford cautions against too much optimism. 'It is important to have the genome', he says, 'but it is not suddenly going to make a successful vaccine.' He says that the reason we still don't have
The TRAP protein, for example, a protein involved in the invasion of liver cells, is a great vaccine candidate, but does not elicit a strong immune response on its own. Over the past few years, however, Hill and his colleagues have developed a method to increase the immune response by so-called 'priming' and 'boosting'. They incorporated the TRAP gene with some other bits of malaria DNA into a plasmid, a circular piece of DNA. When injected into muscle tissue, muscle cells translate this DNA into malaria proteins against which the body produces an important class of immune cells called T-cells. If the researchers then also injected a weakened virus that was modified to contain the same protein and malaria DNA bits, the body generated a much stronger immune response. Hill thinks this is because the virus triggers a stronger response than the plasmid, and giving the priming DNA vaccine causes the induced T-cells to become malaria specific.

Hill points out that these vaccines have already been tested in safety trials and clinical trials in both the UK and the Gambia in Africa. After challenge with sporozoites, he found significant levels of protection in vaccinated people. And that can mean they don't get malaria at all, or they have a significant reduction of infection', he says.

He also mentions results from trials with the RTS,S vaccine, developed by GlaxoSmithKline Biological. This vaccine contains a portion of the circumsporozoite protein, which occurs on the surface of sporozoites, and is combined with a hepatitis B surface antigen to trigger a stronger immune response. In 306 male adult volunteers in the Gambia, it resulted in in 34 per cent protection when given in three doses, and four doses resulted in 47 per cent protection. Even though protection did not last, but waned over time, Hill says: 'there is real progress. Five years ago, there was no vaccine that caused protection in humans. Now, at least two vaccines give clear protection'.

Hill says that a lot more has to be done to increase levels of protection by changing the way that vaccines are administered, because current levels of protection are still far from satisfactory. Now where does the genome come in? 'Maybe once we learn more about how to get these strong responses', Hill says, 'then you would try a variety of vaccine candidates.'

Within reach

With progress both in learning how to increase protection with existing vaccines, and the discovery of more - and maybe better - vaccine candidates emerging from the malaria genome, many researchers in the field now believe a vaccine is within reach. But they warn it will at least be another seven to 23 difficult years before we will see the first African child vaccinated.

The fact is that besides the technical hurdles there are many other obstacles to overcome: once a good enough vaccine is found, it will have to be produced in large quantities, and a whole infrastructure to deliver it to the people who need it most must also be put in place. Of the many problems, however, the most important one might be economic. It is now clear that for a disease that almost exclusively hits the poor, we need guaranteed markets to encourage pharmaceutical firms to license and manufacture vaccines. At the moment, the public support for a malaria vaccine is roughly tenfold less than the support for a vaccine for HIV, a disease of comparable global impact.

For the time being, even the initial follow-up of the genomic research seems a huge task. Keeping a mass spectrometer in the air for only half a year, would cost Henk Stunnenberg £50000. With limited funding for research in general, and for developing world diseases in particular, he says it won't be easy to obtain that amount of money. But with another malaria victim every 30 seconds, and more than 50 potential vaccine candidates, he is determined not to give up.

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