ECOLOGICAL AND EVOLUTIONARY EFFECTS OF INTERVENTION STRATEGIES ON THE TRANSMISSION OF MALARIA PARASITES

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DECLARATION

I declare that the research described within this thesis is my own work and that the thesis is my own composition.

CONTENTS

Chapters 2-7 have been written as self-contained papers, including specific introductions and discussions. Chapter 1 provides a short and general overview of the area. Chapter 8 is a general discussion of the work, and addresses some of the questions arising.

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ABSTRACT

Intervention strategies against malaria have two major aims: to reduce disease prevalence and severity. However, these aims are frequently not achieved. This thesis experimentally addresses how intervention strategies - namely antimalarial chemotherapy and vaccination - affect the transmission and virulence of malaria parasites at the phenotypic level. Most forms of antimalarial chemotherapy and potential vaccines act against the parasite stage responsible for the pathology: the asexually replicating erythrocytic forms. A proportion of these asexual parasites, which increases when conditions are bad for asexual replication, develop into non-replicating gametocytes, the only stages capable of infecting the mosquito vector. Gametocyte densities generally correlate with transmission success.

Studies using the rodent malaria *Plasmodium chabaudi* in laboratory mice revealed that a variety of antimalarial drugs (used in doses that killed most, but not all parasites), administered before or after infection, resulted in an increase in the proportion of asexual parasites that developed into gametocytes (gametocytogenesis). As a result, gametocyte densities (and where assayed, subsequent transmission to *Anopheles stephensi* mosquitoes) did not differ from control infections, or were only slightly less. Similarly, sub-curative treatment with the antimalarial drug chloroquine resulted in a five-fold increase in gametocytogenesis of the human malaria *P. falciparum in vitro*. There was a positive relationship between gametocytogenesis and growth inhibition, which differed between clones, independent of drug resistance. Unlike previous reports, the antimalarial drug chloroquine was not found to increase the per gametocyte infectivity of *P. chabaudi*.

Immunisation against *P. chabaudi* resulted in a four-fold reduction in total numbers of both asexual parasites and gametocytes. Immunity against *P. chabaudi* was found to be strain-specific: inhibition of asexual growth was greater if challenge infections were of the same genotype as used for immunisation (homologous challenge), compared to when the immunising and challenge clones differed (heterologous challenge), although there was no difference in gametocyte numbers. Strain-specific immunity also acted on per gametocyte infectivity: gametocytes in a homologous challenge were five times less infectious (to *A. stephensi* mosquitoes) than gametocytes in a heterologous challenge, which did not differ in per gametocyte infectivity from control infections. Patterns of gametocytogenesis through time in control, immunised and drugged animals, were consistent with the parasite responding in an adaptive manner to changing environmental conditions.

Virulence (as measured by host anaemia) was reduced by both chemotherapy and immunisation. Differences in virulence between immunised, drugged and control animals could be explained by differences in asexual replication. Drugged infections achieved the same level of transmission as control infections, but at reduced virulence. The relationship between virulence and transmission did not differ between control and immunised infections. The evolutionary and epidemiological significance of all these data are discussed in the context of the control of human malaria.

Chapter 1

INTRODUCTION

1.1 HOSTS, PARASITES AND INTERVENTION

Parasites, by definition, reduce host fitness. The costs of parasitism are frequently huge, resulting in host death (e.g. microsporidians parasitising mosquitoes, Agnew & Koella 1997) or loss of reproductive function (e.g. schistosomes parasitising snails, Baudoin 1975). As a result, hosts have evolved numerous mechanisms to resist parasites, even though these defences might themselves be costly (e.g. Boots & Begon 1993, Kraaijeveld & Godfray 1997, Yan *et al.* 1997). Parasite fitness is dependent on successfully evading host defences, so that host resources can be exploited. Consequently, parasites and hosts can be considered to be in an evolutionary arms race: selection for host resistance results in selection on parasites to evade host defences (Anderson & May 1991). For some host-parasite interactions, this arms race has evolved to occur on a phenotypic level. For example, the mammalian immune system is capable of rapidly recognising and responding to parasite antigens, although more time is required if the antigen is novel. Both trypanosomes and malaria parasites constantly change which antigen they express on their surface during an infection, so as to evade the immune system (e.g. Turner 1997, Roberts *et al.* 1992).

Selection on parasites to evade host defences will often be stronger than selection on hosts to resist parasites. First, a parasitised host may still have some reproductive success, whereas a parasite without a host is likely to have none. Second, not all hosts are parasitised, whereas all parasites require a host. Moreover, parasites generally have shorter generation times than their hosts, thus respond to selection more rapidly. Consequently, parasites are likely to stay one step ahead of their hosts.

Artificial control of parasites of medical or economic importance is therefore necessary.

Anti-parasite intervention aims to reduce the prevalence of the parasite (by reducing its transmission) and / or the pathology it causes. Unfortunately, parasite transmission is intimately linked with parasite fitness (Anderson & May 1982), so that there will be intense selection on the parasite to minimise the fitness consequences of intervention. For example, resistance to anti-parasite drugs has been reported in nematodes (Conder & Campbell 1995), HIV (Larder & Kemp 1989), and malaria parasites (Peters 1987), to name but a few.

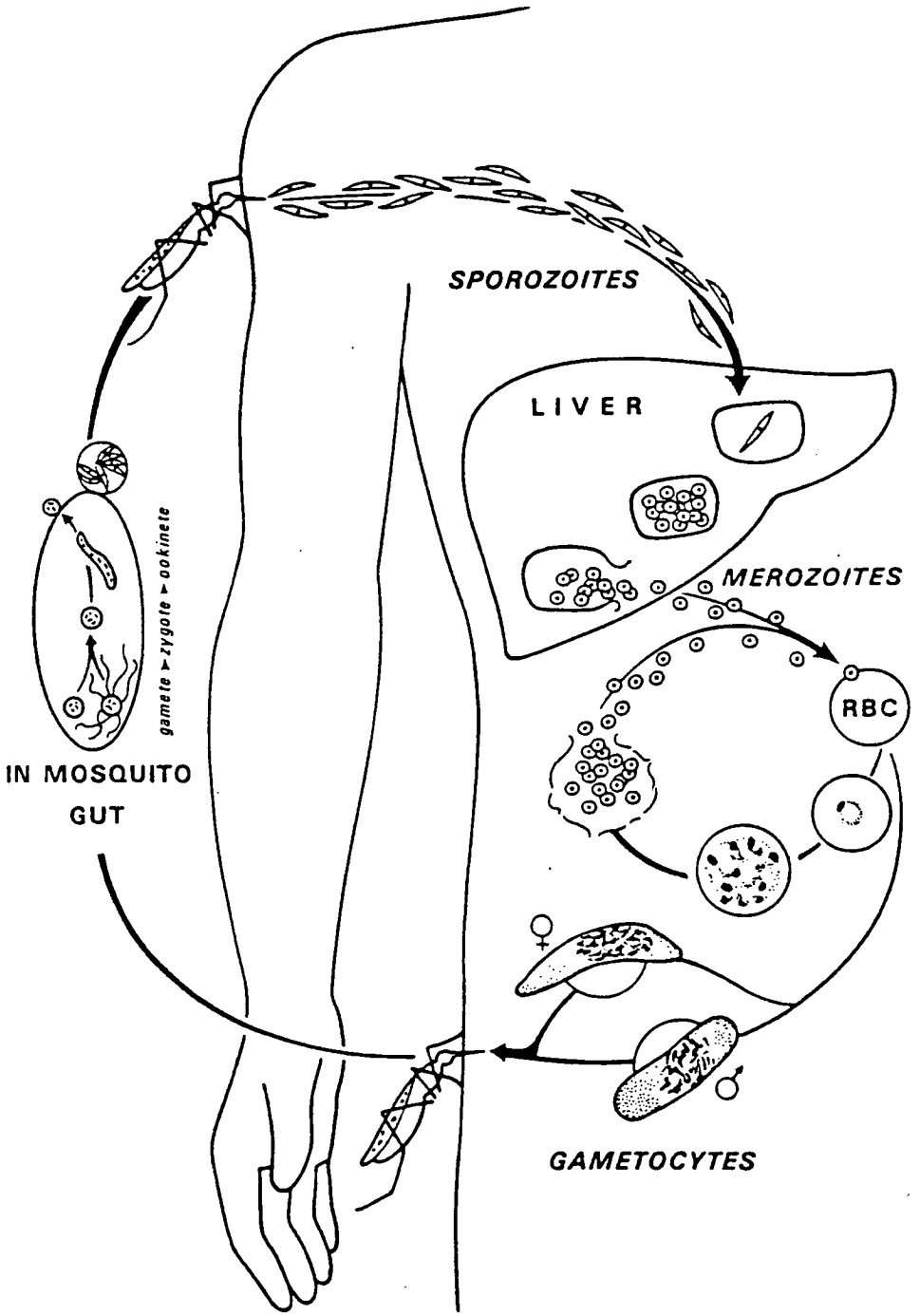
1.2 ANTIMALARIAL INTERVENTION

Intervention strategies used against malaria fall into two main categories. First, intervention which acts against the asexually replicating blood-stage parasites (figure 1.1), which aims to reduce both transmission and pathology. Examples include most antimalarial drugs and most potential vaccines (Greenwood 1997). Second, intervention that solely aims to reduce transmission. This includes reducing the vector population (through the use of insecticides and reducing available mosquito breeding sites), reducing the frequency of mosquitoes biting (e.g. bed nets), and forms of vaccination (transmission-blocking; Sinden 1997) and chemotherapy (e.g. primaquine) that specifically target parasites stages infective to the mosquito (gametocytes or the resultant gametes, figure 1.1).

1.3 ANTIMALARIAL INTERVENTION AND TRANSMISSION

Ideally, intervention strategies will reduce transmission to the extent that the parasite population cannot maintain itself (i.e. the average number of new infections resulting from an established infection (R_0) is reduced below one; Anderson & May 1982).

Figure 1.1 (over): Life-cycle of generalised mammalian malaria parasites. A mammalian malarial infection is initiated by the inoculation of one or more haploid sporozoites by an infected mosquito taking a blood meal. These sporozoites migrate to the liver, where they invade hepatocytes and undergo extensive asexual replication. The resultant parasites make their way to the bloodstream where further cycles of asexual replication occur within red blood cells. It is this parasite stage that is responsible for the pathology of a malarial infection. A small proportion of the asexually replicating parasites, rather than undergo further rounds of asexual replication, develop into non-replicating gametocytes. These are the only stages capable of infecting the mosquito vector. When gametocytes are taken up in a mosquito blood meal, they develop into male and female gametes which fuse into a motile ookinete. The ookinete penetrates into the midgut wall, where it develops into an oocyst. Within the oocyst, meiosis and recombination occur (Walliker *et al.* 1987). The meiotic products undergo mitotic replication, resulting in thousands of haploid sporozoites. These migrate to the mosquito salivary glands, where they are inoculated into the next host that the mosquito feeds on. Reproduced with permission from Hadley *et al.* (1986).


SPOROZOITES
LIVER
MEROZOITES
RBC
GAMETOCYTES
IN MOSQUITO
GUT
gamete → zygote → ookinete

Despite successful eradication of malaria from, for example, Sardinia and Corsica, larger scale eradication programmes in the rest of the world have generally failed (Gramiccia & Beales 1988). As a result, modern control programmes are less ambitious, aiming only to reduce transmission and pathology (Bruce-Chwatt 1985). This has been successfully achieved at times. For example, a meta-analysis of results from a number of field trials using insecticide-impregnated bed nets showed that the incidence of malaria was reduced by approximately 50% (Choi *et al.* 1995). Similarly, malaria prevalence was reduced following a combination of chemotherapy and vector control in the Sudan Savanna (Molineaux & Gramiccia 1980). However, world-wide malaria prevalence certainly has not decreased since the start of consolidated malaria eradication and control programmes, with an estimated 300 million people contracting the disease each year (Bruce-Chwatt 1985). There are numerous explanations for this apparent lack of success, including environmental changes increasing vector populations and control programmes not being sufficiently widespread (Gramiccia & Beales 1988). However, intervention strategies themselves may be less effective than anticipated at reducing transmission for a number of reasons outlined below.

Host changes

The transmission success of malaria is frequently negatively correlated with the degree of antimalarial immunity of the vertebrate host (e.g. Graves *et al.* 1988). However, under some circumstances immunity can actually increase transmission (Targett 1992). More generally, reducing exposure to the parasite by antimalarial intervention can decrease host immunity (Molineaux & Gramiccia 1980), potentially resulting in an increase in transmission from infected individuals. The well documented evolution of mosquito insecticide resistance (Pant 1988) will also greatly reduce the effectiveness of this form of vector control. Physiological or behavioural adaptations in the mosquito may reduce the effectiveness of other vector control strategies.

Parasite changes

Selection on malaria parasites to minimise the fitness costs resulting from antimalarial intervention will be very strong. Indeed, resistance to most antimalarial drugs has evolved, and is spreading rapidly (Peters 1987). Other adaptive changes might also be expected. For example, many free-living organisms minimise fitness costs bought about by environmental changes by either genetic (e.g. Reznick *et al.* 1990, Lafferty 1993) or phenotypic (e.g. Minchella & Loverde 1981, Crowl & Covich 1990, Trussell 1996) changes in their life history strategies. Host immunity and chemotherapy frequently fail to kill all parasites (e.g. Wernsdorfer 1994), resulting in a reduction in circulating parasite numbers and infection longevity. The resultant loss in transmission might be partially compensated for by an increase in the proportion of asexually replicating parasites that develop into transmission stages (gametocytes). Indeed, facultative increases in gametocyte production (gametocytogenesis) appear to be a general response to inhibition of asexual replication (Mons 1985, Carter & Graves 1988, Sinden *et al.* 1996). These responses might be fine-tuned by intervention-imposed selection.

Direct effects

Intervention strategies may have some (surprising) direct transmission-enhancing properties. For example, the antimalarial drug chloroquine has been reported to increase the probability of a given density of gametocytes infecting a mosquito (reviewed in Butcher 1997).

1.4 ANTIMALARIAL INTERVENTION AND VIRULENCE

Any method of antimalarial intervention that kills asexually replicating blood stage parasites (most forms of chemotherapy and potential vaccines) is likely to reduce host morbidity and the probability of host death (e.g. Alles *et al.* 1998). However, like transmission, the virulence of a malarial infection is likely to be negatively correlated

with host immunity. Reducing transmission through intervention strategies may result in a decrease in host immunity and thus an increase in overall levels of virulence in a community (e.g. Snow & Marsh 1995, Trape & Rogier 1996, Alles *et al.* 1998).

Parasite virulence is generally believed to be the result of selection acting on parasite transmission success (reviewed in Bull 1994, Read 1994, Frank 1996, Ebert & Herre 1996, Lipsitch & Moxon 1997). Levels of virulence that maximise transmission are likely to change with some alterations in environmental conditions (e.g. Bull *et al.* 1991). Antimalarial intervention might be one of these.

1.5 EXPERIMENTAL SYSTEMS

Plasmodium chabaudi in laboratory mice

Most of the experiments described in this thesis were conducted using the rodent malaria *P. chabaudi* in laboratory mice. *In vivo* experiments are important because they allow parasites to be studied under relatively natural conditions. Indeed, the immune system is likely to be the strongest selective force acting on *Plasmodium* life histories. However, mice are not the natural host of *P. chabaudi* - the parasite was isolated from arboreal thicket rats (*Thamnomys rutilans*) in Central Africa (reviewed by Landau & Chabaud 1994). It has been suggested that patterns of infections are very different in these hosts. Natural infections in thicket rats are generally chronic, have low parasite densities and are life-long (Landau & Chabaud 1994), whereas laboratory-induced infections in mice tend to be short-lived, reach very high parasite densities and are often fatal in certain mouse genotypes (Stevenson *et al.* 1982).

There are reasons to believe that these striking differences in infection dynamics are a result of sampling bias. First, after the primary infection in mice, parasites remain at low densities, generally only apparent during subsequent small peaks in parasite densities (recrudescences). These recrudescences occur for as long as measurements

have been taken (up to 3 months; A. Read & M. Anwar, unpublished data). Such 'chronic' infections resemble those described in thicket rats. Second, primary infections initiated in thicket rats in the laboratory show similar dynamics to primary infections in mice (Carter & Diggs 1977). It is not surprising that such primary infections have never been observed in thicket rats in the wild, given that parasite densities in primary infections in mice reach low levels within about 20 days (Taylor *et al.* 1997). Moreover, the complete life-cycle of *P. chabaudi* can be completed using mice and a unnatural vector of the parasite, *Anopheles stephensi* (Landau & Chabaud 1994), suggesting that the mouse is not such an artificial host after all.

The previous discussion is important because aspects of this thesis specifically deal with the adaptive basis of *P. chabaudi* life histories: this would be harder if observed parasite life histories were the result of a novel host-parasite association. However, it is also important that the parasite is not specifically adapted to laboratory conditions: it is necessary that the parasite is behaving as much as possible as it would under natural conditions. Fortunately, both clones used in this thesis (CR and ER; Beale *et al.* 1978) have been exposed to a variety of mouse genotypes and transmitted by both mosquito and infected blood at very variable time points in the infection. Adaptation to particular laboratory conditions is therefore unlikely to have occurred to any large extent (Taylor 1997).

P. chabaudi has advantages over other rodent malaria models. First, infections are rarely lethal in 'resistant' mouse genotypes (Stevenson *et al.* 1982). Second, gametocytes are produced in reasonable numbers and are readily identifiable. Third, the infection dynamics (Carter & Graves 1988) and parasite-immune system interactions (Taylor-Robinson *et al.* 1995) bear the closest resemblance to infections with the most common and virulent human malaria, *P. falciparum* . It is important to

note than speculative extrapolations from this model to human malaria are meant to apply only to *P. falciparum* in this thesis.

P. falciparum in vitro

Work was also carried out using *P. falciparum* in culture. This allowed more precise measurements of parasite numbers than is possible in vivo, as well as allowing comparison between human malaria parasites and the rodent malaria model.

1.6 EXPERIMENTAL AIMS

In this thesis I experimentally address the short-term (phenotypic) effects of chemotherapy and host immunity on the transmission and virulence of malaria parasites. Data from short-term effects of intervention are obviously important in their own right, but can also be used to make sensible speculations about longer term effects of these forms of intervention on parasite life histories. Moreover, most of what we know about the adaptive basis of life histories in free-living organisms comes from analogous manipulations (Stearns 1992). Antimalarial chemotherapy is still widely used, thus its effects on transmission and virulence are of great importance, especially in the light of the rapid spread of resistance to most antimalarial drugs (Peters 1987). The effect of host immune status on transmission and virulence are important in understanding epidemiology in general and also the potential effects of first generation vaccines (Greenwood 1997).

The overall aim of this thesis is to determine how sub-curative chemotherapy and partial host immunity affect the transmission and virulence of malaria parasites. The specific questions addressed are as follows:

1. Do sub-lethal (to the parasite) chemotherapy, and host immunity, induce gametocytogenesis? See chapters 2, 4-6.

2. Does chemotherapy and host immune status affect gametocyte infectivity? See chapters 3 and 5.
3. How do these forms of intervention affect overall transmission? See chapters 2-6.
4. How do these forms of intervention affect disease virulence? See chapter 7.

Chapter 2

ADAPTIVE CHANGES IN *PLASMODIUM* TRANSMISSION STRATEGIES FOLLOWING CHLOROQUINE CHEMOTHERAPY

This is a slightly edited version of Buckling et al. (1997). See Appendix.

2.1 SUMMARY

Both theory and data suggest that malaria parasites divert resources to the production of transmission stages (gametocytes) when within-host conditions deteriorate. Increased investment into transmission stages should therefore follow sub-curative treatment with antimalarial drugs, but relevant clinical studies necessarily lack adequate control groups. I therefore carried out controlled experiments to test this hypothesis, using a rodent malaria (*Plasmodium chabaudi*) model. Infections treated with a sub-curative dose of the antimalarial chloroquine showed an earlier peak and a greater rate of gametocyte production relative to untreated controls. These alterations led to correlated changes in infectivity to mosquitoes. Treatment of human malaria commonly does not result in complete parasite clearance. If surviving parasites produce compensatory increases in their rate of gametocyte production, such treatment may have minimal effect on decreasing, and may actually increase, transmission. Importantly, if increased investment in transmission is a generalised stress response, the effect might be observed following a variety of antimalarial treatments, including other drugs and potential vaccines. Similar parasite life-history counter-adaptations to intervention strategies are likely to occur in many disease-causing organisms.

2.2 INTRODUCTION

Strategies of resource allocation which maximise fitness can differ between benign and stressful environments. Consequently, natural selection often favours phenotypic

alteration of reproductive effort in response to stress (Minchella & LoVerde 1981, Crowl & Covich 1990, Stearns 1992, Roff 1992). In disease-causing organisms, such adaptive alterations could render intervention strategies against parasitic diseases less effective than anticipated. For example, during the course of an infection, malaria parasites (*Plasmodium* spp.) are capable of modulating the proportion of replicating parasites (asexuals) that develop into non-replicating transmission stages (gametocytes). If this modulation is stress-induced, medical interventions such as chemotherapy could lead to greater investment in gametocyte production, thus offsetting much of the transmission-reducing benefits of killing parasites. Here I test this idea experimentally using a rodent malaria-mouse model.

Extrapolating from metazoan life-history studies, there are two reasons to expect that malaria parasites will switch investment from asexuals to gametocytes when conditions deteriorate. First, increased reproductive effort should occur following cues associated with decreased probability of future survival or reproduction. For example, enhanced egg-production has been demonstrated in snails following exposure to castrating trematode infections (the 'fecundity compensation' hypothesis; Minchella & Loverde 1981). Increased investment by malaria parasites into gametocytes should therefore occur in response to environmental cues associated with the decline in future transmission potential, such as clearance of the infection or the onset of transmission-blocking immunity (Koella & Antia 1995, Taylor & Read 1997). Second, if conditions change such that one life-history stage becomes relatively more vulnerable than another, increased investment into the least vulnerable stage is predicted (the 'safe harbour' hypothesis; Shine 1978). In the Jerusalem artichoke (*Helianthus tuberosus* L.), for example, inhibition of sexual reproduction by removing flowers results in increased investment in asexual reproduction via tuber development (Westley 1993). If conditions become less favourable for asexuals relative to gametocytes (for example as stage specific immunity develops), increased investment into transmission is again

predicted.

The environmental cues which stimulate gametocyte production (gametocytogenesis) are poorly understood (Sinden 1983, Carter & Graves 1988, Alano & Carter 1990), but there is some evidence that the rate of gametocyte production increases in response to conditions unfavourable for asexual replication. *P. falciparum* (the most common and virulent human malaria parasite) produces more gametocytes *in vitro* when there is high density of parasitised red blood cells (Carter & Miller 1979, Brockelman 1982, Bruce *et al.* 1990). By definition, antimalarial drugs acting against blood-stage parasites impose considerable stress, and some also have a greater inhibitory effect on asexual parasites than on gametocytes. Increased gametocytogenesis following treatment that greatly reduces parasite numbers is therefore to be expected. However, here the evidence is more ambiguous.

Early *in vivo* clinical trials with human *Plasmodium* frequently reported more gametocytes following treatment with drugs inhibiting folate-metabolism and hence DNA-synthesis (e.g. paludrine, pyrimethamine and the sulphonamides; Findlay *et al.* 1946, Mackerras & Ercole 1948, Shute & Maryon 1951, Foy & Kondi 1952, Ramakrishnan *et al.* 1953, McCarthy & Clyde 1973). More recently, increased gametocyte production has also been reported in *P. falciparum* infections following treatment with Fansidar, a synergistic combination of pyrimethamine & sulphadoxine (Tin & Nyunt-Hlaing 1984, Marwoto *et al.* 1986). However, these increases occurred more rapidly than the 8-10 day maturation period of *P. falciparum* gametocytes (Smalley 1976, Jensen 1979), and other studies using Fansidar with longer follow up periods do not support the hypothesis (Strickland *et al.* 1986, Hogh *et al.* 1995). With other blood-stage antimalarials, such as chloroquine or quinine, most clinical studies have found no increases in gametocytogenesis following sub-curative treatment (e.g. Mackerras & Ercole 1949a, Jeffery *et al.* 1956, Jeffery 1958, Hogh *et al.* 1995).

These data have given rise to the conventional wisdom that only drugs that inhibit DNA synthesis are capable of inducing increased gametocytogenesis (Carter & Graves 1988, Alano & Carter 1990), even though there may be exceptions (Mackerras & Ercole 1949b).

But all these data have a common problem: chemotherapy may have been used when increases in gametocyte production would have occurred anyway. This is particularly likely if the conditions stimulating both gametocytogenesis and symptoms (and hence treatment) approximately coincide, as would be the case if, for example, high parasite densities or host stress were a common trigger. Understandably, no studies included appropriate untreated control infections, so that the effect of sub-curative antimalarial chemotherapy on gametocyte production has yet to be resolved.

If enhanced gametocytogenesis can occur following sub-curative treatment, reductions in infectivity might not be nearly as great as reductions in morbidity. This may have implications for malaria control and epidemiology, particularly if increased gametocytogenesis is a generalised stress response stimulated by a variety of treatments. I therefore carried out controlled experiments using a *P. chabaudi*-mouse model to test the life-history prediction of increased gametocytogenesis following sub-curative chloroquine chemotherapy, and to determine any impact on subsequent infectivity to a mosquito vector. Chloroquine (CQ) was used because of its wide availability in malaria-endemic areas and the general view that its use does not result in increased gametocytogenesis (Carter & Graves 1988, Alano & Carter 1990). In addition, it only affects asexual parasites and immature gametocytes (Smalley & Sinden 1977), so that increased gametocytogenesis might be expected on account of both the fecundity compensation and safe-harbour hypotheses.

2.3 MATERIALS AND METHODS

Parasites & hosts

Male C57/BL/6J mice (Harlan-Olac, England) aged 8-14 weeks were infected with $1x10^6$ red blood cells infected with a CQ-naive *P. chabaudi* clone (either CR or ER, from the WHO Registry of Standard Malaria Parasites maintained at the University of Edinburgh, U.K.) in a 0.1 ml intra-peritoneal inoculum of 50 % ringer's solution (27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl), 45 % heat-inactivated calf serum and 5 % 200 units / ml heparin solution. Mice, age-matched within experiments, were housed in cages of 2-4 animals at a temperature of 25±1 °C with a 0700 to 1900 hours light cycle, and provided with 41B rat and mouse maintenance diet (Harlan-Teklad, England) and water containing 0.05 % pABA, *ad libitum.*

CQ preparation and administration

CQ solution was prepared from a stock of 40 mg / ml CQ sulphate (Nivaquine™) diluted in distilled water and administered orally using a lubricated catheter in approximately 0.1 ml doses of 12 mg / kg of mouse weight in all cases. Preliminary experiments revealed this dose to be sub-curative. Control mice were given 0.1 ml distilled water. All treatment took place between 1600 and 1700 hours.

Parasite counts

From day 4 post-infection (p.i.), daily thin blood smears from the tail vein were Giemsa-stained and asexual parasites accurately counted per 1.5×10^3 red blood cells (RBCs). At low asexual densities parasites were counted per 10^4 RBCs. Mature gametocytes were counted per 1.25×10^4 RBCs. Parasite densities were calculated from RBC densities, measured by flow cytometry (Coulter Electronics™) every second day, multiplied by parasites per RBC.

Mosquito feeds & dissections

Mice were anaesthetised by an intra-muscular injection of 0.5 ml per 20 g mice 3:2:1 distilled water: Vetalar^{TM}: Rompun^{TM}, and placed onto pots covered with nylon mesh containing ~40 4-5 day old female *Anopheles stephensi*, which had been starved for the previous 24 hours. Mosquitoes were left to feed in the dark for 30 minutes, between 1845 and 1945 hours. After removal of unfed mosquitoes, the remainder were subsequently maintained at 25-30 °C, 70-80 % humidity, with a 12 hour light cycle and provided with 5 % glucose, 0.05 % pABA solution *ad libitum.* After 8-9 days, approximately 25 mosquitoes per mouse were dissected to determine the presence of oocysts on midguts.

Experiments

Details of individual experiments are shown in table 2.1. In experiments 1, 2 and 4, infectivity was assayed by exposing half the mice in each experimental group to mosquitoes on day 12 p.i., and the other half on day 14 p.i. Preliminary experiments and other data (Taylor *et al.* 1997) showed that infectivity peaks during this period.

Experiment	Clone	Day p.i. of CQ treatment	No. mice infected CQ	Control
1	CR	6	6	5
2	CR	5	6	5
3	CR	4	5	
		5	6	6
		6	5	
4	ER	5	6	6

Table 2.1: Details of experiments. p.i., post-infection; CQ, chloroquine

Statistical analysis

Comparisons of control and CQ-treated infections, which for the most part involve repeated measures on the same infections, were made using univariate analyses of the following summary measures for each infection.

(1) *Total asexuals.* This was estimated from parasite densities between days 8-16 p.i. Parasite densities prior to day 8 were not used because it was unclear whether parasites in the CQ-treated groups were dead or alive at the time of the smear, and by day 18 p.i. parasite densities were at very low levels. Estimates of total parasites between these days were obtained by integrating under the parasite density through time curves for each infection. This is a reasonably accurate measure of total numbers of asexuals (between days 8-16 p.i.) because the asexual cycle of *P. chabaudi* is known to be 24 hours (reviewed in Cox 1988).

(2) *Total gametocytes.* Gametocytes of *P. chabaudi* take approximately two days to mature (Buckling *et al.* unpublished, Gautret *et al.* 1997). An index of the total number of gametocytes produced by asexuals between 8 and 16 days p.i. was therefore obtained by integrating under the gametocyte density through time curves between days 10-18 p.i. This measure is not a direct estimate of total gametocyte numbers because gametocyte longevity is uncertain, though the data presented below suggests few survive longer than 24 hours. However, the sum of the gametocyte densities on each day is likely to be a well correlated with total transmission probability: gametocyte densities at any point in time correlate with both the proportion of mosquitoes infected and densities within mosquitoes (Taylor & Read 1997).

(3) *Proportion of gametocytes* . This was calculated as the proportion of total parasites [(1)+ (2)] that were gametocytes [2] for each infection.

(4) *Day of peak gametocyte density.*

(5) *The proportion of mosquitoes infected.*

Prior to analysis, estimates of total asexuals and total gametocytes were $\log_{10}$-transformed and proportion of gametocytes square-root arcsin-transformed to bring their distributions close to normal. All analyses were carried out using generalised linear models (GLIM; Crawley 1993). The effects of CQ treatment on total asexuals, total gametocytes and proportion of gametocytes were analysed by 2-way ANOVA, with treatment (CQ+, CQ-) and experiment (1-4) fitted as factors. The effect of CQ on the day of peak gametocyte density was analysed separately for each clone by ANOVA. For clone CR, treatment and experiment (1-3) were fitted as factors, and for clone ER, only treatment was fitted, as this clone was used in experiment 4 only. For all 2-way ANOVAs, the interaction term was incorporated into the error term if it expalined a non-significant amount of deviance ($p < 0.05$; Crawley 1993).

A binomial error structure, applying William's correction for over-dispersion, was used for the logistic regression of infection probability (Crawley 1993). Starting with the highest order interactions, all factors (experiment (1,2 & 4), treatment and day of mosquito feed (12 or 14)) and their interactions were individually removed in turn from the complete model. Non-significant terms were removed and significant test statistics obtained from the resulting minimal model.

2.4 RESULTS

Total asexuals and total gametocytes did not differ between experiments (table 2.2). CQ treatment however had a significant effect, reducing asexuals to 20 % of controls and gametocytes to 50 % (figures 2.1 & 2.2, table 2.2). The effect of CQ did not differ between experiments (table 2.2).

The proportion of gametocytes was about 2.5 times greater in CQ-treated infections

than in control infections (table 2.2). There were no differences between experiments, nor did treatment effects differ between experiments (table 2.2). Figure 2.3 shows the relationship between total gametocytes and total asexuals for all infections.

		mean (s.e.) Control	CQ
Total asexuals ($x10^9$)			
Treatment	$F_{1,52} = 114$, $p < 0.001$	2.1 (0.18)	0.45 (0.05)
Experiment	$F_{3,52} = 0.67$, n.s.		
Treat. x Exp.	$F_{3,49} = 1.26$, n.s.		
Total gametocytes ($x10^7$)			
Treatment	$F_{1,52} = 7.1$, $p = 0.01$	1.14 (0.15)	0.67 (0.09)
Experiment	$F_{3,52} = 0.44$, n.s.		
Treat. x Exp.	$F_{3,49} = 1.87$, n.s.		
Proportion gametocytes			
Treatment	$F_{1,52} = 16$, $p < 0.001$	0.007 (0.001)	0.02 (0.003)
Experiment	$F_{3,52} = 0.76$, n.s.		
Treat. x Exp.	$F_{3,49} = 1.54$, n.s.		

Table 2.2: Results of ANOVA for the major summary measures. The treatment by experiment interaction was not significant in all cases, and was incorporated into the error term to determine F-ratios of factor effects. n.s.; non-significant.

Peak gametocyte densities occurred approximately 2 days earlier in CQ-treated infections than in control infections for the experiments using CR (figures 2.2a-c, table 2.3) and 3 days earlier in the experiment using ER (figure 2.2d, table 2.3). For experiments 1-3, the effect of treatment on timing of peak gametocyte densities did not differ between experiments (table 2.3), but the timing of peak gametocyte densities differed between experiments (table 2.3). This was probably due to differences in

timing of CQ treatment between experiments: there was no significant difference between experiments when CQ-treated groups were separated on the basis of specific day of treatment ($p > 0.25$ for controls and days 5 & 6 p.i. CQ treatment).

Interestingly, there was a significant positive relationship between the day of CQ treatment and the day of peak gametocyte density for the days 4, 5 & 6 p.i. CQ-treated groups in experiments 1-3: (ordered heterogeneity test (Rice & Gaines 1994): $r_S P_C = 0.96$, $p < 0.01$).

There was no difference in total gametocytes between the day 4, 5 & 6 p.i. CQ-treated groups in experiment 3 ($F_{2,13} = 0.20$, $p = 0.83$). The proportion of gametocytes however significantly differed between the groups ($F_{2,13} = 4.38$, $p = 0.035$); pair-wise comparisons revealed the proportion of gametocytes to be greater in day 6 p.i. CQ-treated infections than in those treated on day 4 p.i. but there were no other significant differences (scheffe analysis $p = 0.04$, $p > 0.2$ for both other comparisons).

	Clone used	
	CR	**ER**
Treatment	$F_{1,41} = 54.9$, $p < 0.001$	$F_{1,10} = 19.9$, $p < 0.01$
Experiment	$F_{2,41} = 4.46$, $p < 0.05$	
Treat. x Exp.	$F_{2,39} = 0.15$, n.s.	

Table 2.3: Results of ANOVA for day of peak gametocyte densities, which were between 2 and 3 days earlier in CQ-treated infections. The interaction term for clone CR was incorporated into the error term for calculation of factor F-ratios. n.s.; non-significant.

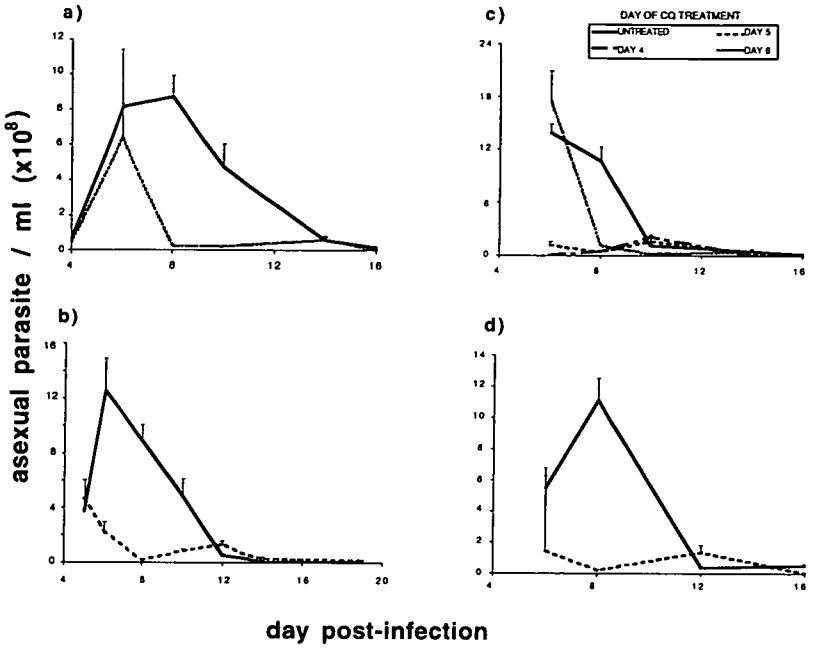


Figure 2.1: Mean asexual parasite densities (+ 1 s.e.) during infections for experiments 1-4 (a-d respectively). CQ, chloroquine

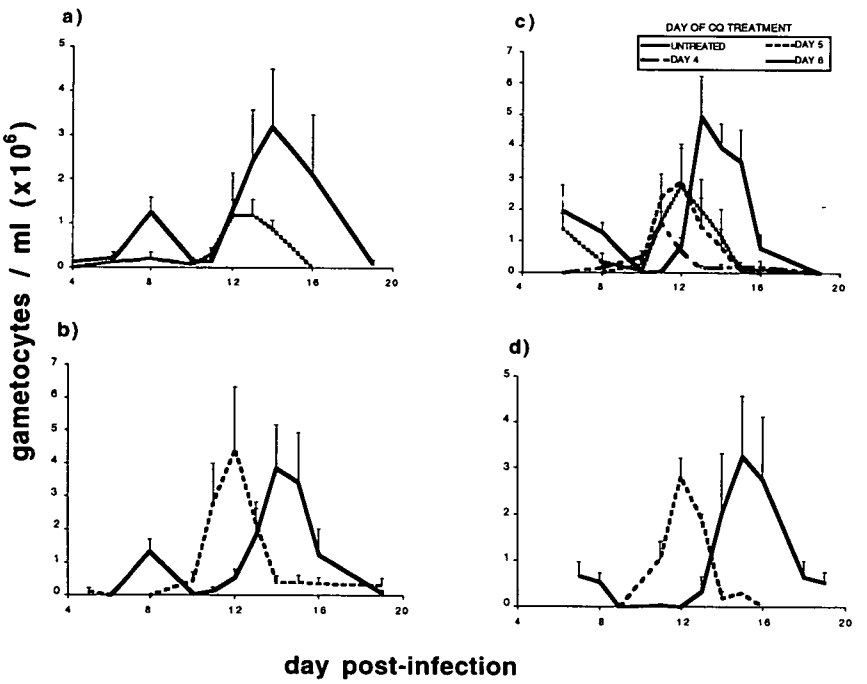


Figure 2.2: Mean gametocyte densities (+ 1 s.e.) during infections for experiments 1-4 (a-d respectively). CQ, chloroquine.

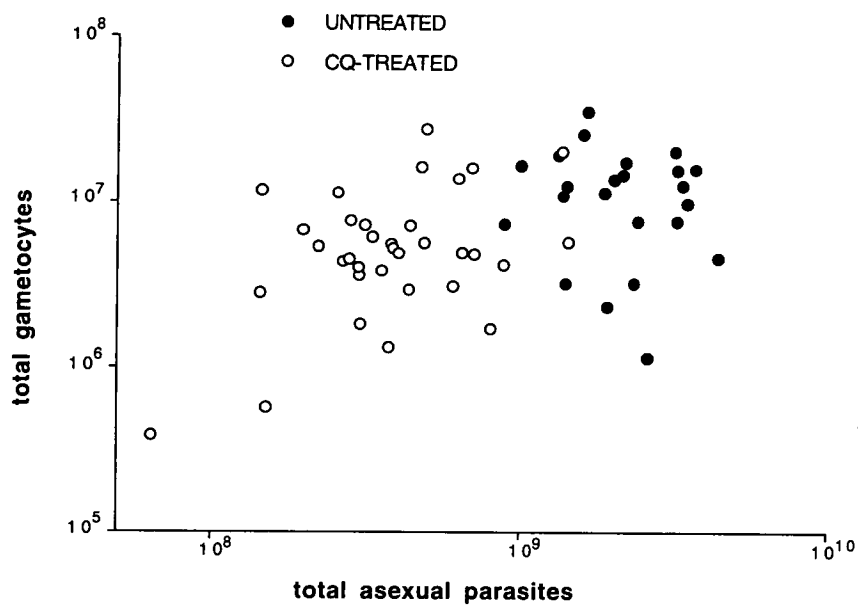


Figure 2.3: Total asexuals and total gametocytes (between days 8-16 and 10-18 p.i., respectively) for all chloroquine-treated and untreated infections in experiments 1-4.

Proportions of mosquitoes infected were greater in CQ-treated mice than control mice on day 12 p.i. and vice versa on day 14 p.i. (figure 2.4, table 2.4). There were no significant differences in overall proportion of mosquitoes infected between experiments, day and treatment, and neither treatment or day effects, and their interaction, differed between experiments (table 2.4).

	χ^2_1	p
Treatment	15.7	<0.001
Day of mosquito feed	0.8	n.s.
Treatment x day	1.2	n.s.

Table 2.4: Minimal model for logistic regression of mosquito infection probability. Experiment and the other interactions were removed from the model as they did not explain a significant amount of deviance ($p > 0.2$). n.s.; non-significant.

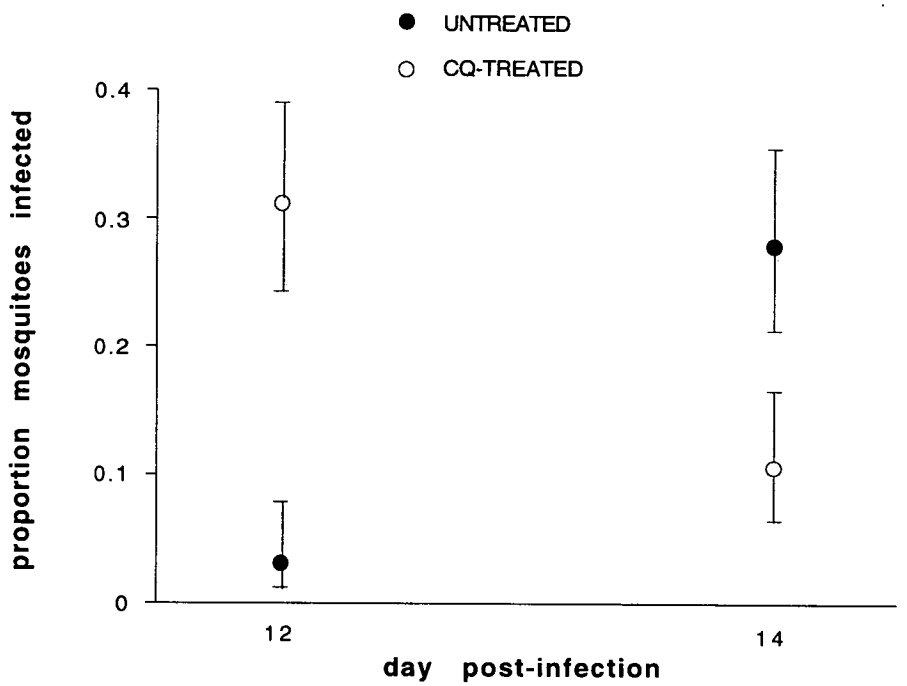


Figure 2.4: Mean proportions of mosquitoes infected (±1 s.e.) for chloroquine-treated and untreated mice on days 12 & 14 post-infection

2.5 DISCUSSION

Infections treated with CQ produced significantly fewer asexuals and gametocytes than untreated infections, but for a given number of asexuals, the number of gametocytes was about 2.5 times greater in CQ-treated infections. This is most likely the result of increased gametocytogenesis following sub-curative CQ treatment, consistent with the hypothesis.

Changes in the relative mortality rates of gametocytes and asexuals can not adequately explain the results. First, stage-specific mortality caused by CQ can not be involved. The half-life of whole-blood CQ-concentration in mice heavily infected with *P. chabaudi* (21-25 % parasitised RBCs) is in the order of 7 hours (Cambie *et al.* 1994). Parasite numbers were first assayed at least two days after treatment, when the CQ level must have been less than 0.5 mg / kg; preliminary experiments revealed twice this concentration to have no noticeable effect on parasite numbers or infection dynamics. Moreover, virtually all the gametocytes counted must have been produced from post-treatment asexuals: any long-lived gametocytes present during the period of CQ activity would have made up only a tiny fraction of the gametocytes used to estimate subsequent gametocytogenesis because they were at such low densities when infections were drug-treated (figure 2.2). Second, differential mortality resulting from differences in immune response between the groups not only requires a gametocyte-specific clearance mechanism, for which there is currently no evidence (Taylor & Read 1997), but also one which is suppressed by CQ.

The best explanation for the relative increase in numbers of gametocytes following CQ treatment is therefore increased gametocytogenesis, as predicted by both the safe harbour and the fecundity compensation hypotheses. Nevertheless, the delay between CQ treatment (hence peak asexual density) and gametocyte production (figures 2.1 & 2.2) is not entirely consistent with these adaptive hypotheses. Increased

gametocytogenesis in response to stress might be expected to be immediate, resulting in mature gametocytes 2-3 days later. Instead, we observed a delay of about a week. However, it is striking that in control infections there is also a delay of approximately a week between peak asexual density and peak gametocyte density. An intriguing possibility, consistent with the hypotheses, is that a sudden drop in parasite numbers might be acting as the cue for delayed gametocytogenesis in both CQ-treated and control mice. In untreated mice, such a strategy might have the function of minimising production of gametocytes during crisis, an immune mediated response associated with anaemia which down regulates parasite numbers (Jarra & Brown 1989) and during which gametocyte infectivity is greatly suppressed (Wery 1968). It is feasible that CQ treatment results in an artificially early occurrence of the same cue that stimulates this crisis-avoiding delay.

Gametocytogenesis frequently follows a substantial reduction in overall parasite numbers (Sinden 1983, Carter & Graves 1988, Alano & Carter 1990, Sinden *et al.* 1996, figures 2.1 and 2.2), suggesting a positive relationship between gametocytogenesis and factors that correlate with parasite destruction. Such factors might explain how gametocytogenesis was triggered in control and CQ-treated infections despite radically different parasite dynamics (figures 2.1 and 2.2) and host condition in the two groups. Increased relative gametocytogenesis following CQ treatment might be explained by the resulting short-term very high rate of parasite destruction. This relationship would also explain why gametocytogenesis was greater in mice treated with CQ on day 6 p.i. compared with day 4 p.i. The later treatment inevitably resulted in greater parasite death, both because by that stage there were more for CQ to kill (figure 2.1c), and because of enhanced immune activity as a result of greater exposure, in terms of both time and numbers, to parasite antigens.

Whatever the mechanism involved, these results clearly demonstrate increased gametocytogenesis following sub-curative CQ treatment. As far as I am aware, this is the first fully controlled demonstration that sub-curative treatment with an antimalarial drug can increase gametocytogenesis in *Plasmodium*, and thus alter patterns of infectiousness. The dose of CQ used in this study is equivalent to half the commonly recommended dose for treatment of people with *P. falciparum* (25 mg / kg over three days; Desjardins *et al.* 1988). Because of treatment terminating following clinical improvement, and the high frequency of drug-resistant parasites, sub-curative treatment often occurs (Wernsdorfer 1994). The results are therefore potentially relevant to the treatment of *Plasmodium* with CQ in the field. First, even large reductions in the numbers of asexual parasites may have a much less dramatic effect on infectivity because of compensatory investment into gametocytes. It is notable that in our transmission experiments, the proportion of mosquitoes infected were similar from treated and untreated infections (figure 2.4). Second, transmission between hosts might actually be faster where sub-curative treatment is common, because of the earlier timing of peak infectivity (figure 2.4). Transmission may be further enhanced by the general reduction in host immunity because of decreased exposure to the parasite (Graves *et al.* 1988) and by the increased infectiousness of gametocytes apparently induced by very low level CQ treatment (Ramkaran & Peters 1969, Wilkinson *et al.* 1976, Ichimori *et al.* 1990). Finally, if the effect is a generalised stress-response, it might be observed following treatment with most blood-stage antimalarials and even potential vaccines.

Here I have only considered phenotypic modifications of gametocytogenesis. Consistent differences in gametocyte production between isolates of *P. falciparum in vitro* (Burkot *et al.* 1984, Graves *et al.* 1984) may imply natural genetic variation in gametocytogenesis on which drug-imposed selection could act to generate long-term changes in resource allocation between asexuals and gametocytes. Parasites will

probably increase their fitness under drug pressure by producing gametocytes in greater quantities earlier in the infections. Lines *et al.* (1991) suggested that just such adaptation may underlie increases in malaria transmission in Tanzania following long-term CQ use. This is supported by a recent study in Sri Lanka which showed that infections of CQ-resistant parasites are more likely to be gametocyte positive than infections caused by sensitive parasites (Handunnetti *et al.* 1996): under drug pressure, adaptive life-history changes are likely to evolve in parallel with the development of drug resistance. Both short- and long-term parasite life-history changes in response to intervention strategies are to be similarly expected in many disease-causing organisms.

Chapter 3

THE EFFECT OF CHLOROQUINE TREATMENT ON THE INFECTIVITY OF *PLASMODIUM CHABAUDI* GAMETOCYTES

3.1 SUMMARY

The antimalarial drug, chloroquine (CQ), has been reported to increase the infectivity of the forms of blood-stage malaria parasites (gametocytes) that are capable of infecting mosquito vectors. This effect has been convincingly demonstrated in the short-term (12 hours post treatment), although several authors have suggested infectivity enhancement a week or more after treatment. I carried out experiments to investigate the effects of CQ on the longer term infectivity of gametocytes of the rodent malaria parasite, *Plasmodium chabaudi*, to *Anopheles stephensi* mosquitoes. Gametocytes of CQ-treated infections were significantly more infectious than untreated infections 6 and 7 days post treatment, although not on days 8 and 9. However, this effect was most likely the result of a reduction in infectivity in untreated infections, caused by immune activity which was not so pronounced in CQ-treated infections. Gametocytaemia (gametocytes / red blood cell) showed a strong positive and linear relationship with infectivity. Infectivity was not influenced by either asexual parasitaemia, asexual density or anaemia. Parsimonious interpretations of the effect of CQ on gametocyte infectivity are discussed.

3.2 INTRODUCTION

Antimalarial chemotherapy has frequently failed to significantly reduce malaria prevalence, and resistance to many antimalarial drugs has spread at an alarming rate (Butcher 1997). This is partly because some of the commonly used antimalarial drugs have little parasite killing activity against the transmission stages of a *Plasmodium*

infection, gametocytes (Smalley & Sinden 1977, Klein *et al.* 1991). However, two other mechanisms by which chemotherapy might enhance transmission have been suggested. First, chemotherapy may induce greater gametocyte production, both through genotypic (Bishop 1954, Lines *et al.* 1991, Handunnetti *et al.* 1996) and short-term phenotypic changes (Buckling *et al.* 1997, chapter 2). Given the generally positive correlation between gametocyte densities and infectivity to mosquitoes (Carter & Graves 1988, Taylor & Read 1997), such changes are likely to increase transmission. Second, the antimalarial drug chloroquine has been reported to increase the infectiousness of gametocytes, independent of gametocyte densities (Butcher 1997).

Data on infectivity enhancement of gametocytes by chloroquine is ambiguous. It is clear that CQ *per se* does not increase gametocyte infectivity: the addition of CQ to cultures prior to feeding mosquitoes did not increase either the proportions of mosquitoes infected, or the number of parasites (oocysts) that subsequently developed (Chutmongkonkul *et al.* 1992, Rosario *et al.* 1988, Butcher 1997). Enhanced gametocyte infectivity 12 hours post sub-curative chloroquine (CQ) treatment has been demonstrated *in vivo* for the rodent malaria parasites *Plasmodium yoelli* (Ramkaran & Peters 1969, Hamidi 1977) and *P. y. nigeriensis* (Ichimori *et al.* 1990). However, there was no reported increase in the infectivity of *P. falciparum* gametocytes when mixed with sera collected 24 hours after CQ had been taken, compared with sera before treatment (Ponnundurai *et al.* 1989). Similarly, there was no infectivity enhancement of *P. vivax* in human volunteers between 0 and 72 hours post-treatment (Klein *et al.* 1991).

There has been suggestions that CQ can enhance per gametocyte infectivity a week or more after treatment, but here the data are even more ambiguous. Curative CQ-treatment of *P. falciparum*-infected humans resulted in greater median parasite burdens

in *Anopheles balabacensis* mosquitoes (but not in *A. dirus*) fed 2 & 7 days after treatment, compared to before treatment. However, mean oocyst burdens and the proportions of mosquitoes infected did not change (Wilkinson *et al.* 1976). Hogh *et al.* (1998) recently reported that 'serum from chloroquine-treated, uninfected, non-immune volunteers enhanced gametocyte infectivity with increasing efficiency for 21 days following treatment', using cultured gametocytes of *P. falciparum* and of the rodent malaria, *P. berghei*. They report a significant increase in infectivity through time following CQ treatment with *P. berghei* gametocytes, and a non-significant increase with *P. falciparum* gametocytes. Infectivity of gametocytes obtained from naturally infected CQ-treated volunteers was not significantly greater when mixed with the patients own (CQ-containing) plasma compared to European control plasma (Hogh *et al.* 1998). A field study investigating factors influencing human infectivity to mosquitoes also found no *P. falciparum* infectivity-enhancing effects of CQ (Tchuinkam *et al.* 1993).

It is clearly important to determine whether CQ enhances gametocyte infectivity for more than a matter of hours. I therefore carried out experiments to investigate gametocyte infectivity of the rodent malaria, *P. chabaudi, in vivo* 6 to 9 days after CQ-treatment, at the time of peak gametocyte production.

3.3 MATERIALS AND METHODS

Parasitology

Details of methodology are given in chapter 2. Male C57/BL/6J mice were infected by intra-peritoneal inoculation with 10^6 parasites on day 0. Parasite clones and sample sizes are shown in table 3.1. On day 5 or 6 post infection (p.i.; 1 to 2 days before the average peak asexual parasite density (Buckling *et al.* 1997, chapter 2)), half the mice were orally treated with a sub-curative dose of chloroquine sulphate (12 mg / kg); the others received distilled water (controls). On day 12 and 14 p.i. (the days of peak

gametocyte densities in CQ-treated and untreated infections, respectively (Buckling *et al.* 1997, chapter 2)), half the mice from each experimental group were anaesthetised and fed to ~40 4-5 day old *A. stephensi.* Asexual parasites and gametocytes per red blood cell (RBC) and RBC density were determined on both feed days; parasite densities were calculated from the product of these variables. Approximately 25 mosquitoes per mouse were dissected 8-9 days after feeding and the number of parasites (oocysts) on their midguts determined.

Experiment	Clone	Day p.i. of CQ treatment	No. mice infected	
			CQ	Control
1	CR	6	6	5
2	CR	5	6	6
4	ER	5	6	6

Table 3.1: Details of experiments. p.i., post-infection; CQ, chloroquine

Statistical analyses

All analyses were carried out using GLIM 4. Proportions of mosquitoes infected per mouse were analysed by logistic regression with a binomial error structure. Over-dispersion in the data was corrected by using a heterogeneity factor (HF) of 3.05, determined from the ratio of Pearson's χ^2 to the degrees of freedom in the minimal model (Crawley 1993). Day p.i. of feed (12 or 14), treatment (CQ+, CQ-) and experiment (1,2 or 3) were fitted as factors, and angular-transformed gametocytes / RBC (gametocytaemia) fitted as a covariate. The minimal model was determined by

stepwise deletion, starting with the highest order interactions, with non-significant terms discarded (Crawley 1993). The explanatory power of remaining terms was determined by deletion from the minimal model. Additional covariates (log_{10}-transformed gametocyte densities, asexual densities and RBC densities, and angular-transformed asexuals / RBC (asexual parasitaemia)) were substituted with gametocytaemia in the minimal model to determine if they explained a significant amount of additional deviance.

Further analyses were carried out in the same way within days (HF = 3, 3.6 for days 12 & 14 p.i., respectively) and treatments (HF = 1.8, 4.1 for CQ+ & CQ-, respectively), using only covariates that were significant in the previous model. Analyses of oocyst burdens were also carried out within days and treatments using a negative binomial structure, with the over-dispersion parameter, *k*, estimated separately within each analysis (Crawley 1993, Wilson & Grenfell 1997). Data from individual mosquitoes were nested within mouse to avoid psuedo-replication. Non-significant interactions were combined with the error term to determine significance of main effects. Differences in (log_{10}-transformed) gametocyte densities between days and treatments were determined by ANOVA, using stepwise deletion as above, with treatment, day and experiment fitted as factors.

3.4 RESULTS

Gametocyte densities were 3.8 times higher on day 12 p.i than day 14 p.i. in CQ-treated infections, and 4.4 times higher on day 14 p.i. than day 12 p.i. in untreated infections (figure 3.1, day by treatment interaction: $F_{1,29} = 5.03$, $p < 0.05$). In the full logistic regression model, gametocytaemia explained 46 % of the deviance associated with proportions of mosquitoes infected (figure 3.2, table 3.2), which was significantly greater than the deviance explained by gametocyte density (difference between gametocytaemia and gametocyte density: $\chi^2_1 = 4.87$, $p < 0.05$). The

relationship between (untransformed) gametocytaemia and proportion of mosquitoes infected was approximately linear (figure 3.2); allowing the best fit regression to curve, by the addition of the quadratic function, did not explain significantly more deviance ($p > 0.1$).

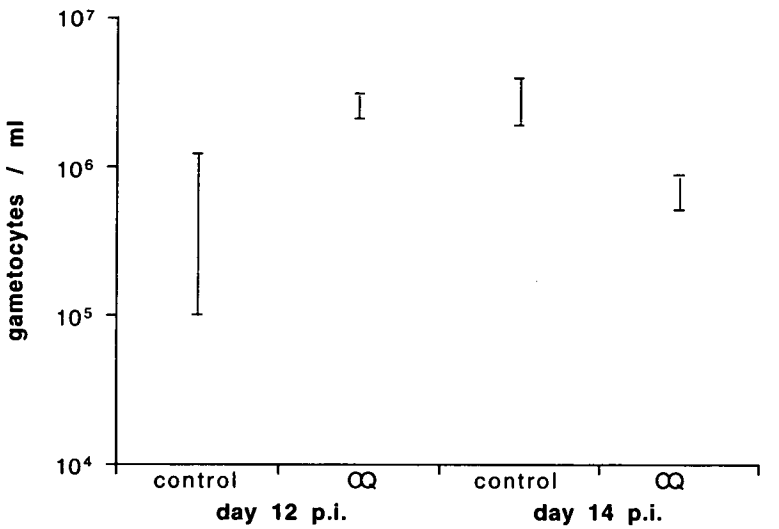


Figure 3.1: Mean ± 1 s.e. gametocyte densities in CQ-treated and untreated infections on days 12 & 14 post infection.

To determine the effect of CQ on per gametocyte infectivity, gametocytaemia had to be controlled for. When this was done, the effect of CQ-treatment on proportions of mosquitoes infected differed on days 12 and 14 p.i. (table 3.2). No main effects of the factors (day, experiment or treatment), covariates (gametocyte, asexual and RBC densities, and asexual parasitaemia) or their other interactions explained a significant additional amount of deviance.

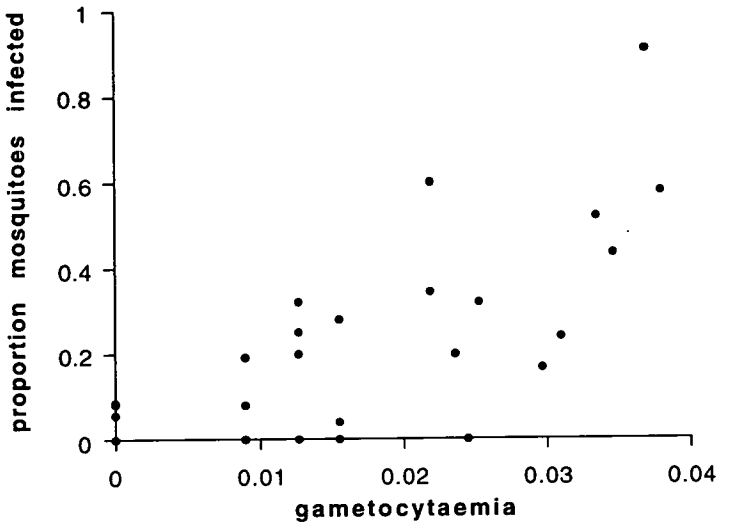


Figure 3.2: Relationship between proportion of mosquitoes infected and gametocytaemia (gametocytes / 1000 RBCs) for all data (35 mice). Some points are multiple data points.

	χ^2_1	p
Gametocytaemia	25.12	< 0.001
Day of mosquito feed	0.74	n.s.
Treatment	0.95	n.s.
Day x treat.	4.19	< 0.05

Table 3.2: Minimal model for logistic regression of mosquito infection probability. Experiment, additional covariates and the other interactions were removed from the model as they did not explain a significant amount of deviance ($p > 0.1$). n.s.; non-significant.

Because of the significant treatment by day interaction in the full model, data were separately analysed within days and treatments. When the data from only day 12 p.i was analysed, infectivity per gametocyte was 3.2 times greater (at the mean gametocytaemia) in CQ-treated than untreated infections (figure 3.3a, table 3.3). Mean oocyst burdens (per gametocyte) were 3.1 times greater (at the mean gametocytaemia) in CQ-treated compared to untreated infections (table 3.3). Neither proportions of mosquitoes infected or mean oocyst burdens (per gametocyte) on day 14 p.i. differed between CQ-treated and untreated infections (figure 3.3b, table 3.3).

	Proportion infected		**Oocyst burdens**	
	Day 12	Day 14	Day 12	Day 14
Gametocytaemia	$\chi^2_1 = 12.1^{**}$	$\chi^2_1 = 16.1^{**}$	$F_{1,10} = 88.6^{**}$	$F_{1,13} = 27.8^{**}$
Treatment	$\chi^2_1 = 4.3^{*}$	$\chi^2_1 = 0.56$	$F_{1,10} = 12.1^{**}$	$F_{1,13} = 1.8$
Experiment	$\chi^2_2 = 0.5$	$\chi^2_2 = 1.48$	$F_{2,10} = 0.4$	$F_{1,13} = 1.5$
Gam. x exp.			$F_{2,10} = 6.1^{*}$	
mean+s.e. control	0.065+0.03	0.22+0.1	0.48+0.31	1.32+0.3
mean+s.e. CQ	0.21+0.05	0.20+0.09	1.49+0.35	1.17+0.24

Table 3.3: Minimal models for logistic regression of proportion of mosquitoes infected and negative binomial analysis of oocyst burdens on days 12 and 14 p.i.; non-significant interactions were removed from the model. $^{*}p < 0.05$; $^{**}p < 0.01$. Arithmetic mean (+ 1 s.e.; corrected for error distribution) oocyst burdens, proportions of mosquitoes infected at the mean gametocytaemia (= 0.35 gametocytes / 1000 RBCs) are shown for CQ (chloroquine)-treated and control infections

When only untreated infections were considered, the proportion of mosquitoes infected and mean oocyst burdens (per gametocyte) were, at the mean gametocytaemia,

respectively 3.4 and 2.9 times greater on day 14 p.i. than day 12 p.i. (figure 3.3c, table 3.4). However, within CQ-treated, gametocyte infectivity as measured by both proportions of mosquitoes infected and mean oocyst burdens, did not differ between days 12 & 14 p.i. (figure 3.3d, table 3.4).

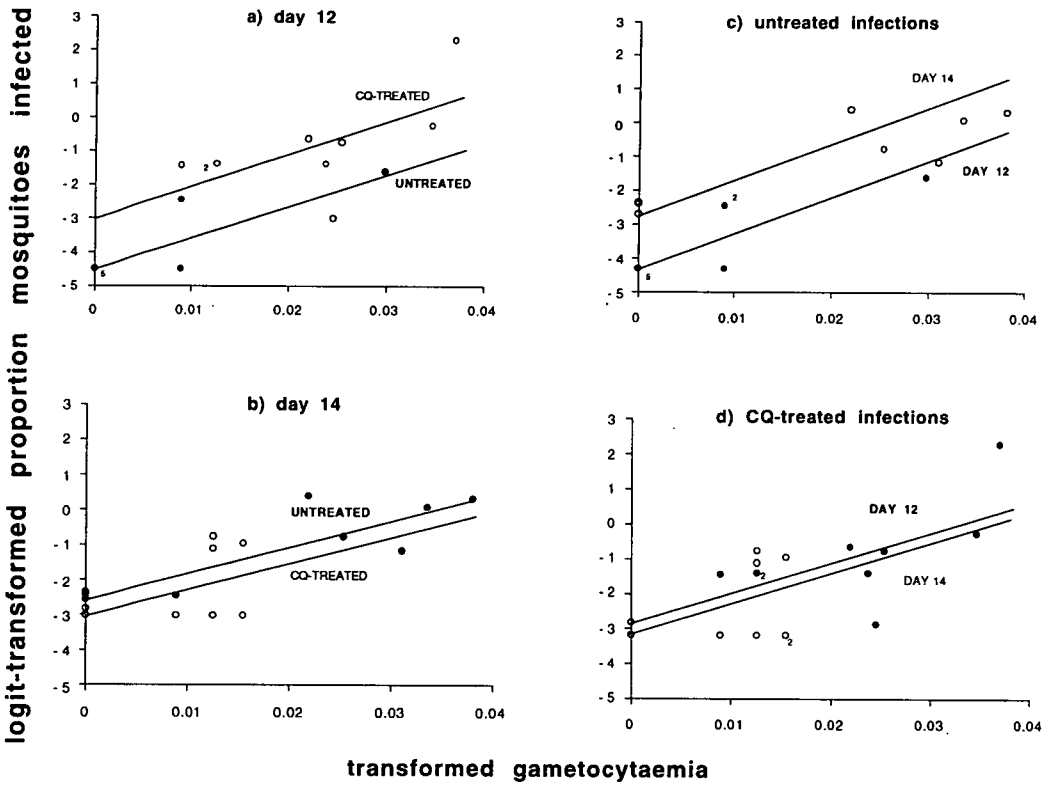


Figure 3.3: Relationship between logit-transformed proportions of mosquitoes infected and square-root arcsin-transformed gametocytaemia. Lines show ordinary least square regressions from statistical models. Legends in bold correspond to solid symbols. Small numbers in body of figures represent multiple data points. (a) data from day 12 post-infection (p.i.). (b) data from day 14 p.i. (c) data from all untreated infections. (d) data from all CQ-treated infections.

	Proportion infected		Oocyst burdens	
	Control	CQ-treated	Control	Cq-treated
Gametocytaemia	$\chi^2_1 = 26.7^{**}$	$\chi^2_1 = 13.2^{**}$	$F_{1,12} = 30.3^{**}$	$F_{1,11} = 12.9^{**}$
Day	$\chi^2_1 = 7.9^{*}$	$\chi^2_1 = 0.2$	$F_{1,12} = 10.9^{**}$	$F_{1,11} = 1.33$
Experiment	$\chi^2_2 = 0.7$	$\chi^2_2 = 0.2$	$F_{2,12} = 1.8$	$F_{1,11} = 0.5$
Gam. x Exp.				$F_{2,11} = 5^{*}$

Table 3.4: Minimal models for logistic regression of proportion of mosquitoes infected and negative binomial analysis of oocyst burdens for control and CQ-treated infections; non-significant interactions were removed from the model. $^{*}p < 0.05$; $^{**}p < 0.01$.

3.5 DISCUSSION

Gametocytaemia was the best predictor of infectiousness in this study (figure 3.2), but did CQ enhance per gametocyte infectiousness? On day 12 p.i., gametocytes in CQ-treated mice were more infectious than those in untreated infections (figure 3.3a), both in terms of proportion of mosquitoes infected and mean oocyst burdens. Two days later, any effects of CQ on gametocyte infectivity were no longer detectable (figure 3.3b). These data can be interpreted in two ways. First, CQ enhanced infectivity on day 12 p.i., but the effect was lost by day 14 p.i. Second, infectivity was suppressed on day 12 p.i. in untreated infections, but not on day 14 p.i. That infectivity was greater on day 14 p.i. than day 12 p.i. in untreated infections (figure 3.3c), but there was no difference in infectivity between days in CQ-treated infections (figure 3.3d), leads to acceptance of the latter hypothesis. Thus, any effect of CQ on infectivity was not direct.

The reduction in infectivity in untreated infections relative to CQ-treated infections on day 12 p.i. is consistent with the infection dynamics. In untreated infections a

phenomenon called 'crisis' occurs. This is a rapid reduction in parasite numbers associated with low RBC densities and strong immune activity (Jarra & Brown 1989), during which gametocyte infectivity is suppressed (Wery 1968, and see Carter *et al.* 1997 for a related phenomenon in the human malaria parasite, *P. vivax*). Crisis occurred between days 8-10 p.i. in untreated infections in these experiments, and it is entirely plausible that gametocyte infectivity-suppressing 'crisis factors' are still present by day 12 p.i., but not day 14 p.i. Crisis did not occur in CQ-treated infections because of the greatly reduced peak asexual parasite densities (Buckling *et al.* 1997).

In untreated infections, reduced gametocyte infectivity on day 12 p.i. was also associated with low gametocyte densities. There was a similar pattern on day 14 p.i. in CQ-treated infections (figures 3.1 & 3.3). The infectivity of individual gametocytes may therefore be a positive function of gametocyte density, as would be the case if the probability of gamete fusion in the mosquito midgut was considerably reduced at low gametocyte densities. There is however no evidence that per gametocyte infectiousness increased non-linearly at high gametocytaemias (figure 3.2).

The results imply that if CQ does increase the infectiousness of *P. chabaudi* gametocytes, the effect is lost by 6-7 days post-treatment. This is before the vast majority of gametocytes were produced in both untreated and CQ-treated infections (Buckling *et al.* 1997, chapter 2). The plasma half-life of CQ in malaria infected mice is approximately 7 hours (Cambie *et al.* 1994), and thus would be at very low concentrations after 6-7 days. The current results are therefore inconsistent with the suggestion that long-term metabolites of CQ are responsible for the reported infectivity-enhancing effects of CQ (Butcher *et al.* 1997, Hogh *et al.* 1998). Ultimately, our data are consistent with most previously published data: there is no

strong evidence to suggest that CQ has long-term infectivity-enhancing effects on gametocytes.

Infectivity enhancement by CQ has only been convincingly demonstrated in the short-term (12 hours post-treatment), in controlled experiments using rodent malaria parasites *in vivo* (Ramkaran & Peters 1969, Hamidi 1977, Ichimori *et al.* 1990). A plausible explanation for these data is an immunosuppressive effect of CQ. CQ is known to inhibit antigen processing and presentation (Fox & Kang 1993), and leukocyte function in general (reviewed in Targett 1992a). Such mechanisms could conceivably reduce anti-gametocyte immune activity, resulting in increased gametocyte infectivity in the short term. Infectivity enhancing effects would be expected to last only as long as CQ (or the major antimalarial metabolite, desethyl-CQ) were at sufficient concentrations to have an immunosuppressive effects. Infectivity enhancement would be expected to increase with CQ dose, up to a point where growing gametocytes were being destroyed - mature gametocytes are unaffected by CQ (Smalley & Sinden 1977). This prediction is supported by intermediate doses of CQ having the greatest infectivity-enhancing effect (Ramkaran & Peters 1969). Although a short-term immunosuppressive effect of CQ could be of potential importance to malaria epidemiology and the spread of drug-resistant genotypes, longer term effects, if they occur, would be of great significance. We find no evidence of such long term effects in *P. chabaudi*. Further work on *P. falciparum* is required to resolve this important issue.

Chapter 4

INCREASED GAMETOCYTOGENESIS IN *PLASMODIUM CHABAUDI* FOLLOWING ANTIMALARIAL CHEMOTHERAPY

4.1 SUMMARY

The proportion of asexual blood-stage malaria parasites that develop into transmission stages (gametocytes) can increase in response to stress. I investigated whether stress imposed by a variety of antimalarial drugs, used sub-curatively as treatment or prophylaxis, increased gametocyte production (gametocytogenesis) in the rodent malaria parasite, *Plasmodium chabaudi, in vivo*. All methods of chemotherapy greatly reduced the numbers of asexual parasites produced during an infection, but increased overall rates of gametocytogenesis: there was either no reduction in gametocyte densities, or a smaller reduction than experienced by asexuals. I used a simple model to estimate temporal variation in gametocyte production. In untreated and prophylaxis infections, gametocytogenesis always increased as the infection progressed, showing a negative relationship with asexual multiplication. Drug treatment resulted in high rates of gametocytogenesis earlier in the infection, resulting in earlier peak gametocyte densities relative to untreated infections. Given the correlation between gametocyte densities and infectivity to mosquito vectors, and the high frequency of sub-curative drug treatment and prophylaxis, these data suggest that chemotherapy may frequently have only a small effect on reducing malaria transmission and may help to explain the rapid spread of drug resistant genotypes.

4.2 INTRODUCTION

Mammalian malaria parasites (*Plasmodium* spp.) undergo cycles of asexual replication in red blood cells (RBCs), with a small proportion of the asexual parasites producing

non-replicating transmission stages, gametocytes. *In vitro* studies have demonstrated that gametocyte production (gametocytogenesis) of the human malaria parasite *P. falciparum* is upregulated in response to a variety of stimuli, most of which create conditions unfavourable for asexual replication (Mons 1985, Carter & Graves 1988, Sinden *et al.* 1996). If increased gametocytogenesis is a general response to unfavourable conditions, it may be expected following novel stresses, such as antimalarial chemotherapy (Buckling *et al.* 1997, chapter 2).

If drugs are used in doses which kill some parasites, the survivors will inevitably suffer some ill effects from the drugs and may be stimulated to produce gametocytes. Indeed, sub-curative treatment is common (Wernsdorfer 1994), as is partially protective prophylaxis (drugs taken before infection), given the high proportion of individuals with low drug concentrations detected in blood and urine (e.g. Koella *et al.* 1990, Hogh *et al.* 1995). Because of the generally positive correlation between gametocyte density and infectivity to the mosquito vector (Carter & Graves 1988, Taylor & Read 1997, Taylor *et al.* 1997, Buckling *et al.* 1997, chapter 2), stress-induced gametocyte production may render antimalarial chemotherapy ineffective at markedly reducing malaria transmission.

A number of clinical studies have investigated the effect of antimalarial drugs on gametocytogenesis in humans (reviewed in Buckling *et al.* 1997, chapter 2). The data are however necessarily ambiguous. For ethical reasons, curative drug doses were used. In addition, there were no untreated controls, so that any increases in gametocyte densities may represent the natural progression of the infection. However, a series of controlled experiments on the effect of treatment with the antimalarial chloroquine on gametocyte production in the rodent malaria, *Plasmodium chabaudi*, showed that following chloroquine treatment, the proportion of circulating parasites

that were gametocytes was more than twice that in untreated infections, peak gametocyte densities occurred two days earlier and, importantly, treated infections were as infectious to mosquitoes as untreated infections (Buckling *et al.* 1997, chapter 2).

Here I report further investigations into chemotherapy-induced gametocytogenesis of *Plasmodium chabaudi in vivo*, which address the following issues. First, is increased gametocytogenesis and the earlier appearance of peak gametocyte densities in response to sub-curative drug treatment independent of the mode of action of antimalarial drugs? I used pyrimethamine, quinine & mefloquine, which have different parasite-killing mechanisms. Pyrimethamine ultimately inhibits DNA synthesis. The mechanisms of action of the other drugs are not fully understood, but, like chloroquine, they are believed to interfere with haemoglobin metabolism in some way (reviewed in Butcher 1997). Second, does increased gametocytogenesis occur following different types of sub-lethal prophylaxis? I used pyrimethamine and chloroquine to test this. Third, how does gametocytogenesis temporally vary during the course of an untreated *in vivo* infection, and how is this pattern altered by chemotherapy?

4.3 MATERIALS AND METHODS

Parasites & hosts

Male C57/BL/6J mice (Harlan-Olac, England) aged 8-14 weeks were infected with $1x10^6$ red blood cells infected with a drug-naive *P. chabaudi* clone (either CR or ER (Beale *et al.* 1978), from the WHO Registry of Standard Malaria Parasites maintained at the University of Edinburgh, UK) in a 0.1 ml intra-peritoneal inoculum of 50 % ringer's solution (27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl), 45 % heat-inactivated calf serum and 5 % 200 units / ml heparin solution. Mice, age-matched within experiments, were housed in cages of 2-4 animals at a temperature of 25±1 °C with a 0700 to 1900 hours light cycle, and provided with 41B rat and mouse maintenance diet

(Harlan-Teklad, England) and water containing 0.05 % pABA, *ad libitum.*

Drug preparation and administration

Chloroquine solution was prepared from a stock of 40 mg / ml Chloroquine sulphate (Nivaquine^{TM}) diluted in distilled water. Quinine & mefloquine solutions were prepared by dissolving their hydrochlorides in distilled water, except in the cases where pyrimethamine (base) was used in experiments, in which case all drugs were dissolved in Dimethyl sulfoxide (DMSO). Drug solutions were administered orally using a lubricated catheter in approximately 0.1 ml volumes; doses are shown in table 4.1. Drug treatment occurred on day 5 post-infection (p.i.) between 1600 and 1700 hours, and prophylaxis approximately 1 hour before parasite inoculation (day 0 p.i.). Control mice were given 0.1 ml distilled water or DMSO, depending on the solvent used for the drugs in each experiment.

Parasite counts

Thin blood smears from the tail vein were taken approximately every second day from day 4 or 5 p.i., Giemsa-stained and asexual parasites counted per 1.5 x 10^3 red blood cells (RBCs). At low asexual densities parasites were counted per 10^4 RBCs. Mature gametocytes were counted per 1.25 x 10^4 RBCs. Parasite densities were calculated from RBC densities, measured by flow cytometry (Coulter Electronics^{TM}) every second day, multiplied by parasites per RBC.

Experiments

Details of individual experiments are shown in table 4.1.

Experiment	Experimental block	Clone	Treatment	dose (mg/kg)*	n
1	1	CR	untreated	-	5
			qu-treated	200	4
2	2	ER	untreated	-	4
			qu-treated	200	4
3	3	ER	untreated	-	4
			qu-treated	200	6
1	1	CR	untreated	-	5
			py-treated	2	4
3	2	ER	untreated	-	4
			py-treated	5	5
5	1	CR	untreated	-	4
			mf-treated	9	4
6	2	ER	untreated	-	4
			mf-treated	9	3
1	1	CR	untreated	-	5
			py proph	1	4
4	2	ER	untreated	-	6
			py proph	2	5
4	1	ER	untreated	-	6
			cq proph	6	5
5	2	CR	untreated	-	4
			cq proph	4	5
6	3	ER	untreated	-	4
			cq proph	4	3

Table 4.1: Details of experiments and analyses. Each 'Experiment' was carried out at a different time, resulting in 2 or 3 'Experimental blocks' for each method of chemotherapy. qu = quinine, py = pyrimethamine, mf = mefloquine, cq = chloroquine; proph = prophylaxis; n = number of mice. * mg / kg mouse weight.

Estimation of daily rates of gametocytogenesis

I used a simple exponential growth model to estimate the proportion of asexual parasites that produced gametocytes on individual days (see figure 4.1).

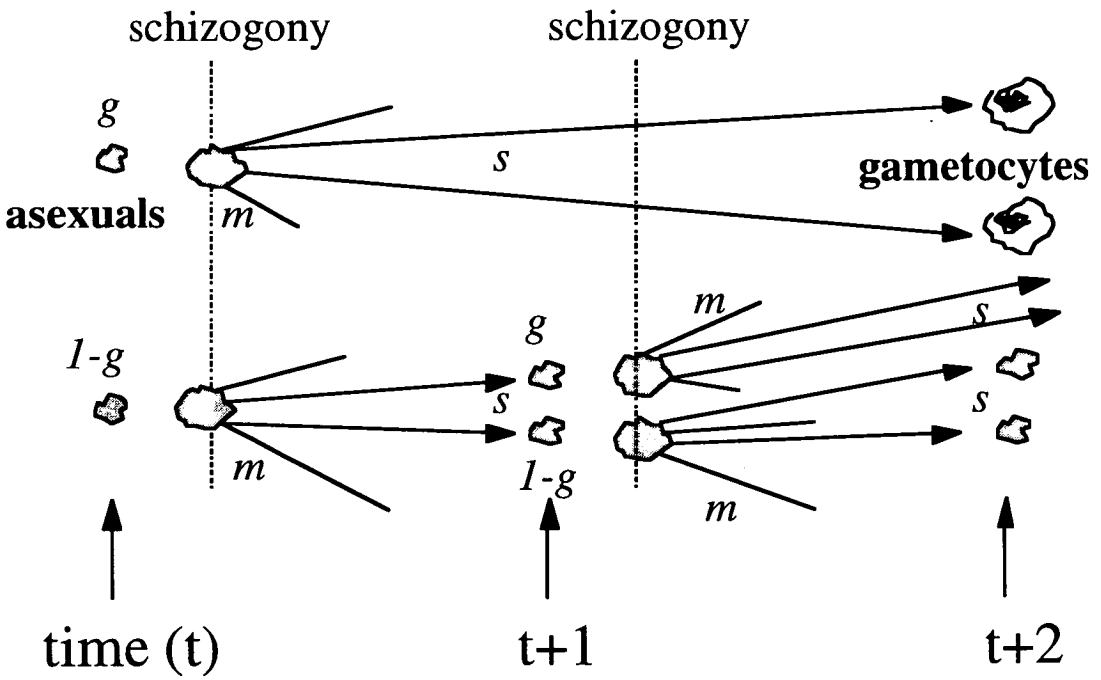


Figure 4.1: Schematic showing parameters of the model (see text). A proportion (g) of asexual parasites measured at time t will produce gametocytes when they reach maturity. Mature parasites undergo schizogony, producing m progeny. A proportion of parasites (s) will survive until $t+1$. Surviving asexual parasites then repeat the cycle. Surviving gametocytes undergo no further mortality, reaching maturity at $t+2$.

The synchrony of parasite development in *P. chabaudi* infections (Cox 1988) simplifies matters. The maturation period of *P. chabaudi* gametocytes is

approximately 2 days (Gautret *et al.* 1997), such that a proportion, g, of the asexual parasites at day t will produce gametocytes that will mature on day $t+2$. *P. falciparum* gametocytes become less vulnerable to immune and drug clearance as they mature, probably because they express less immunogenic antigens and have reduced metabolic activity (Carter & Graves 1988). Age-dependent mortality of *P. chabaudi* gametocytes is likely to be similar. It is therefore assumed that survival probability (s) in the first 24 hours after asexual densities were measured is the same for gametocytes and asexuals. This period encompasses the time taken for asexual parasites to mature and rupture (schizogony), releasing the gametocyte or asexual progeny. Schizogony occurs around midnight in clones ER and CR under the employed light-dark cycle (McLean 1986), approximately 7 hours after parasite densities at time t were measured. Zero gametocyte mortality is assumed in the second 24 hour period.

The half-life of mature *P. chabaudi* gametocytes is estimated to be less than 12 hours (A. Buckling, unpublished data), so that few mature gametocytes are likely to contribute to the gametocyte population of the following day. If we assume that asexual parasites produce m progeny parasites which develop into all gametocytes or asexuals (as is the case for *P. falciparum*; Bruce *et al.* 1990), the number of gametocytes on day $t+2$ (G_{t+2}) can be written as

$$G_{t+2} = s\, g\, m\, A_t \qquad (1),$$

where A_t refers to the number of asexuals on day t..

The asexual cycle of *P. chabaudi* is 24 hours (Cox 1988), so that two asexual cycles occur during the maturation time of a gametocyte. If we assume that both asexual mortality, s, and the probability of an asexual parasite producing gametocytes, g , are constant during the two day period, the number of asexuals on day $t+2$ (A_{t+2}) can be

written as

$$A_{t+2} = s^2 (1-g)^2 m^2 A_t \qquad (2).$$

Combining equations 1 and 2,

$$g = \frac{\frac{G_{t+2}}{A_t}}{\sqrt{\frac{A_{t+2}}{A_t} + \frac{G_{t+2}}{A_t}}} \qquad (3).$$

We can thus estimate g from observed densities of asexuals and gametocytes. There are potential problems with some of the assumptions outlined above. In particular, it is likely that rates of gametocytogenesis (g) and survival of parasites (s) will differ between the two rounds of asexual replication that occur for each estimate of g. Values of g were generally very low (see below); differences in g between the two periods will thus have only a small effect on the estimates. Differences in parasite survival may however be very large, potentially under-estimating or over-estimating g when mortality is greatest during the first or second period, respectively. However, based on biologically feasible corrections to the assumptions, Crooks *et al.* (in preparation) demonstrate that the current estimates of gametocytogenesis are a good approximation, although accuracy is likely to markedly decrease towards the end of infections.

Statistical analysis

The following summary measures were determined for each infection.

(1) *Total asexuals*. This was estimated from parasite densities between days 7-18 p.i. for drug treatment comparisons, and between days 0-18 p.i. for prophylaxis comparisons. Parasite densities prior to day 7 p.i. were not used for the former

because the effects of drug treatment on gametocytogenesis would not be apparent, and by day 18 p.i., the vast majority of parasites had been cleared by their hosts. Estimates of total parasites between these days were obtained by integrating under the parasite density through time curves for each infection. This is a reasonably accurate measure of total numbers of asexuals (between the appropriate time periods) because the asexual cycle of *P. chabaudi* is known to be 24 hours (reviewed in Cox 1988).

(2) *Total gametocytes.* An index of the total number of gametocytes was estimated between the same time periods as for the asexual parasites by integrating under the gametocyte density through time curves. This measure will be reasonably accurate, as the half-life of gametocytes is less than 12 hours and measures were taken every second day. The sum of the gametocyte densities on each day is likely to be a well correlated with total transmission probability: gametocyte densities at any point in time correlate with both the proportion of mosquitoes infected and parasite densities within mosquitoes (Taylor & Read 1997).

(3) *Proportion of gametocytes* . This was calculated as the proportion of total parasites [(1)+ (2)] that were gametocytes [2] for each infection.

(4) *Day of peak gametocyte density.*

(5) *Daily rates of gametocytogenesis.* Rates of gametocytogenesis were calculated for all drug-treated, and the appropriate untreated infections, on days 5, 8, 10, 12 p.i., except where asexual densities on those days were determined to be zero. Rates were additionally calculated for days 14 & 16 p.i. in prophylaxis experiments, when, unlike the drug-treated infections, parasite densities were consistently at a detectable level.

Prior to analysis, estimates of total asexuals and total gametocytes were $\log_{10}$-

transformed, and proportions of gametocytes were square-root arcsin-transformed, to bring the distributions close to normal. The effect of each method of chemotherapy relative to the appropriate control infections (i.e. separate analyses were carried out for quinine-treated, pyrimethamine-treated, mefloquine-treated, pyrimethamine prophylaxis and chloroquine prophylaxis infections) on each of these measures was determined by ANOVA using GLIM 4 (Crawley 1993), with TREATMENT (drug+, drug-) and EXPERIMENTAL BLOCK (each method of chemotherapy was compared with untreated infections 2-3 times; see table 4.1) fitted as factors. The interaction term was removed from the statistical model if non-significant ($p > 0.05$) (Crawley 1993). Analyses of days of peak gametocyte density for each infection were carried out as above, except the data for all drug treatment experiments was pooled together, and likewise for all the prophylaxis data.

Daily rates of gametocytogenesis (g, in equation 3) were highly over-dispersed and in no way approximated a normal distribution, regardless of transformation. The data consisted of many zeros and very low values, as well as much higher values (figure 4.2). Even the use of non-parametric ranking procedures is unlikely to provide an appropriate solution to this problem, given that variation in the very low values will often represent the presence or absence of a single gametocyte in the surveyed microscopic fields. A nominal classification system was therefore used, with each rate of gametocytogenesis classified as high or low, based on whether the value was less or greater than the median of the data set. (The drug treatment and prophylaxis data sets were analysed separately). To control for multiple sampling from the same infection, the median of a data set was calculated as the median of the medians for each infection within the data set. Data for each day was analysed using chi-squared tests of independence (with Yates's correction for continuity), to determine whether the proportion of infections that had high rates of gametocytogenesis (median > 0.008 and 0.003, for drug treatment and prophylaxis comparisons, respectively) differed

between all drug-treated and the appropriate untreated infections, and all prophylaxis and the appropriate untreated infections.

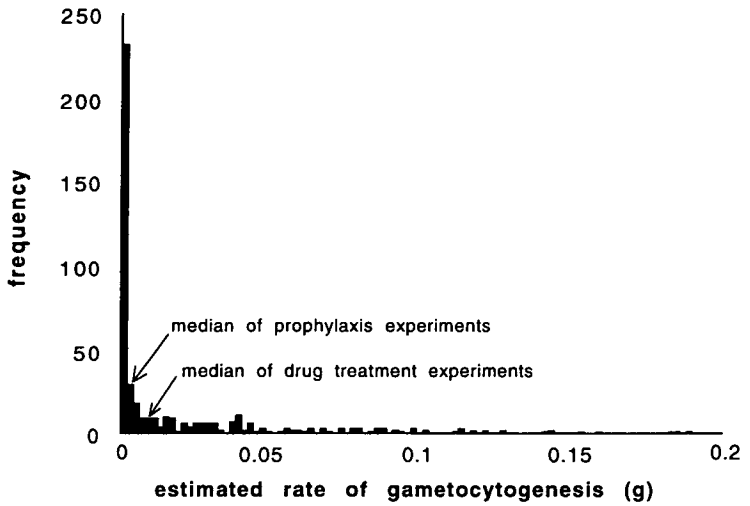


Figure 4.2: Histogram of rates of gametocytogenesis at all time points, in all infections.

4.4 RESULTS

Total parasites

Quinine, pyrimethamine and mefloquine treatment reduced total asexuals by approximately 75 % in all cases (table 4.2). Quinine treatment reduced total gametocytes by approximately 25 %; the other methods of drug treatment did not result in a significant decrease (table 4.2). The proportion of parasites that were gametocytes was approximately 4 times higher following quinine and pyrimethamine treatment, than they were in untreated infections; there was no significant difference between mefloquine-treated and untreated infections (figures 4.3 & 4.4, table 4.2).

Method of chemotherapy	Total asexuals ($x10^8$)	Total gametocytes ($x10^6$)	Proportion gametocytes ($x10^{-3}$)
qu-treated			
Treatment	$F_{1,23} = 51.2^{**}$	$F_{1,23} = 6.17^{*}$	$F_{1,23} = 5.18^{*}$
Experiment	$F_{2,23} = 2.48$	$F_{2,23} = 1.10$	$F_{2,23} = 2.92$
Treat. x exp.	$F_{2,21} = 0.36$	$F_{2,21} = 0.29$	$F_{2,21} = 1.06$
mean (s.e.) drug-	25.6 (1.7)	12.8 (1.52)	6.1 (0.9)
mean (s.e.) drug+	5.36 (1.10)	8.75 (3.20)	29 (16)
py-treated			
Treatment	$F_{1,15} = 23.8^{**}$	$F_{1,15} = 2.07$	$F_{1,15} = 16.2^{**}$
Experiment	$F_{1,15} = 3.05$	$F_{1,15} = 1.51$	$F_{1,15} = 8.4^{**}$
Treat. x exp.	$F_{1,14} = 1.24$	$F_{1,14} = 0.2$	$F_{1,14} = 3.8$
mean (s.e.) drug-	23.71 (1.32)	10.87 (1.4)	5.6 (0.8)
mean (s.e.) drug+	7.08 (2.37)	7.96 (2.12)	22 (6.6)
mf-treated			
Treatment	$F_{1,12} = 26.3^{**}$	$F_{1,12} = 2.2$	$F_{1,12} = 2.67$
Experiment	$F_{1,12} = 1.67$	$F_{1,12} = 5.54^{*}$	$F_{1,12} = 9.38^{**}$
Treat. x exp.	$F_{1,11} = 1.41$	$F_{1,11} = 0.95$	$F_{1,11} = 1.16$
mean (s.e.) drug-	24.51 (1.11)	12.92 (1.61)	6.4 (1.3)
mean (s.e.) drug+	5.96 (1.75)	12.94 (6.76)	14 (4.7)
py proph.			
Treatment	$F_{1,16} = 22.7^{**}$	$F_{1,16} = 0.76$	$F_{1,17} = 5.67^{*}$
Experiment	$F_{1,16} = 8.46^{*}$	$F_{1,16} = 23.0^{**}$	$F_{1,17} = 12.14^{**}$
Treat. x exp.	$F_{1,16} = 6.24^{*}$	$F_{1,16} = 5.82^{*}$	$F_{1,16} = 2.67$
mean (s.e.) drug-	40.9 (2.1)	13.1 (2)	2.9 (0.5)
mean (s.e.) drug+	23.4 (4.5)	12 (3.7)	4.6 (1.2)
cq proph.			
Treatment	$F_{1,21} = 61.74^{**}$	$F_{1,23} = 3.93$	$F_{1,23} = 0.15$
Experiment	$F_{2,21} = 5.46^{*}$	$F_{2,23} = 6.01$	$F_{2,23} = 5.23$
Treat. x exp.	$F_{2,21} = 6.72^{*}$	$F_{2,21} = 1.23$	$F_{2,21} = 0.57$
mean (s.e.) drug-	37.2 (3.5)	10.9 (2.3)	2.8 (0.5)
mean (s.e.) drug+	19.55 (2)	7.35 (1.5)	3.2 (0.4)

Table 4.2: Results of the analyses of total asexuals, gametocytes and the proportion of gametocytes. All methods of chemotherapy were analysed against the appropriate untreated infections. qu = quinine, py = pyrimethamine, mf = mefloquine, cq = chloroquine; proph. = prophylaxis. * $p < 0.05$; ** $p < 0.01$.

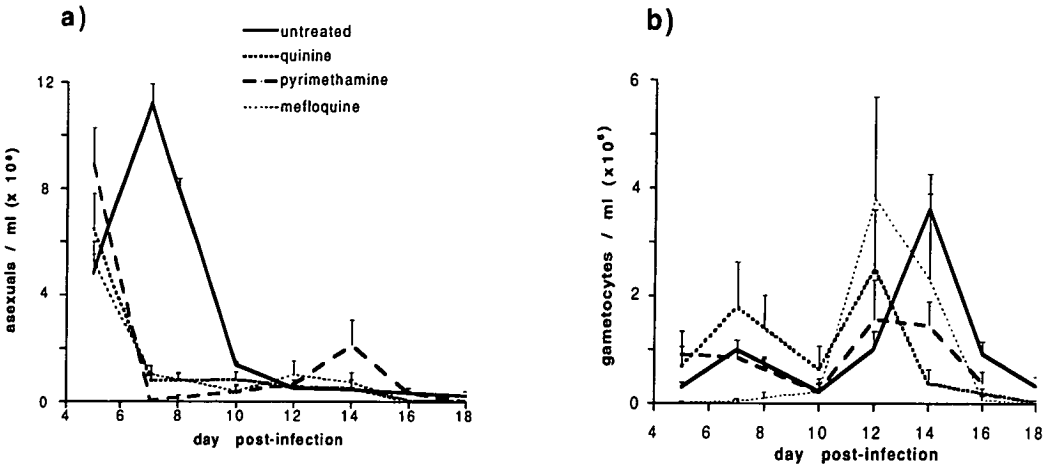


Figure 4.3: Mean + 1 s.e asexual densities (a) & gametocyte densities (b) through time for drug-treated and control infections. Data are pooled across experiments.

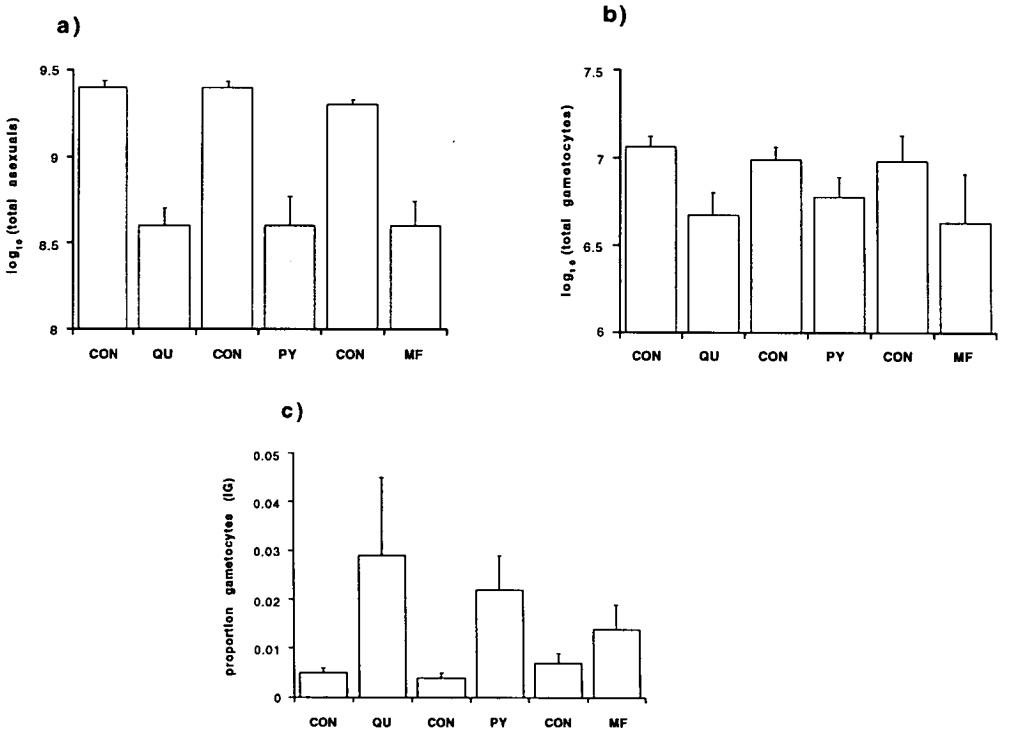


Figure 4.4: Mean + 1 s.e total asexual parasites (a), total gametocytes (b) & proportion of gametocytes for all drug-treated and appropriate control infections.

There was an approximately 50% reduction in total asexuals, but no significant reduction in total gametocytes following both pyrimethamine and chloroquine prophylaxis, relative to untreated infections (table 4.2). The resultant proportion of parasites that were gametocytes was not significantly different from untreated infections following chloroquine prophylaxis, but was almost twice that of untreated infections following pyrimethamine prophylaxis (figures 4.5 & 4.6, table 4.2).

Both clones CR and ER were used in at least one experimental block each for all comparisons between individual methods of chemotherapy and the appropriate untreated infections. Significant differences between experimental blocks ($p < 0.05$) occurred for total gametocytes and the proportion of gametocytes in the mefloquine treatment experiments and for total asexuals, total gametocytes and the proportion of gametocytes for both prophylaxis experiments. In all cases, these effects could be attributed to significantly higher values in clone CR than clone ER. There were no other significant differences between experimental blocks for either of these measures. The magnitude of the difference between untreated and prophylaxis infections differed significantly between experimental blocks (experimental block by treatment interactions) for total asexuals and total gametocytes in the pyrimethamine prophylaxis experiments, and for total asexuals in the chloroquine prophylaxis experiments, although the treatment effects were always in the same direction within-experimental block. These effects can be attributed to different drug doses being used in the different experimental blocks. There were no other significant experimental block by treatment interactions.

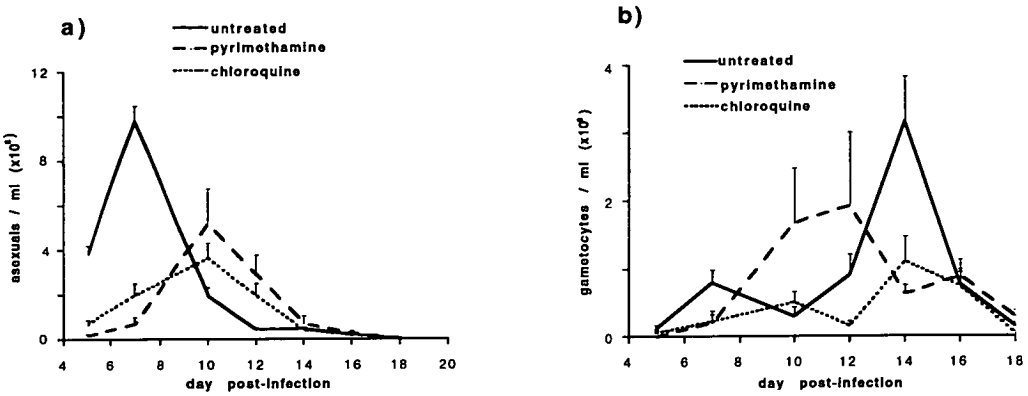


Figure 4.5: Mean + 1 s.e. asexual densities (a) & gametocyte densities (b) through time for drug prophylaxis and control infections. Data are pooled across experiments.

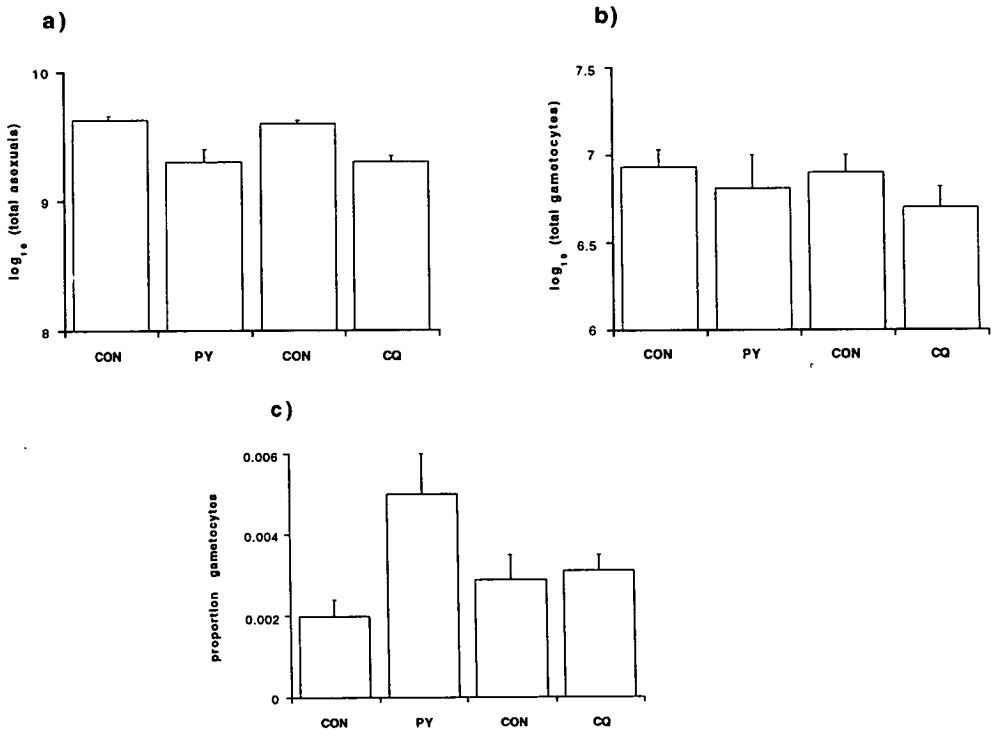


Figure 4.6: Mean + 1 s.e total asexual parasites (a), total gametocytes (b) & proportion of gametocytes for all prophylaxis and appropriate control infections.

Timing of peak gametocyte densities

Drug treatment resulted in peak gametocyte density on average occurring 3 days earlier than in untreated infections (day 11 p.i. and day 14 p.i, respectively; figure 4.3, $F_{1,45} = 9.0$, $p < 0.01$). Prophylaxis did not induce a change in the timing of peak gametocyte densities, (peaks in untreated and prophylaxis infections occurring on average between days 13 and 14 p.i.; figure 4.5, $F_{1,36} = 0.06$, $p > 0.1$). There were no significant experimental block or interaction effects ($p > 0.1$ in all cases).

Estimates of daily rates of gametocytogenesis

The results of the analyses of conversion rates through time for drug treatment and prophylaxis experiments are shown in table 4.3. Rates of gametocytogenesis increased through time, with most conversion rates below the overall median early in the infection, and most above the median towards the end (figures 4.7 & 4.8). This pattern differed quite markedly for drug-treated infections: almost 50 % of infections had rates of gametocytogenesis above the median immediately following drug treatment (on day 5 p.i.) and 80 % had rates above the median (compared with 40 % in untreated infections) on day 10 p.i. (figure 4.7). In general, the different methods of drug treatment resulted in similar patterns of gametocytogenesis through time, except on day 5 p.i. when, unlike quinine- and pyrimethamine-treated infections, no mefloquine-treated infections had high rates of gametocytogenesis (figure 4.7). Patterns of gametocytogenesis through time were similar for prophylaxis and untreated infections. The only exception was on day 10 p.i., when the percentage of infections with rates of gametocytogenesis above the median were 60 % and 20 % for untreated and prophylaxis infections, respectively (figure 4.8). Patterns of gametocytogenesis based on the proportion of infections that had high rates of gametocytogenesis were very similar to patterns of mean gametocytogenesis (compare figures 4.7 & 4.8 with figure 4.9).

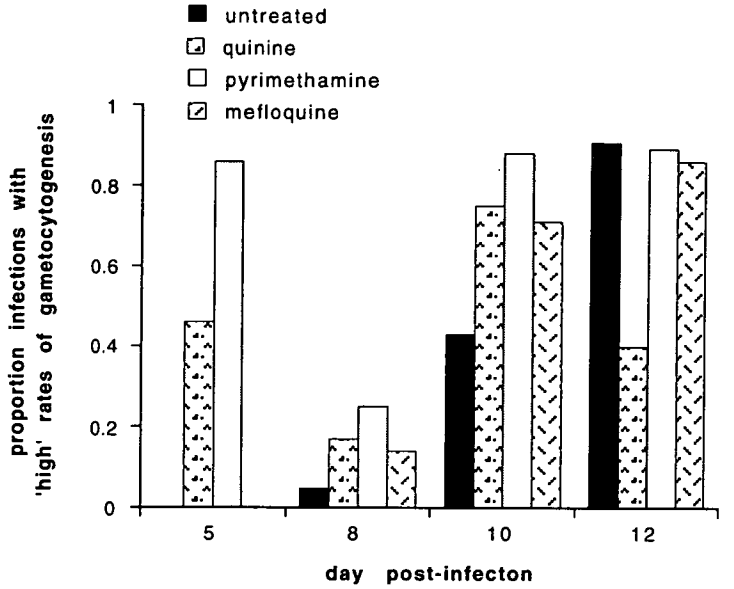


Figure 4.7: Proportion of infections in drug treatment experiments with 'high'(greater than the median) rates of gametocytogenesis through time.

Day p.i.	drug-treated Vs untreated	prophylaxis Vs untreated
5	$\chi^2_1 = 10.2$**	$\chi^2_1 = 0.91$
8	$\chi^2_1 = 0.98$	$\chi^2_1 = 0.27$
10	$\chi^2_1 = 4.75$*	$\chi^2_1 = 3.96$*
12	$\chi^2_1 = 2.01$	$\chi^2_1 = 1.31$
14		$\chi^2_1 = 0.033$
16		$\chi^2_1 = 0$

Table 4.4: Results of analyses of the proportions of infections with 'high' (greater than the median) rates of gametocytogenesis on each day. * $p < 0.05$; ** $p < 0.01$.

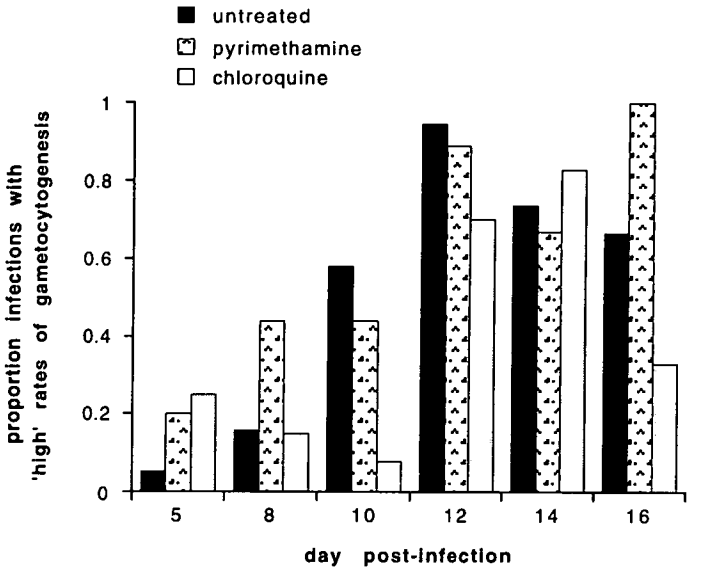


Figure 4.8: Proportion of infections in drug prophylaxis experiments with 'high' (greater than the median) rates of gametocytogenesis through time.

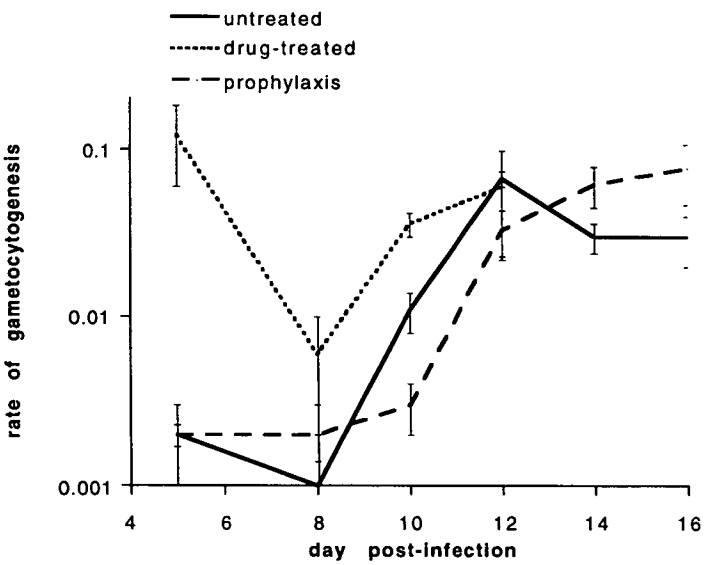


Figure 4.9: Mean ± 1 s.e. rates of gametocytogenesis through time for pooled drug-treatment, prophylaxis & untreated infections.

4.5 DISCUSSION

All methods of chemotherapy (i.e. different drugs, used as treatment or prophylaxis) reduced the overall numbers of asexual parasites produced during infections, although there was not a notable reduction in gametocytes following pyrimethamine and mefloquine treatment and both methods of prophylaxis. Quinine treatment did result in a small decrease in total gametocytes (approximately 25 %, relative to untreated infections), although the proportion of gametocytes to asexuals was approximately four times greater than in untreated infections. These results suggest that all methods of chemotherapy resulted in an overall increase in gametocytogenesis: more gametocytes are produced from a given number of asexuals. These increases in gametocytogenesis were apparent in two clones of *P. chabaudi*, with slightly different asexual and gametocyte dynamics.

Could these large decreases in asexual parasites and no (or disproportionately small) decreases in gametocytes be explained by increased mortality of asexual parasites relative to gametocytes following chemotherapy? All the antimalarial drugs used are more damaging to asexual parasites than mature gametocytes (Desjardins *et al.* 1988), but this is unlikely to explain the results. Although detailed pharmacokinetic data in mice of all the drugs are not available, the effective half-life of mefloquine in mice is estimated to be 18 hours (Desjardins & Trenholme 1984), and extrapolation from human data suggests that this is likely to be the longest half-life of the drugs used (Peters 1987). For all the drugs, preliminary studies showed that concentrations 25 % of those actually used had no noticeable effect on asexual growth. Changes in the relative death rates of gametocytes and asexuals through drug use is therefore only of any potential importance during the first 2 days following drug administration, a period excluded from the calculation of total numbers of parasites (assuming a maximum half-life of 18 hours, after 36 hours maximum drug concentration, = original concentration x 0.5^2). It seems unlikely that there are other sources of

differential mortality that may affect our interpretations. Differences in the relative mortality rates of gametocytes and asexuals resulting from differences in immune responses between drug-treated and untreated groups requires an as yet unknown gametocyte-specific clearance mechanism (Taylor & Read 1997), which is suppressed by all the antimalarial drugs used.

Temporal variation in gametocytogenesis

In untreated infections, gametocytogenesis occurred at a low rate during the start of infections (until day 8 p.i.), and then rapidly increased to a plateau by day 12 p.i (figures 4.7 - 4.9). The temporal variation in gametocytogenesis in prophylaxis infections was almost identical to that of untreated infections. A noteable difference was a delay in reaching high rates of gametocytogenesis, as shown by the greater proportion of infections with high rates of gametocytogenesis in untreated infections on day 10 p.i. (figures 4.8 & 4.9). In contrast, drug treatment resulted in greater alterations in patterns of gametocytogenesis. There was a massive increase in gametocytogenesis immediately following drug treatment (day 5 p.i.). Such a rapid response to stressful conditions is consistent with studies on *P. falciparum in vitro* (Carter & Miller 1979, Brockelman 1982, Bruce *et al.* 1990). Drug-treated infections also showed greater rates of gametocytogenesis than untreated infections on day 10 p.i. This was largely responsible for the earlier peak gametocyte densities following drug treatment, relative to untreated infections.

An important finding of this study is the immediate increase in gametocytogenesis following drug treatment (on day 5 p.i.). The drugs were clearly at sufficient concentrations to kill parasites (figure 4.3). Would drug-imposed increased mortality of asexual parasites relative to gametocytes then affect the estimates of day 5 p.i. conversion rate in drug-treated infections? The calculation of gametocytogenesis makes the assumption that gametocytes between 24 and 48 hours (when they reach

maturity) have zero mortality, but that mortality before this is the same as for asexual parasites. These assumptions are probably not greatly violated by drug-imposed mortality. Specific data for *P. chabaudi* is not available, although all the drugs used are inactive against mature *P. falciparum* gametocytes but kill immature forms (Desjardins *et al.* 1988). Moreover, mortality during the first 24 hours includes the approximately 7 hours it takes for asexual parasites to mature. Given the rapid absorption and subsequent parasite-killing activity of these drugs (at least in people; Desjardins *et al.* 1988), drug-induced mortality is likely to be greatest for these pre-schizont asexual parasites, probably over-shadowing any possible differences in mortality of the gametocyte or asexual progeny produced by the survivors of the drug treatment. The possibility that gametocytes that reached maturity on day 6 p.i. (which might not have been affected by the drug treatment on day 5 p.i.) would result in a significant over-estimation of drug-induced gametocytogenesis is also unlikely. The half-life of *P. chabaudi* gametocytes is less than 12 hours (A. Buckling, unpublished data), thus very few of the small number of mature gametocytes present on day 6 p.i. would contribute to the population on day 7 p.i.

Broadly speaking, rates of gametocytogenesis increased when parasite multiplication rate was low. In all treatments, gametocytogenesis increased towards the end of the infection, when parasites were being effectively controlled by the immune system. This pattern is consistent with *P. falciparum* data: parasites from longer established infections of Gambian children (as determined by the presence of mature gametocytes) had rates of gametocytogenesis six times higher than parasites from younger infections (Smalley *et al.* 1981). Drug treatment (which greatly inhibited parasite replication) resulted in an immediate increase in gametocytogenesis, and these infections subsequently had high rates of gametocytogenesis sooner than in untreated infections (figures 4.3, 4.7 & 4.9). The delay, relative to untreated infections, in reaching high levels of gametocytogenesis following prophylaxis seems counter-intuitive, given that

prophylaxis also inhibited asexual growth (figures 4.8 & 4.9). However, parasites in untreated infections started to decrease in number and showed increased rates of gametocytogenesis sooner than in prophylaxis infections (figure 4.5).

Temporal patterns of gametocytogenesis were largely independent of the drug used, although the method of therapy - whether treatment or prophylaxis - had a major effect. It is notable however that the immediate increase in gametocytogenesis following drug treatment did not occur in mefloquine-treated infections. It is possible that for some reason the parasites do not respond to mefloquine-imposed stress. Alternatively, the effect occurred but was not detected because gametocyte densities in mefloquine-treated infections were generally below the detection threshold on day 7 p.i. Mefloquine is much faster acting than quinine and especially pyrimethamine (Desjardins *et al.* 1988) and there will therefore have been fewer parasites surviving on day 5 p.i. to produce gametocytes observed on day 7 p.i. Treatment with chloroquine, a similarly fast acting drug, resulted in almost identical gametocyte dynamics as with mefloquine treatment (Buckling *et al.* 1997, chapter 2). More generally, daily rates of gametocytogenesis will have been under-estimated if mortality of asexual parasites is greater during the first 24 hours than the second. This was likely the case immediately following treatment with all the antimalarial drugs used.

Field implications

P. chabaudi infections in mice have qualitatively similar dynamics to *P. falciparum* infections in humans, with peak gametocyte densities occurring after peak asexual densities (Carter & Graves 1988). In addition, the reported changes in gametocytogenesis in these experiments are consistent with patterns of gametocytogenesis in *P. falciparum in vitro* : gametocytogenesis increases when asexual growth is inhibited in general (Carter & Miller 1979, Brockelman 1982, Carter & Graves 1988, Bruce *et al.* 1990, Sinden *et al.* 1996), or specifically by antimalarial

drugs (chapter 6). Our results may therefore have implications for antimalarial drug use against *P. falciparum* in the field. First, the reduction in transmission bought about by sub-lethal chemotherapy may be much less than expected because of compensatory increases in gametocyte production. Second, chemotherapy may result in high rates of gametocytogenesis occurring earlier than in untreated infections, resulting in earlier high infectivity. Such a phenotype will potentially increase transmission under conditions where the speed of transmission is a major component in parasite fitness (e.g. during epidemics). The use of gametocyte-sterilising drugs, such as primaquine, following periods of high gametocytogenesis (whether induced by chemotherapy or by natural host factors as the primary parasitaemia is controlled) might have a significant effect on reducing malaria transmission.

Partially effective vaccines may also stress parasites, at least in the short term. If so, they too may generate compensatory gametocyte production similar to that observed in this study. *In vitro*, *P. falciparum* gametocyte production increased following the addition of serum and lymphocytes from *P. falciparum* infected children (Smalley & Brown 1981) and anti-*P. falciparum* antibody (Ono *et al.* 1986). More generally, facultative changes in parasite life histories may render medical and veterinary intervention less effective than anticipated.

Chapter 5

THE EFFECT OF HOST IMMUNITY ON THE TRANSMISSION OF MALARIA PARASITES

5.1 SUMMARY

I investigated how partial immunisation against the rodent malaria parasite, *Plasmodium chabaudi*, affected the transmission of the parasite from subsequent infections. Immunisation resulted in similar reductions in both asexual parasite and gametocyte (the stage infective to the mosquito vector) densities, although there were compensatory increases in gametocyte production during the early parts of the infection. There was also strong evidence of strain (genotype)-specific immunity: asexual densities were lower following immunisation with an identical clone as the challenge infection (homologous challenge), compared with when the immunising and challenge clones were different (heterologous challenge). The infectivity of gametocytes, as measured by the proportions of mosquitoes infected, was approximately five times greater in controls and heterologous challenges than homologous challenges. This suggests the occurrence of strain-specific gametocyte infectivity-reducing immunity. The results are discussed in the context of epidemiology and evolution.

5.2 INTRODUCTION

In areas with high rates of malarial transmission, individuals generally develop clinical immunity to malaria by about five years of age (Greenwood 1997). However, in younger children and in areas where malaria is less endemic, immunity to malaria is frequently only partially protective (McGregor & Wilson 1988). Similarly, partial protection against malaria is all that can be realistically expected of vaccines that may

be in use in the near future (Greenwood 1997). It is therefore of great interest to know how partial host immunity against malaria might affect the transmission of the disease.

Malarial infections are maintained by continual cycles of invasion of red blood cells, asexual replication and release of the progeny. A small proportion of parasites, which can be modulated by environmental conditions (Carter & Graves 1988), develop into non-replicating transmission stages (gametocytes). These are the only stages capable of infecting the mosquito vector. The number of gametocytes produced during the course of an infection is a good measure of parasite transmission success: both the probability of mosquito infection and the parasite burden per mosquito increase with gametocyte density (Carter & Graves 1988, Taylor & Read 1997, Taylor *et al.* 1997, chapter 3).

Incomplete immunity to malaria, both in humans and laboratory models, generally results in a reduction in the total numbers of asexual parasites that are present during the course of an entire infection, by reducing asexual densities and / or infection longevity (Graves *et al.* 1988, Jarra & Brown 1985, Jarra *et al.* 1986, Bates *et al.* 1988). This is likely to result in a reduction in the total number of gametocytes produced during the infection for two reasons. First, there will be fewer asexuals to produce gametocytes. Second, there is likely to be some cross-immunity between asexuals and gametocytes (Motard *et al.* 1995, Goodier & Targett 1997). Partial immunity can further reduce *Plasmodium* transmission success, by 'sterilising' gametocytes and / or the resultant gametes in the mosquito midgut ('transmission-blocking' immunity; Mendis *et al.* 1987, Graves *et al.* 1988, Graves *et al.* 1991, Mulder *et al.* 1994, Sinden 1997).

Studies using the rodent malaria parasites *P. chabaudi* and *P. berghei* have shown that partial anti-parasite immunity is in part strain (genotype)-specific. Both peak asexual

densities and the duration of the patent infection are less for animals challenged with the same genotype as used for immunisation (homologous challenge), compared with animals challenged with a genotype different from the immunising genotype (heterologous challenge) (McLean *et al.* 1982, Jarra & Brown 1985, Jarra *et al.* 1986, Bates *et al.* 1988). Strain-specific immunity has also been experimentally demonstrated for the human malaria parasites *P. falciparum* and *P. vivax* (Boyd 1942, Jeffery 1966, Sadun *et al.* 1966, Cadigan & Chaicumpa 1969). Whether strain-specific transmission-blocking immunity also occurs is unkown.

Host immune status may also influence the proportion of asexual parasites that produce gametocytes. Increased gametocytogenesis has been reported in response to conditions that do not favour asexual multiplication, including high parasite densities (Brockelman 1982, Bruce *et al.* 1990) and antimalarial chemotherapy (Buckling *et al.* 1997, chapters 2, 4 & 6). Assuming poor asexual growth *in vivo* to be a good indicator that the infection will soon be cleared, an increase in gametocyte production in response to stress would increase the overall transmission success of the parasite, and thus be favoured by natural selection (Stearns 1992, Roff 1992, Buckling *et al.* 1997, chapter 2). Partial immunity against malaria parasites reduces asexual growth and infection longevity. Increased gametocyte production of the rodent malaria, *P. yoelii*, *in vivo* has been reported following immunisation with a recombinant malarial heat shock-like protein that is expressed throughout the life cycle (Motard *et al.* 1995), and in *P. falciparum* (the most common and virulent human malaria) after the addition of immune serum and lymphocytes (Smalley and Brown 1981) and anti-*P. falciparum* antibody (Ono *et al.* 1986).

I carried out experiments with the rodent malaria *P. chabaudi* to address the following questions. First, does partial immunity reduce transmission? Second, does strain-specific immunity acting against asexual parasites result in strain-specific transmission

success? Third, does immunisation change gametocyte infectivity, and if so does it do so in a strain-specific manner? Finally, does immunisation change patterns of gametocytogenesis, and if so does it do so in a strain-specific manner?

5.3 MATERIALS AND METHODS

Immunisation and infection

Male C57/BL/6J mice (Harlan-Olac, England) aged 8-14 weeks were immunised with $1x10^4$ red blood cells (RBCs) infected with a *P. chabaudi* clone (either CR or ER, from the WHO Registry of Standard Malaria Parasites maintained at the University of Edinburgh, UK) in a 0.1 ml intra-peritoneal inoculum of 50 % ringer's solution (27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl), 45 % heat-inactivated calf serum and 5 % 200 units / ml heparin solution. ER and CR probably differ at a number of loci, and are known to differ at the immunogenic surface antigen MSP1 (McLean *et al.* 1991). Control (non-immunised) animals were inoculated with uninfected RBCs in the same volume of inoculum solution. Four days post-infection (p.i.), all mice were treated orally, using a lubricated catheter, with 25 mg / kg mefloquine solution, prepared by dissolving mefloquine sulphate in 60 °C distilled water with a magnetic stirrer. Treatment was repeated over the next 2 days, to ensure infection clearance, which was confirmed by extensive examination (< 500 microscopic fields) of Giemsa-stained weekly blood-smears (see below), until reinfection. Four weeks after the first day of drug treatment, mice were infected with $1x10^6$ parasites of either clone CR or ER. The length of the immunising infection and the amount of time before challenge were determined from preliminary experiments to result in partial protection against the subsequent challenge. Mice infected with each clone could be classified into 3 treatment groups: non-immune (naive), homologous challenge and heterologous challenge. Mice, age-matched within experiments, were housed in cages of 3-5 animals at a temperature of 25±1 °C with a 0700 to 1900 hours light cycle, and provided with 41B rat and mouse maintenance diet (Harlan-Teklad, England) and

water containing 0.05 % pABA, *ad libitum*. Details of the sample sizes used in the two replicate experiments and the experimental design can be found in table 5.1.

Immunising clone	**Challenge clone**	**Sample size: Expt 1**	**Expt 2**
CR	CR	3	5
ER	CR	3	3
none	CR	4	3
ER	ER	3	5
CR	ER	3	4
none	ER	3	5

Table 5.1: Details of experiments. Expt, Experiment. Sample size refers to number of animals within each treatment.

Parasite counts

Every second day, between days 5 and 17 p.i. and 1700 - 1800 hours, thin blood smears from the tail vein were Giemsa stained and asexual parasites counted per 1.5 x 10^3 RBCs. At low asexual densities parasites were counted per 1.25 x 10^4 RBCs. Mature gametocytes were counted per 1.25 x 10^4 RBCs. Parasite densities were calculated from the product of RBC densities (measured every second day using flow cytometry; Coulter Electronics^{TM}) and parasites / RBC.

Mosquito feeds & dissections

On day 7 p.i. (when preliminary experiments revealed the presence of gametocytes in the majority of mice) in each experiment, 2 mice per treatment group per clone were assayed for infectivity by allowing *Anopheles stephensi* mosquitoes to feed on them. Mice were anaesthetised by an intra-muscular injection of 0.5 ml per 20 g mice 3:2:1 distilled water: Vetalar^{TM}: Rompun^{TM}, and placed onto pots covered with nylon mesh containing ~40 4-5 day old female *A. stephensi*, which had been starved for the previous 24 hours. Mosquitoes were left to feed in the dark for 30 minutes, between 1845 and 1945 hours. After removal of unfed mosquitoes, the remainder were maintained at 25-30 °C, 70-80 % humidity, with a 12 hour light cycle and provided with 5 % glucose, 0.05 % pABA solution *ad libitum.* After 8-9 days, approximately 25 mosquitoes per mouse were dissected to determine the presence of oocysts on midguts.

Statistical analyses

Comparisons of naive and immunised hosts were made by analyses of the following summary measures for each infection. A number of mice in each experiment died (from severe anaemia) between days 8 and 12 p.i. These were excluded from all analyses except infectivity to mosquitoes, which was assayed only on day 7 p.i.

(1) *Total asexuals.* This was estimated from parasite densities between days 5-17 p.i. by integrating under the parasite density through time curves for each infection. This is a reasonably accurate measure of total numbers of asexuals (between days 5-17 p.i.) because the asexual cycle of *P. chabaudi* is known to be 24 hours (Cox 1988). Before day 5 p.i. and after day 17 p.i., parasites densities are negligible relative to the total produced in the intervening period.

(2) *Total gametocytes.* This was estimated for the same time period as for total asexuals (days 5-17 p.i.), during which the bulk of gametocyte production occurs. This measure is a reasonable estimate of total gametocyte numbers because the half life of a mature gametocyte is estimated to be less than 12 hours (A. Buckling, unpublished data). In addition, the sum of the gametocyte densities on each day is likely to be a well correlated with total transmission probability: gametocyte densities at any point in time correlate with both the proportion of mosquitoes infected and densities within mosquitoes (Taylor & Read 1997).

(3) *Proportion of gametocytes* . This was calculated as the proportion of total parasites [(1)+ (2)] that were gametocytes [2] for each infection.

(4) *The proportion of mosquitoes infected*

(5) *Daily rates of gametocytogenesis*

We used a simple exponential growth model to estimate the proportion of asexual parasites that produced gametocytes on individual days. Rates of gametocytogenesis (g) on day t were calculated for days (t =) 5, 7, 9, 11 and 13 p.i. using

$$g = \frac{\frac{G_{t+2}}{A_t}}{\sqrt{\frac{A_{t+2}}{A_t} + \frac{G_{t+2}}{A_t}}} \qquad \textbf{(1)},$$

where A and G refer to asexual and gametocyte densities, respectively, on a given day. Equation 1 takes into account differential mortality between asexual parasites and gametocytes, and differences in their maturation periods and longevity. Details of the model can be found in section 4.3.

Prior to analysis, estimates of total asexuals and total gametocytes were $\log_{10}$-transformed and proportion of gametocytes square-root arcsin-transformed, to bring their distributions close to normal. All analyses were carried out using generalised linear models (GLIM; Crawley 1993). Starting with the highest order interactions, all factors, covariates and their interactions were individually removed in turn from the complete model. Non-significant interaction terms were removed and test statistics obtained from the resulting minimal model.

The effect of immunisation *per se* on (1) total asexuals, (2) total gametocytes and (3) the proportion of gametocytes was determined by ANOVA, with TREATMENT (immunised or naive), CLONE (ER or CR) and EXPERIMENT (1 or 2) fitted as factors. The effect of strain-specific immunity on these measures was determined by carrying out separate analyses using only immunised animals, with IMMUNISING CLONE (ER or CR), CHALLENGE CLONE (ER or CR) and EXPERIMENT fitted as factors. Detection of a significant interaction between the immunising clone and the challenge clone is evidence that strain-specific immunity has occurred (Read & Viney 1996).

A binomial error structure was used for the logistic regression of probability of a mosquito becoming infected (Crawley 1993). Square-root arcsin-transformed gametocytaemia on the day of the feed (gametocytes / RBC; the best correlate of infection probability on an individual day, chapter 3) was fitted as a covariate. The data did not require correction for over-dispersion.

Daily rates of gametocytogenesis (g, in equation 1) were highly over-dispersed and in no way approximated a normal distribution, regardless of transformation. The data consisted of many zeros and very low values, as well as much higher values. Even the use of non-parametric ranking procedures is unlikely to provide an appropriate

solution to this problem, given that variation in the very low values will often represent the presence or absence of a single gametocyte in the surveyed microscopic fields. A nominal classification system was therefore used, with each rate of gametocytogenesis classified as high or low, based on whether the value was less or greater than the median of the entire data set. To control for multiple sampling from the same infection, the median of a data set was calculated as the median of the medians for each infection within the entire data set. Data for each day was analysed using chi-squared tests of independence (with Yates's correction for continuity), to determine whether the proportion of infections that had high rates of gametocytogenesis (median > 0.0006) differed between immunised and naive animals.

5.4 RESULTS

Infection dynamics

Immunisation resulted in a three-fold reduction in the mean numbers of total asexual parasites (figures 5.1a, 5.2a & 5.3a, table 5.2), and a four-fold reduction in total gametocytes (figures 5.1b, 5.2b & 5.3b, table 5.2). The resultant proportion of gametocytes did not significantly differ between immunised and naive animals (figure 5.3c, table 5.2). There was no difference between the two clones in either total asexuals (figure 5.3a,table 5.2) or gametocytes (figure 5.3b, table 5.2), although the ratio of gametocytes to asexuals in clone CR was approximately twice that of clone ER (figure 5.3c, table 5.2).

Animals challenged with the same clone that they were immunised with (homologous challenge) had fewer total asexual parasites than animals challenged with the other clone (heterologous challenge) (figure 5.3a, table 5.3). In other words, asexual parasites were noticeably affected by strain-specific immunity. There was no evidence that strain-specific immunity affected either total gametocytes (figure 5.3b, table 5.3) or the proportion of gametocytes (figure 5.3c, table 5.3). Apart from strain-specific

immunity, whether clone ER or CR were used as the immunising or challenge clone had no effect on total asexuals, total gametocytes or the ratio of the two (figure 5.3, table 5.3). In other words, the clones did not appear to differ in their immunogenecity or how much they were affected by immunisation.

	Total Asexuals	**Total Gametocytes**	**Proportion Gametocytes**
Treatment	$F_{1,40} = 58.8^{**}$	$F_{1,40} = 7.8^{**}$	$F_{1,40} = 1.7$
Clone	$F_{1,40} = 0.01$	$F_{1,40} = 1.84$	$F_{1,40} = 8.9^{**}$
Experiment	$F_{1,40} = 4.8^{*}$	$F_{1,40} = 5.7^{*}$	$F_{1,40} = 1.4$

Table 5.2: Results of analyses of the effect of immunisation (treatment) on various summary measures. All interaction terms were removed from the model, as they were non-significant ($p > 0.1$). * $p < 0.05$; ** $p < 0.01$.

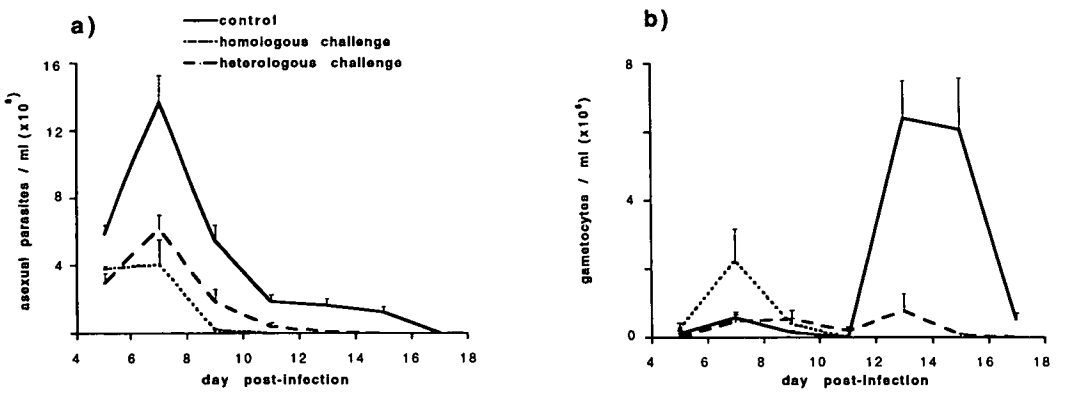


Figure 5.1: Dynamics of infections with clone CR. Mean (+1 s.e.) asexual densities (a), gametocyte densities (b).

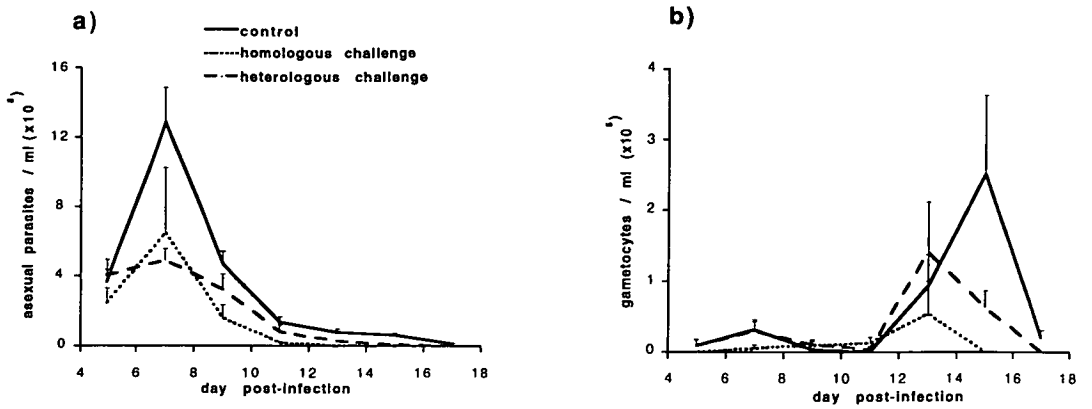


Figure 5.2: Dynamics of infections with clone ER. Mean (+1 s.e.) asexual densities (a), gametocyte densities (b).

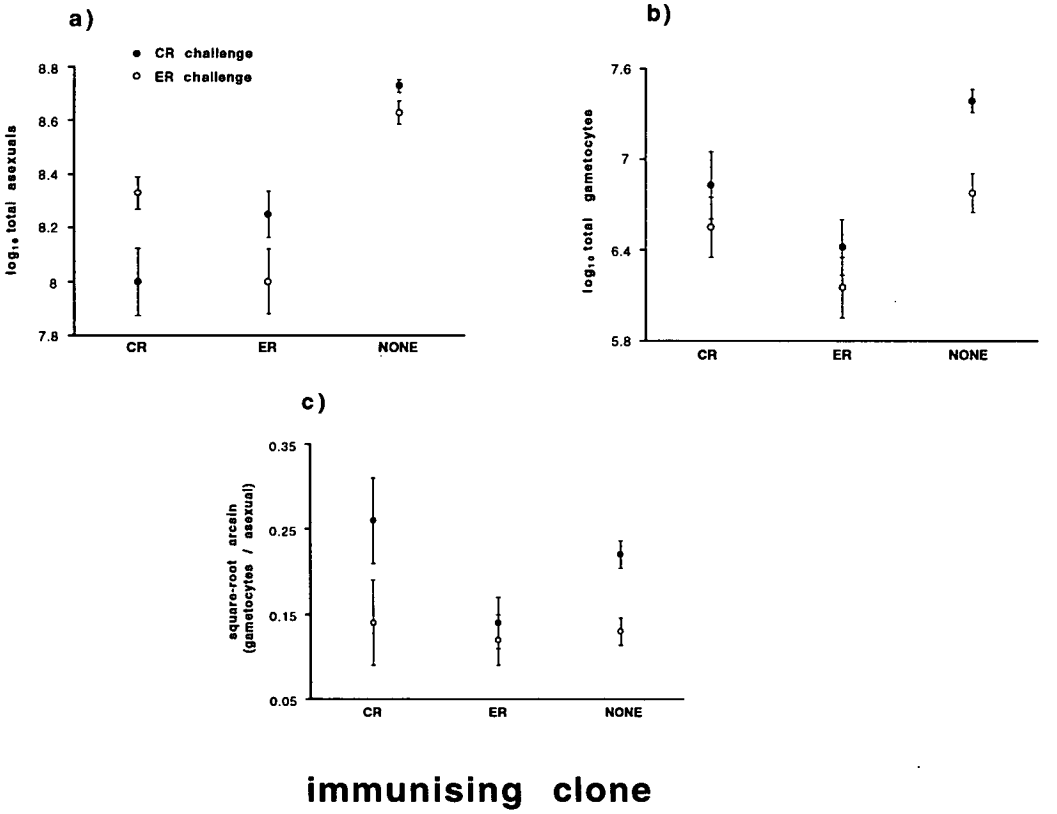


Figure 5.3: Estimates of (a) total asexual parasites, (b) total gametocytes and (c) the proportion of gametocytes, between days 5 - 17 p.i. for all treatments. Plotted points are means ± 1 s.e.

	Total Asexuals	Total Gametocytes	Proportion Gametocytes
Imm. clone	$F_{1,23} = 0.68$	$F_{1,24} = 0.35$	$F_{1,24} = 2$
Chall. clone	$F_{1,23} = 0.67$	$F_{1,24} = 0.8$	$F_{1,24} = 3.4$
Imm. x chal.	$F_{1,23} = 10.6$*	$F_{1,23} = 1.42$	$F_{1,24} = 0.27$
Experiment	$F_{1,23} = 8.5$*	$F_{1,24} = 3.6$	$F_{1,24} = 0.06$

Table 5.3: Results of analyses of infections in immunised animals only. the effect of dimmunisation (treatment) on various summary measures. All other interaction terms were removed from the model, as they were non-significant ($p > 0.1$). * $p < 0.01$.

Rates of gametocytogenesis in control animals were at a low level up until day 11 p.i., when they rapidly increased. There was a similar pattern in immunised animals, although rates of gametocytogenesis were generally higher than in control animals before day 11 p.i. - noteably so on day 7 p.i. (figure 5.4, $\chi^2_1 = 5.4$, $p = 0.02$). There were no other significant differences in rates of gametocytogenesis between control and immunised animals on the other days (figure 5.4, $p > 0.1$ in all cases).

Infectivity

The proportion of mosquitoes infected increased with gametocytaemia at the time of the feed (figure 5.5, table 5.4), and at the same rate for both clones and all three types or treatment (table 5.4). The relationship between the proportion of mosquitoes infected and gametocytaemia was linear: the addition of the quadratic term to the untransformed data did not explain a significant amount of deviance ($p > 0.1$). After gametocytaemia had been controlled for, infectivity per gametocyte was reduced five-fold (at the mean gametocytaemia) in homologous challenges, compared to that in heterologous challenges and controls (figure 5, table 5.4), which did not differ (figure 5.5, table 5.4). Gametocyte infectivity was between 2 - 3 times higher for clone ER than CR (table 5.4).

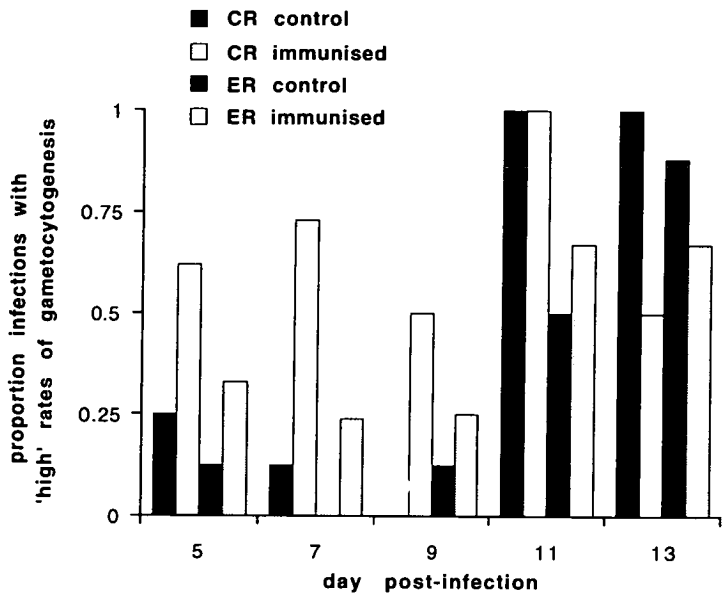


Figure 5.4: Proportion of control and immunised infections (challenged with clone CR or ER) with 'high' (greater than the median) rates of gametocytogenesis through time. See text for details.

	χ^2_1
Gametocytaemia	37.98**
Treatment	15.31**
Clone	6.8*
Experiment	9.1*

Table 5.4: Minimal model of logistic regression of mosquito infection probability. Treatment was simplified to a 2 level factor: (control + heterologous challenge) and homologous challenge with a non-significant increase in deviance ($\chi^2_1 = 0.73$). No interactions were significant ($p > 0.1$). *$p < 0.01$; **p , 0.001.

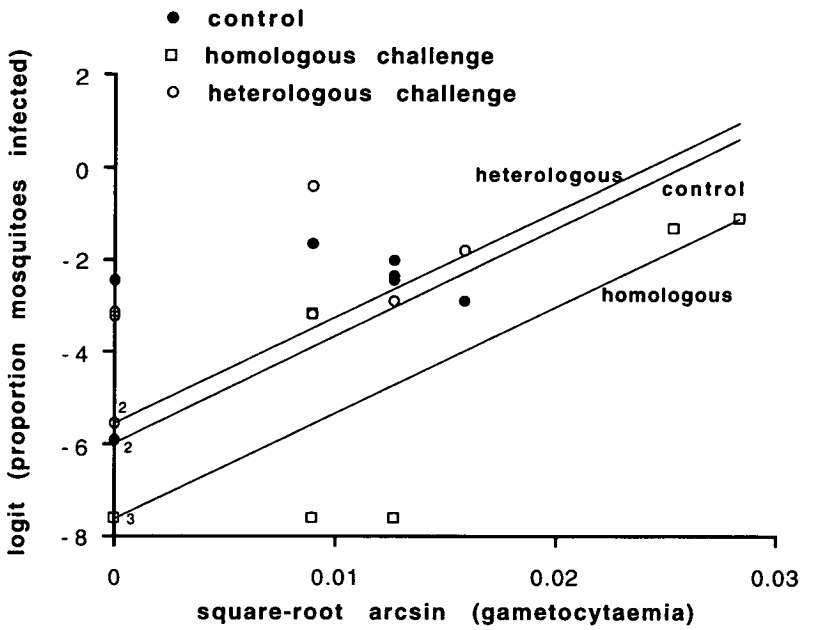


Figure 5.5: The relationship between the proportion of mosquitoes infected and gametocytaemia on day 7 p.i. Lines are ordinary least squares regression lines. Numbers within the graph show multiple data points.

5.5 DISCUSSION

Immunisation against *P. chabaudi* resulted in reductions in both peak asexual densities and infection longevity, and as a result, the total number of asexual parasites during the infection. Consistent with previous studies (McLean *et al.* 1982, Jarra & Brown 1985, Jarra *et al.* 1986, Bates *et al.* 1988), we found evidence of strain-specific immunity: total numbers of asexuals were less in animals that had been challenged with the homologous compared with the heterologous clone (figure 5.3a). The occurrence of strain-specific asexual immunity is unsurprising given the difference between clones ER & CR at a known immunogenic antigen, MSP1, to which allele-specific protective antibody responses are well documented (McLean *et al* 1991). There was also a reduction in the total number of gametocytes following immunisation, although this did not differ between the two methods of immunisation

(5.3b). Based on the within-host data, the low-level immunisation in these experiments would reduce parasite transmission success four-fold, independent of whether the challenge was homologous or heterologous.

The infectivity of gametocytes in a heterologous challenge was not reduced. However, in homologous challenges, gametocyte infectivity was reduced five-fold (figure 5.5). As with strain-specific asexual immunity, the magnitude of this effect was independent of whether clone CR or ER were used as the challenge infection. Given that total numbers of gametocytes did not differ between the two methods of immunisation, gametocyte infectivity is likely to be the major factor in determining the overall transmission success of a parasite strain in a partially immune host.

How could these differences in gametocyte infectivity between homologous and heterologous challenges arise? Asexual parasite density has been reported to negatively correlate with gametocyte infectivity in the rodent malaria, *P. berghei* (Dearsley *et al.* 1990, Sinden 1991). This is clearly not the case in this study, as asexual densities were higher in heterologous than homologous challenges on the day of the mosquito feed (day 7 p.i., figures 5.1a & 5.2a). The most likely explanation is therefore immunity reducing gametocyte infectivity in a strian-specific manner: gametocytes in a host that are of the same genotype as used for immunisation have greatly reduced infectivity relative to that in hosts immunised with an alternative genotype. Such strain-specific immunity requires polymorphisms in immunogenic gametocyte / gamete antigens. This possibility cannot be addressed for *P. chabaudi*, as there is a paucity of relevant data. We therefore consider data from *P. falciparum*, as it is likely that *P. chabaudi*, like *P. berghei*, possesses many of the equivalent *P. falciparum* gametocyte / gamete antigens (Sinden 1997).

Transmission-blocking activity has been reported for antibodies against two *P. falciparum* gametocyte / gamete-specific antigens, Pfs 48 / 45 (Carter *et al.* 1990, Targett *et al.* 1990) & Pfs 230 (Graves *et al.* 1988). Polymorphisms have been reported in Pfs 48 / 45 (Foo *et al.* 1991, Roeffen *et al.* 1996), Pfs 230 (Williamson & Kaslow 1993), and Pfg 27 / 25 (Alano *et al.* 1996), another potential transmission-blocking target antigen (Sinden 1997), suggesting that variation exists for strain-specific transmission-blocking immunity to operate. The transmission-blocking activity of antibody to both these antigens is, however, by no means ubiquitous (Roeffen *et al.* 1996, Healer *et al.* 1997). In addition, it would be surprising if an effective transmission-blocking immune response could by raised against the *P. chabaudi* equivalent of these antigens, given the short duration of the immunising infection and the inevitably small numbers of gametocytes that would have been present. A more parsimonious explanation is that the strain-specific reduction in gametocyte infectivity is a result of strain-specific anti-asexual immunity cross reacting with shared (polymorphic) gametocyte antigens. Highly polymorphic and immunogenic antigens (encoded by *var* genes; Roberts *et al.* 1992) are expressed on the surface of asexual-infected RBCs. If also expressed on gametocytes, immunity acting against these antigens on asexual parasites could potentially mediate the observed reduction in gametocyte infectivity following homologous challenge. If this interpretation is correct, the phenonemena is not, by definition, 'transmission-blocking immunity', which is mediated by antibody raised specifically against gametocyte / gamete antigens.

Averaged across the whole infection, there was no difference in the proportion of parasites that were gametocytes between control and immunised animals. Similarly, there was no difference between homologous and heterologous challenges (figure 5.3c). This implies that neither method of immunisation resulted in compensatory increases in gametocyte production. The effect of immunisation differed from that of

antimalarial drug treatment and prophylaxis (using doses that did not kill all parasites), which resulted in large reductions in asexual parasites but either no, or a disproportionately small, reduction in gametocytes (Buckling *et al.* 1997, chapters 2 and 4).

Consideration of the estimates of daily rates of gametocytogenesis suggest that gametocytogenesis was however higher in immunised animals. In both immunised and control animals, rates of gametocytogenesis increased towards the end of the infection (from day 11 p.i., figure 5.4). Before this period, parasites had higher rates of gametocytogenesis in immunised than control animals. These data support the hypothesis that conditions unfavourable for asexual growth result in increased gametocytogenesis: in all treatments asexual growth decreased as the infection progressed, and asexual growth for the first nine days of the infection was less in immunised than control animals (figure 5.1a & 5.2a). This pattern was the same for both clones.

As daily rates of gametocytogenesis were generally higher in immunised than control animals, why was there no difference in gametocytogenesis estimated across the entire infection? Rates of gametocytogenesis were at their highest, and did not differ between infections in immunised and control animals, between days 11 and 15 p.i. (figure 5.4). As a result, the bulk of gametocytes produced during the course of a control infection are produced after day 11 p.i., even though the asexual population at this time is much less than earlier in the infection (figures 5.1 & 5.2). Asexual densities after day 11 p.i. were disproportionately smaller in immunised than control animals, relative to before day 11 p.i. There was thus no overall increase in the proportion of gametocytes in immunised animals.

When comparing estimates of gametocytogenesis between immunised and control animals, the possibility that relative mortality rates of asexuals and gametocytes may differ between the treatments needs to be considered. Indeed, immunity is likely to be greater against asexual parasites than gametocytes, given that the immune system would have encountered very few gametocytes before the brief immunising infection was drug cleared. Two points strongly suggest this possibility would not bias our estimates of gametocytogenesis in immunised relative to control animals. First, asexual parasites have to survive to produce gametocytes. Second, the half life of a mature gametocyte is less than 12 hours (A. Buckling, unpublished data), thus surviving gametocytes would contribute little to the gametocyte population when the next measure was taken, two days later.

Implications

Strain-specific immunity against asexual parasites has been demonstrated for both *P. falciparum* and *P. vivax* (Boyd 1942, Jeffery 1966, Sadun *et al.* 1966, Cadigan & Chaicumpa 1969). If the data from this study on *P. chabaudi* are of general relevance, the results may have implications for understanding the epidemiology of human malaria and the effects of partially protective vaccines. Although partial host immunity against malaria may result in compensatory increases in gametocytogenesis, it is likely to have a large impact on reducing malaria transmission. These data would suggest a reduction between 75 % and 90 % depending on whether the immunising strain / antigen was heterologous or homologous to the challenge infection. Further reductions would be expected at higher levels of immunity. It is important to note that the difference in infectivity between heterologous and homologous immunisation was primarily brought about by strain-specific gametocyte infectivity-reducing immunity.

Strong strain-specific, in addition to strain-independent, immunity is likely to have a large effect on the population structure of *Plasmodium*, by reducing the probability of

multiple clones being transmitted. The subsequent decrease in the average number of clones per host has a number of potentially important implications, including reductions in outcrossing rates, further reductions in transmission (Taylor *et al.* 1997), and a decrease in the probability of parasites evolving resistance to multiple antimalarial drugs (Mackinnon & Hastings 1997). Partially effective vaccines may therefore have a large impact on reducing malaria transmission. This conclusion ignores possible long term parasite adaptations to varying levels of host immunity, as well as strain-specific immunity allowing different parasite strains to be transmitted largely independently of each other, resulting in a reduction in the selection against highly virulent genotypes (Gupta *et al.* 1994a, b, Gupta *et al.* 1996).

The occurrence of strong strain-specific immunity will result in a short-term selective advantage of *Plasmodium* strains that have newly invaded a host population. This advantage will soon be lost after host immunity has developed against the new strains. If intragenic recombination is sufficiently common, or there is some epistasis between genes encoding antigens, recombinant progeny are also likely to have a short-term selective advantage relative to the parental genotypes. This advantage of the continuous production of antigenically novel genotypes may help to explain why sexual reproduction is ubiquitous in the life cycle of malaria. Sex as an immune evasion mechanism has been previously suggested for a facultatively sexual gastrointestinal nematode of rats, *Strongyloides ratti* (Gemmill *et al.* 1997).

Chapter 6

CHLOROQUINE INCREASES *PLASMODIUM FALCIPARUM* GAMETOCYTOGENESIS *IN VITRO*

6.1 SUMMARY

Malaria parasites are capable of modulating the diversion of resources from asexual growth to the production of stages infective to mosquitoes (gametocytes). Increased rates of gametocytogenesis appear to be a general response to stress, both naturally encountered or novel. I have previously reported earlier and greater gametocytogenesis in response to sub-curative antimalarial chemotherapy in the rodent malaria, *Plasmodium chabaudi, in vivo*. Using an immunofluorescent assay to detect parasites that had invaded red blood cell monolayers, I demonstrate a five-fold increase in gametocytogenesis in the human malaria, *P. falciparum, in vitro*, in response to treatment with the antimalarial drug chloroquine. In all clones used, gametocytogenesis increased with increasing inhibition of asexual growth by chloroquine. Furthermore, there were clone differences in the relationship between stress and gametocyte production, implying the response was genetically variable. This was not however associated with chloroquine resistance. The epidemiological significance of these results are discussed.

6.2 INTRODUCTION

Malaria parasites (*Plasmodium* spp.) are capable of modulating the proportion of blood-stage asexual parasites that develop into non-replicating transmission stages (gametocytes). Both the probability of mosquito infection and the parasite burden per mosquito increase with gametocyte density (Carter & Graves 1988, Taylor & Read 1997, Taylor *et al.* 1997, Buckling *et al.* 1997, chapter 2). Conditions unfavourable for asexual growth, and thus future transmission opportunities, result in an increase in

short-term gametocytogenesis (the proportion of asexual parasites that produce gametocytes; Mons 1985, Carter & Graves 1988, Sinden *et al.* 1996). By showing such compensatory increases in gametocytogenesis, malaria parasites are likely to increase the total number of hosts infected from the current infection.

If increased gametocyte production is a general response to stress, it is predicted to occur following human-imposed stresses, such as antimalarial chemotherapy. Most antimalarial drugs kill asexual parasites. However, if some parasites survive (sub-curative treatment is common in the field; Wernsdorfer 1994), they will inevitably suffer some damage (the extent of which is likely to correlate with the drug-imposed mortality), and may be expected to have a higher probability of producing gametocytes.

A number of clinical trials with *P. falciparum* in people have investigated drug-induced gametocytogenesis (reviewed in Buckling *et al.* 1997, chapter 2). However, for ethical reasons, there were no untreated control groups, and drugs were given in curative doses. Detection of increased gametocytogenesis is clearly impossible from these experiments, and the data are, not surprisingly, very ambiguous. I have previously reported increased gametocyte production (and subsequent infectivity to *Anopheles stephensi* mosquitoes) following sub-curative chemotherapy in the rodent malaria, *P. chabaudi, in vivo* (Buckling *et al.* 1997, chapters 2 and 4). In these experiments, a variety of drugs with different parasite-killing mechanisms were used against drug-naive parasite clones, suggesting the response is a general adaptation to stress, the drugs mimicking naturally encountered stresses, such as host immunity. Precise assessment of gametocytogenesis is however difficult *in vivo*, given the possibility of different mortality rates and spatial distributions of asexual parasites and gametocytes. *In vitro* experiments overcome these problems as well as allowing the study of the human malaria parasite, *P. falciparum.*

I carried out experiments to determine whether the most commonly used antimalarial drug, chloroquine, increases gametocytogenesis in *P. falciparum in vitro*. Antimalarial drugs by definition kill asexual parasites, radically changing culture conditions, therefore a simple comparison of the parasite dynamics between untreated and chloroquine-treated continuous cultures can not adequately test the hypothesis. Commitment of parasite progeny to developing either as asexual parasites or gametocytes occurs prior to release from the parent schizont (Bruce *et al.* 1990). We were able to isolate the progeny of parasites exposed to short-term control and drug treatments by allowing controlled invasion of red blood cell (RBC) monolayers. The phenotypes of the progeny were then determined after two days using an immunofluorescence assay, rather than potentially ambiguous morphological characterisation. Using these techniques we were also able to investigate the precise relationship between drug-induced stress and gametocytogenesis and possible differences in such responses between chloroquine-sensitive and -resistant parasite clones.

6.3 MATERIALS & METHODS

Parasite culturing and chloroquine treatment

Parasites were cultured following a modification of the methods of Trager & Jensen (1976). Four *P. falciparum* clones were used in the study: chloroquine-sensitive 3D7A and HB3B and chloroquine-resistant 7G8 and SUD124/8 (all from the WHO Register of Standard Strains of Malaria Parasites, maintained at the University of Edinburgh). 3D7A is a clone (Walliker *et al.* 1987) made from isolate NF54 (Ponnundurai *et al.* 1981), which was obtained from a patient in the Netherlands. The geographical origin is unknown, although there is some suggestion that the parasite is from Africa (Collins *et al.* 1986). HB3B, 7G8 and SUD124/8 was cloned from the Honduran isolate H1 (Bhasin & Trager 1984), the Brazilian isolate IMTM22 (Burkot *et al.* 1984), and the Sudanese isolate SUD124 (Bayuomi *et al.* 1993), respectively.

Parasite cultures were prepared at 5 % haematocrit using freshly washed group O +, group RhD + human RBCs (Edinburgh and South East Scotland Blood Transfusion Service) in complete RPMI medium (RPMI 1640 supplemented with 25 mM HEPES, 80 mg / l gentamicin sulphate, 50 mg / l hypoxanthine, 0.2 % w/v $NaHCO_3$ and 10 % v/v pooled, heat-inactivated human serum). Cultures were established with an initial parasitaemia (parasites / RBC) of approximately 1 % in 5 ml volumes in 25 ml sterile polystyrene tissue culture flasks (Corning). Cultures were maintained at 37 °C and gassed daily with a mixture of 3 % CO_2, 1 % O_2 & 96 % N_2. The medium was also replaced daily, with fresh pre-warmed medium.

When a large proportion of the parasites were at the 'late ring' stage (less than 24 hours old) and the culture parasitaemia was at least 4 %, two identical subcultures with parasitaemias ranging between 2 - 4 % were established from the original culture. One was treated with chloroquine sulphate (Nivaquine™; see table 6.1 for doses) diluted in distilled water, and the other treated with an equal volume of distilled water (10 µl). The medium was replaced with untreated medium approximately 17 hours later. Parasites were cultured for a further 48 hours, at a volume of 4.5 ml because of removal of culture for RBC monolayer invasion (see below). From the day of the addition of chloroquine (day 0), daily thin blood smears were taken, Giemsa-stained and the number of asexual parasites counted per 1.5 x 10^3 RBCs. One pair of cultures was termed one experiment. Either 6 or 7 experiments were carried out for each clone (see table 6.1); experiments were carried out by themselves or two at a time.

Preparation and infection of RBC monolayers

The method for creating RBC monolayers was based on that of Jackie Williams (Inselburg 1983). Corning polystyrene tissue culture dishes (35 x 10 ml) were incubated with 1.5 ml 10 µg / ml Concanavalin A (Sigma). Except when being

manipulated, culture dishes were kept in a modulator incubator at 37 °C at all times. The Concanavalin A was then removed and the dishes washed twice with incomplete RPMI medium (RPMI 1640 supplemented with 25 mM HEPES and 50 mg / l hypoxanthine). The dishes were incubated for a further 60 minutes with 1.5 ml of washed, uninfected RBCs at 0.5 % haematocrit suspended in incomplete RPMI medium. Unbound RBCs were carefully resuspended and removed by aspiration, and the monolayer was washed twice more with incomplete RPMI medium, to completely remove any unbound RBCs.

Shortly after removal of the chloroquine, 0.5 ml of each culture was removed from the culture flasks and diluted in complete RPMI medium to 0.5 % haematocrit and 1.5 ml of this culture was added to each monolayer (three monolayers per culture). The modular incubator was gassed and the parasites kept at 37 °C for 18-22 hours, to allow invasion of the monolayer. Unbound RBCs were resuspended in complete medium and removed. The monolayers were washed twice more with complete RPMI medium, to remove any unbound cells. This method allows only the progeny of the cohort of parasites that had been exposed to the experimental treatments to invade the monolayers. Because the progeny in the monolayer were never directly exposed to chloroquine, chloroquine-induced differential mortality between gametocytes and asexual parasites was controlled for. The culture dishes were gassed and left for a further 24 hours. This prevented a further asexual cycle occurring in the monolayer, which would have resulted in parasites whose predecessors would not have been exposed to the appropriate treatments. The complete RPMI medium was then removed and the monolayers left to air dry. After determining whether parasite invasion was suitable for the subsequent assay by methanol-fixing and Giemsa-staining one of the three monolayers per culture, the remaining dishes were wrapped in foil and stored anhydrously at -70 °C until the immunofluorescence assay was carried out. A schematic of the basic methodology is shown in figure 6.1.

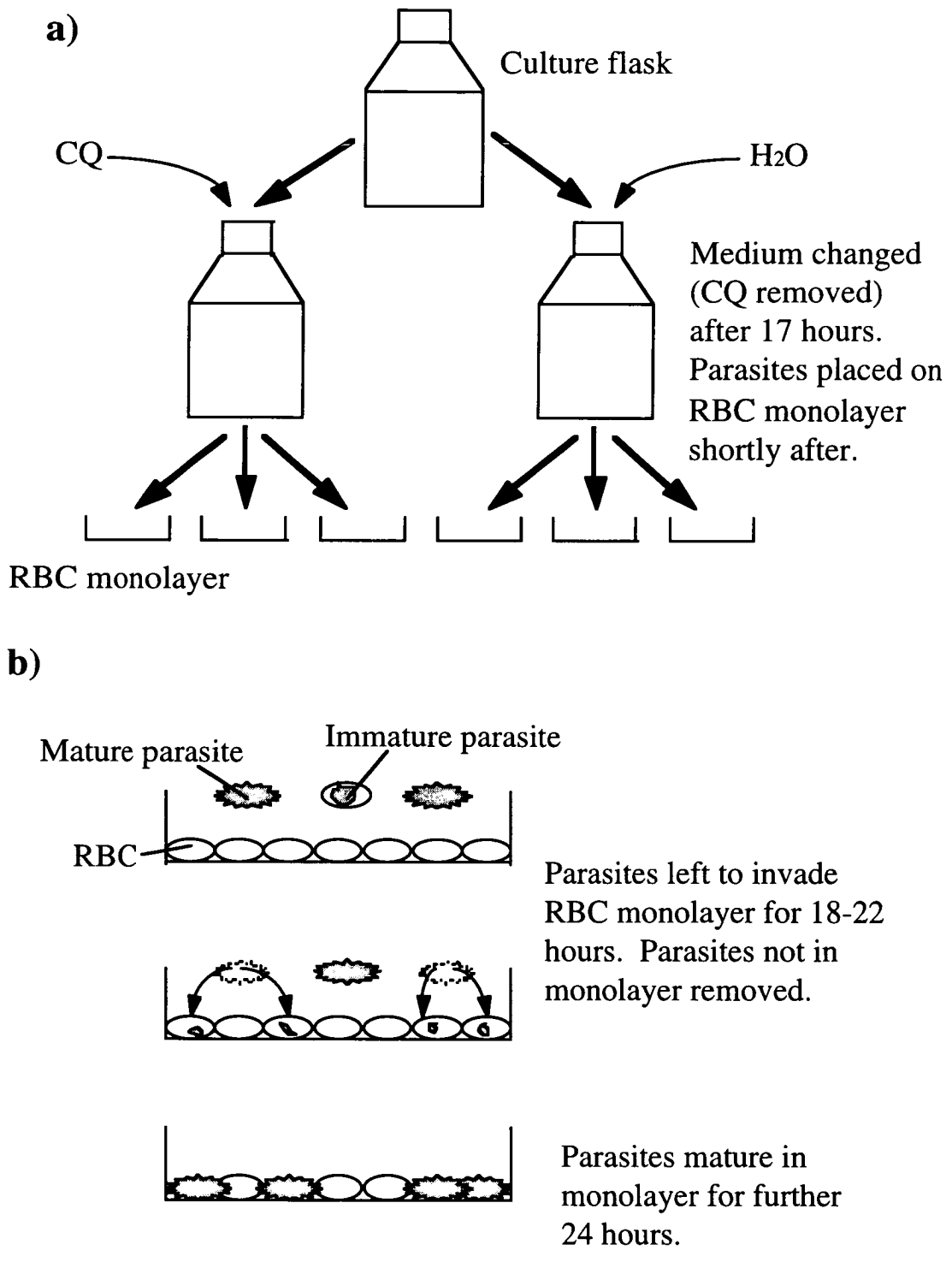


Figure 6.1: Schematic of methods for basic experimental design (a), and events within RBC-monolayers (b).

Clone	CQ concentration (μM)	n
3D7	0.06 - 0.067	6
HB3	0.06 - 0.07	6
7G8	0.075 - 0.4	7
124/8	0.2 - 0.275	7

Table 6.1: Range of chloroquine (CQ) concentrations used for each clone. n; number of experiments carried out per clone.

Immunofluorescence assay (IFA)

Monoclonal antibodies (mAbs) used were mAb 93a3a2 (IgG1), which reacts to the gametocyte-specific Pfs 16 antigen (Baker *et al.* 1994), and mAb 12.8.2.1 (IgG2b), which reacts to the 16 kDa segment of MSP2 (McBride & Heidrich 1987), an asexual-specific antigen (Stanley *et al.* 1985). Both were mouse hybridoma culture supernatants. Pfs16 is expressed approximately 30 hours after parasite invasion of the RBC (Bruce *et al.* 1994). The 16 kDa segment of MSP2 is present on both young ring stages and is expressed again in the developing schizont (from about 30 hours post-invasion; McBride & Heidrich 1987). mAb 12.8.2.1 also showed limited cross reactivity to young gametocytes.

In all cases, IFA of monolayers from paired chloroquine-treated and control cultures was carried out at the same time. After methanol-fixing of the monolayers, 1 ml of 'block' buffer (1 x PBS containing 1 % w/v Fraction V bovine serum albumin (sigma), 0.05 % NaAzide) was added to each dish, which were then left in a humidity chamber for 20 minutes at room temperature. The buffer was then aspirated off and

the dishes incubated for 30 minutes in a humidity chamber at room temperature with 80 µl of block with 1:50 and 1:300 dilutions of mAb 93a3a2 and mAb 12.8.2.1, respectively. Dishes were then washed 6 times with 1 x PBS and incubated for a further 20 minutes in a humidity chamber at room temperature with 80 µl of 1 x PBS containing 10 µg / ml DAPI (4,6-diamidino-2-phenylindole; Sigma), 0.05 % Na Azide and 1:40 dilutions of tetramethylrhodamine conjugated goat anti-mouse IgG1 and fluorosceinisothiocyanate conjugated goat anti-mouse IgG2b (Southern Biotechnology Associates Inc.). The dishes were again washed 6 times with 1 x PBS and a drop of glycerol containing 2.5 mg / ml DABCO [1,4-diazabicyclo(2,2,2)octane; to prevent fading of the fluorescence] added. A cover slip was placed over the monolayer and sealed at the edges with nail polish, allowing the monolayers to be stored for a few days without fading of the fluorescence.

Fluorescence microscopy was carried out on the RBC monolayers using a Leitz Dialux 20 microscope with u.v. illumination. Parasites were observed under three different filters. Through Leitz filter blocks A, I2 & N2, the blue staining of parasite nuclei by DAPI, the red of rhodamine associated with asexuals and the green of fluorescein associated with gametocytes, respectively, could be observed. In each monolayer, between 50 & 400 parasites (based on DAPI-staining and morphology under white light) were counted. Only parasites that were old enough to contain haem pigment (detected by white light) were counted, to avoid inclusion of parasites that died at an early stage of their development. Parasites that did not contain haem frequently expressed the asexual-specific antigen but never the gametocyte-specific antigen, thus inclusion of young parasites would under-estimate the proportion of gametocytes if mortality in a particular monolayer was higher than normal. Included parasites were then observed under the other two filters to determine whether they were gametocytes or asexuals. Morphology under white light was used to confirm the phenotype where

possible. Given the observed limited cross reactivity of the asexual-specific mAb to gametocytes, but not vice versa with the gametocyte-specific mAb, on the occasions when both mAbs seemed to react strongly to a parasite, the parasites were determined to be gametocytes. The counting of parasites in a monolayer from a chloroquine-treated culture was carried out at the same sitting as a monolayer from the paired control culture, in a randomised order. The number of parasites counted in replicate monolayers were combined, to obtain one estimate of the proportion of gametocytes to total parasites for each culture. In five experimental culture pairs, only one monolayer per culture was assayed.

Statistical analysis

Where appropriate, all response variables were transformed to bring their distributions close to normal. All analyses were carried out using GLIM 4. Starting with the interactions, terms were removed from the full model by stepwise deletion (Crawley 1993). Non-significant terms were pooled with the residual deviance to obtain the error deviance. Significance of terms was determined from F-ratios of the deviance explained by each term to the error deviance.

The effect of chloroquine on gametocyte production was analysed by logistic regression using a binomial error structure (heterogeneity factor = 3.2; the ratio of residual deviance to degrees of freedom after all non-significant terms had been removed) with number of gametocytes used as the numerator and the sum of gametocytes and asexuals as the denominator (Crawley 1993). The analysis was carried out using a split-plot design (Sokal & Rohlf 1995). TREATMENT (chloroquine+, chloroquine-) was fitted within-EXPERIMENT (pairs of cultures, 'plot', of which there were 6 or 7 per clone) and CLONE (3D7, HB3, 7G8 & SUD124/8) fitted as a between-EXPERIMENT factor. Between EXPERIMENT differences in the proportion of gametocytes to total parasites were very large. These

differences are however of little biological interest and are not reported in this and subsequent analyses where TREATMENT was fitted within-experiment.

The effect of chloroquine on asexual growth was analysed by ANOVA of square-root arcsin-transformed asexual parasitaemia (asexual parasites / RBC) 2 days post-treatment, with TREATMENT fitted within-EXPERIMENT, and CLONE fitted between-EXPERIMENT. Asexual parasitaemia 2 days post-treatment represented the population of parasites after having undergone one more round of replication, and was thus the most appropriate time point to measure inhibition of asexual growth.

In determining clone differences in the effect of chloroquine on gametocyte production, it is important to know whether clones differed in gametocytogenesis in the absence of chloroquine. This was determined by analysis of only untreated cultures. Because of over-dispersion in the data, proportion of gametocytes to total parasites were square-root arcsin-transformed, and analysed by ANCOVA with CLONE fitted as a factor and angular-transformed asexual parasitaemia on day 0 (when commitment of the progeny to gametocyte and asexual development likely took place) fitted as a covariate.

The effect of chloroquine-induced destruction and / or growth inhibition of asexual parasites on investment into gametocytes was analysed by ANCOVA, with the response variable being,

$$\frac{a - b}{(a + b) / 2} \qquad \textbf{(1)},$$

the difference between proportion of gametocytes in chloroquine-treated (a) and the paired untreated culture (b) (standardised by dividing by the mean proportion of gametocytes within-pair, to control for the large differences in proportions of

gametocytes between experiments). CLONE was fitted as a factor and the standardised difference between asexual parasitaemia (2 days post-treatment) of untreated (*a*) and chloroquine-treated cultures (*b*) (see equation 1) fitted as a covariate. Actual chloroquine concentrations were not used as the covariate, because the effect on asexual inhibition of a given concentration is dependent on overall asexual parasitaemia (Gluzman *et al.* 1987).

Differences in successful binding of mAbs between chloroquine-treated and untreated cultures was analysed by logistic regression using a binomial error structure (heterogeneity factor = 4), with the number mAb- (and DAPI-) positive parasites used as the numerator and the number of DAPI-positive parasites as the denominator. TREATMENT was fitted within EXPERIMENT, and CLONE fitted between-EXPERIMENT.

6.4 RESULTS

Two days after the addition of chloroquine to cultures, asexual parasitaemias were on average 40 % that of paired control cultures (figure 6.2, table 6.1). The effect of chloroquine on asexual parasitaemia did not differ between clones (figure 4.2, table 6.2).

Between-experiment	
Clone	$F_{3,22} = 1.9$
Within-Experiment	
Treatment	$F_{1,25} = 69.2$*
Clone x treat.	$F_{3,22} = 2.56$

Table 6.1: Results of analysis of the effect of chloroquine on asexual parasitaemia two days post-treatment in the culture flasks. *$p < 0.0001$

The greater the asexual parasitaemia in untreated cultures prior to invasion of the monolayer (day 0), the greater the proportion of parasites that subsequently developed into gametocytes (figure 6.3, table 6.3). Neither the relationship between proportion of gametocytes and asexual parasitaemia, or proportion of gametocytes differed between clones (figure 6.3 & 6.4, table 6.3).

Asexual parasitaemia	$F_{1,21} = 6.12$*
Clone	$F_{3,21} = 0.18$
Asex. x clone	$F_{3,18} = 2.77$

Table 6.3: Results of the analysis of the effect of asexual parasitaemia in untreated cultures (at the time monolayers were overlaid) on the subsequent proportion of gametocytes in the monolayer. * $p < 0.05$

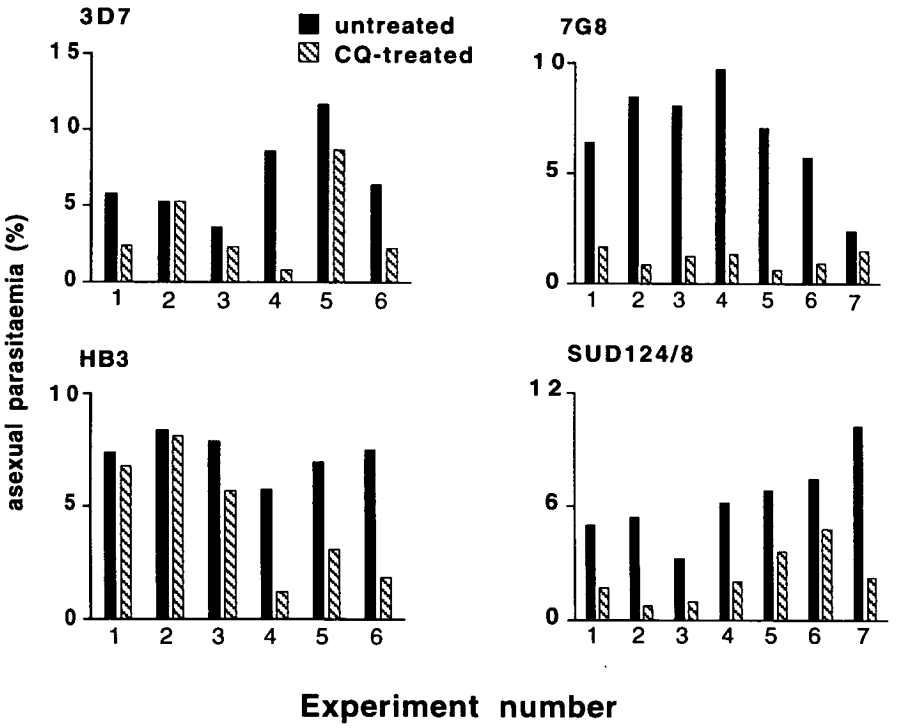


Figure 6.2: Asexual parasitaemias 2 days post-treatment for all clones. Data are shown in culture pairs (experiments). CQ; chloroquine.

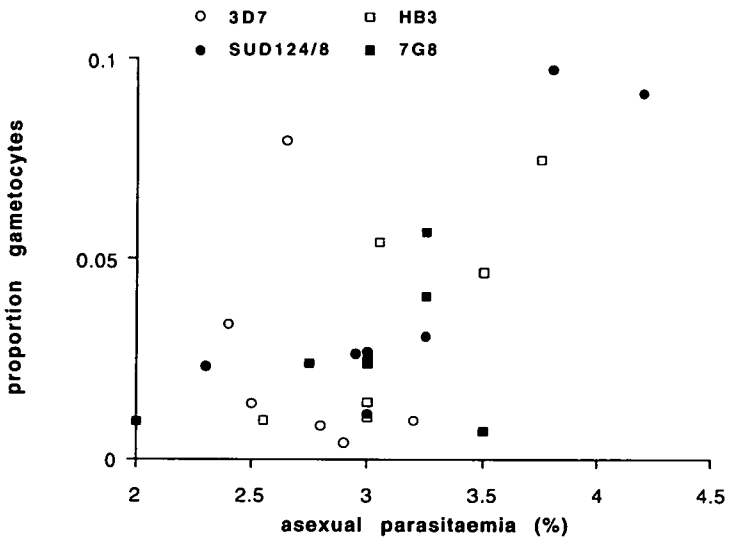


Figure 6.3: The relationship between proportion of parasites that developed into gametocytes in the RBC monolayer and asexual parasitaemia on day 0 (time of commitment to producing gametocytes or asexual progeny) in untreated cultures.

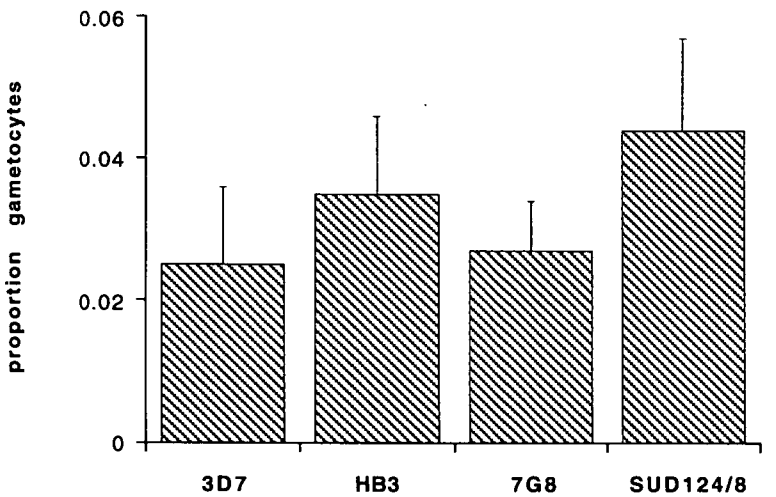


Figure 6.4: Mean (+1 s.e) proportion of parasites that developed into gametocytes for untreated clones.

In all experiments, the proportion of gametocytes to total parasites was greater in monolayers from chloroquine-treated compared to untreated cultures, on average a 5-fold increase across all experiments (figure 6.5, table 6.4). The effect of chloroquine-treatment on proportion of gametocytes differed between clones (table 6.4).

Between-experiment	
Clone	$F_{3,22} = 1.3$
Within-Experiment	
Treatment	$F_{1,25} = 81.3^{**}$
Clone x treat.	$F_{3,22} = 4.3^{*}$

Table 6.4: Results of analysis of the effect of chloroquine on the proportion of parasites that were gametocytes in the monolayers. *p < 0.05; **p < 0.0001.

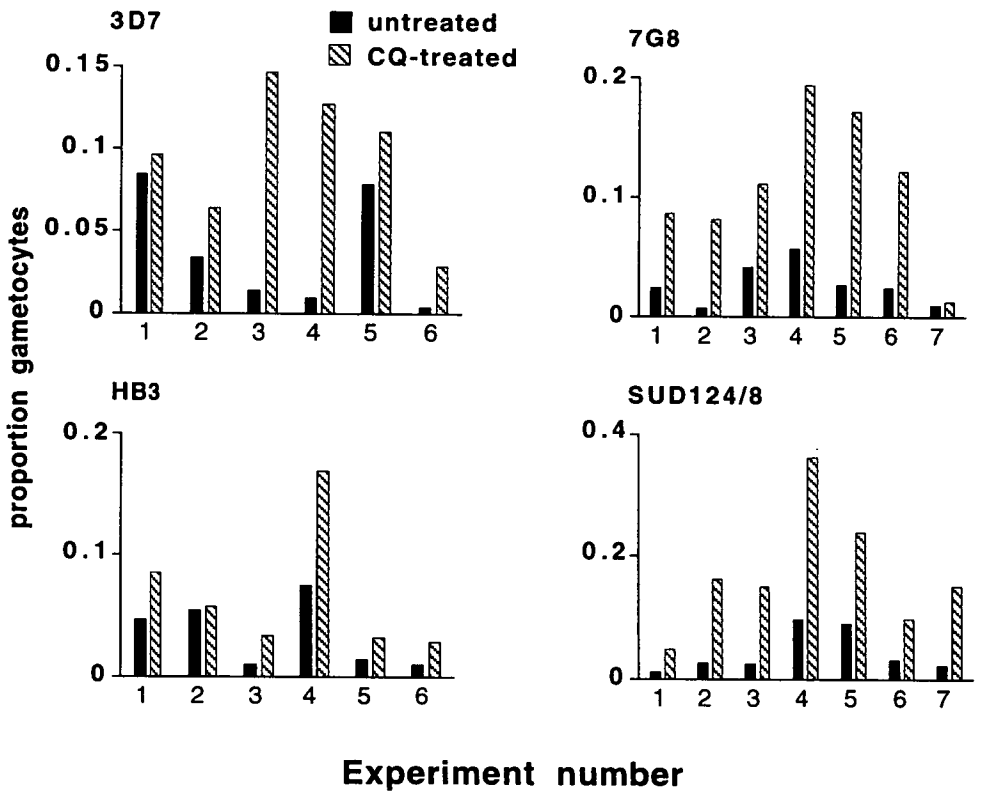


Figure 6.5: Proportion of parasites that developed into gametocytes in the RBC monolayer for all clones. Data are shown in culture pairs (experiments). CQ; chloroquine.

The level of gametocytogenesis induced by chloroquine treatment increased with the extent of chloroquine-induced asexual grwoth inhibition (figure 6.6, table 6.5). For a given value of relative asexual growth inhibition, proportions of gametocytes in chloroquine-treated cultures relative to untreated cultures were more than 2 times greater in clones 3D7 and 124/8 than HB3 and 7G8 (figure 6.6, table 6.5).

Asexual inhibition	$F_{1,21} = 22.89^{**}$
Clone	$F_{1,23} = 11.4^{*}$
Asex. x clone	$F_{3,18} = 1.6$

Table 6.5: Results of the analysis of the effect of asexual growth inhibition (in chloroquine-treated cultures relative to untreated cultures) on the increase in the proportion of gametocytes in monolayers from chloroquine-treated cultures relative to untreated culture. Clone could be simplified to from a four- to a two-level factor, without a significant increase in deviance. * $p < 0.01$; ** $p < 0.001$.

Successful mAb detection (the percentage of DAPI-positive parasites that were also TRITC- or FITC-positive) were 79 % and 85 % for the progeny of chloroquine-treated and untreated cultures, respectively (table 6.6). Even if the additional 6% of undetected parasites in chloroquine-treated cultures were all asexual parasites, this would not qualitatively change the results. Detection did not differ between clones, nor did the effect of treatment on detection differ between clones (table 6.6).

Between-experiment	
Clone	$F_{3,22} = 0.48$
Within-Experiment	
Treatment	$F_{1,25} = 10.42^{*}$
Clone x treat.	$F_{3,22} = 1.83$

Table 6.6: Results of analysis of succesful mAb detection. * $p < 0.01$.

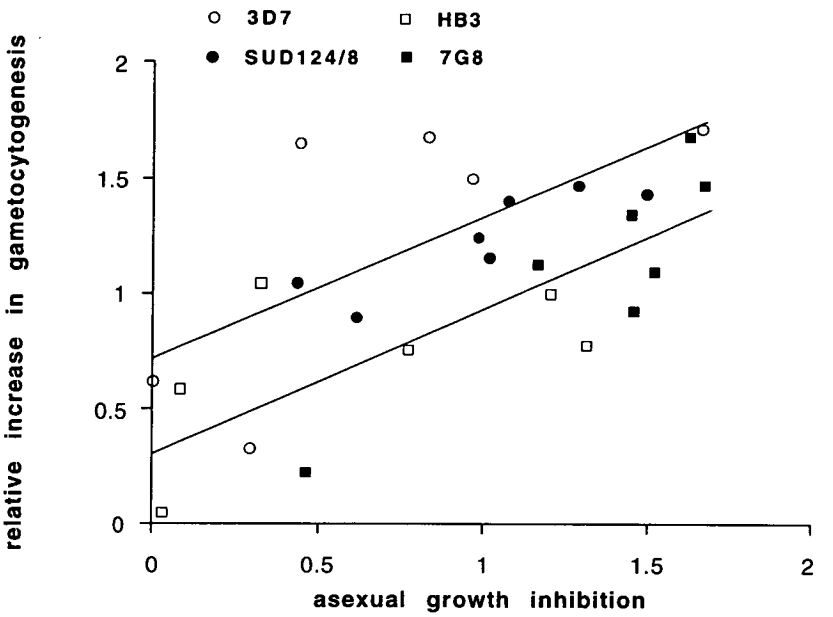


Figure 6.6: The relationship between the relative increase in gametocytogenesis and inhibition of asexual growth following chloroquine treatment (see text). The upper line is the ordinary least-squares regression line for 3D7 and SUD124/8, the lower for HB3 and 7G8. CQ-sensitive and -resistant clones are shown by open and filled symbols, respectively.

6.5 DISCUSSION

The results demonstrate that sub-curative chloroquine treatment increases gametocytogenesis in *P. falciparum* (figure 6.5). *P. falciparum*, like *P. chabaudi* (Buckling *et al.* 1997, chapters 2 and 4) appears to increase its investment into gametocytes when conditions are unfavourable for asexual growth. Such a response is presumably adaptive in that it is likely to increase transmission from harsh environments.

In all clones, gametocytogenesis increased with increasing inhibition of asexual growth. There were genetic differences in this relationship, with two clones (3D7 and

SUD124/8) showing a more than 2-fold increase in gametocytogenesis, for a given level of drug efficacy, than the other two clones (HB3 and 7G8). That gametocytogenesis did not differ between clones when untreated (figure 6.4) suggests that the observed clone differences in the relationship between chloroquine-imposed stress and gametocytogenesis represent differences in sensitivity to environmental stress.

These differences were independent of drug resistance (figure 6.6): 3D7 & HB3 are chloroquine-sensitive, and SUD124/8 & 7G8 are chloroquine-resistant. Unlike previous field reports (Handunnetti *et al.* 1996, Robert *et al.* 1996), there was also no difference in gametocyte production of untreated chloroquine-resistant and -sensitive clones. It therefore appears that chloroquine-imposed selection *per se* has not modified *P. falciparum* gametocytogenesis, either intrinsically or in response to chloroquine. This supports the hypothesis that increased gametocyte production is a general response to retarded asexual growth in unfavourable conditions, with chloroquine treatment being an example of such. Consistent with previous data (Carter & Miller 1979, Brockelman 1982, Bruce *et al.* 1990), increased resource competition, through increased asexual parasitaemia, resulted in increased gametocytogenesis, when all the data was considered. Probably because of small sample sizes for each individual clone, this trend was not apparent for clones 3D7A and 7G8 (figure 6.3).

Demonstration of chloroquine-enhanced gametocyte production *in vivo* in *P. chabaudi* (Buckling *et al.* 1997, chapters 2 and 4) and *in vitro* in *P. falciparum* strongly suggests that the phenomenon may be widespread. Given the positive relationship between gametocyte densities and infectivity to mosquitoes (Carter & Graves 1988, Taylor & Read 1997, Taylor *et al.* 1997, Buckling *et al.* 1997, chapter 2), such compensatory

increases in gametocyte production may help to explain the relative ineffectiveness of chloroquine chemotherapy, which is frequently sub-curative (Wernsdorfer 1994), to reduce malaria transmission. Increased *P. falciparum* gametocyte production is induced by a wide variety of stresses (Mons 1985, Carter & Graves 1988, Sinden *et al.* 1996), including a variety of antimalarial drugs in *P. chabaudi* (Buckling *et al.* 1997, chapters 2 and 4). It may be that any intervention strategy that inhibits asexual growth, including most methods of sub-curative antimalarial treatment and prophylaxis, as well as potential blood-stage vaccines, may induce compensatory increases in transmission stage production by the parasites.

Chapter 7

ANTIMALARIAL INTERVENTION AND THE EVOLUTION OF *PLASMODIUM* VIRULENCE

7.1 SUMMARY

Experiments were carried out to determine the effects of partially protective immunity and antimalarial drug prophylaxis (drug administered before infection) on virulence, within-host parasite replication and transmission of the rodent malaria parasite, *Plasmodium chabaudi, in vivo*. Both methods of intervention reduced virulence relative to control infections. Virulence was less in animals challenged with the immunising genotype rather than a different genotype. Strong positive correlations were found between within-host parasite replication and virulence (measured by host anaemia), and parasite replication and the number of transmission stages (gametocytes) produced by the parasite. The relationship between virulence and transmissability did not differ from control infections, except transmission for a given level of parasite replication (and virulence) was increased in drugged infections. These data suggest that both selection for increased transmission and competitive ability within-hosts may be important determinants of the evolution of virulence in malaria parasites. It is speculated that the use of intervention strategies is likely, if anything, to select for a reduction in parasite virulence. The study also highlights the significance of separate parasite stages for within-host replication and transmission on the evolution of virulence.

7.2 INTRODUCTION

Disease virulence (defined as the reduction in host fitness) is generally believed to be the result of selection optimising parasite transmission success (Bull 1994, Read 1994, Frank 1996, Ebert & Herre 1996, Lipsitch & Moxon 1997). Most models of the

evolution of microparasite virulence assume that virulence *per se* is detrimental to parasite fitness, but is the unavoidable consequence of selection for increased transmission (the 'adaptive trade-off' models, e.g. Levin & Pimentel 1981, Anderson & May 1982, Bremermann & Pickering 1983). This is because of an assumed positive correlation between within-host parasite replication rate and both transmission and virulence. When virulence reaches a certain level, the costs of virulence (usually assumed to be host mortality) are greater than the benefits resulting from increased transmission. Transmission will thus be maximised at an intermediate level of virulence.

Most forms of anti-parasite intervention (for example chemotherapy and vaccination) directly inhibit parasite replication, resulting in a reduction in transmission. This reduction in transmission might result in selection for increased parasite replication and thus increased virulence if the parasite infects individuals not exposed to the intervention (Read 1994). How the intervention extrinsically affects the relationship between within-host replication and transmission, and replication and virulence is also likely to affect how selection will act on within-host replication (Frank 1996): changing these relationships will affect the costs and benefits of a given level of within-host replication. For example, if an intervention strategy caused per parasite virulence to decrease, there will be a reduction in the cost (e.g. host mortality) of a given level of within-host replication. As a result, the level of within-host replication which balances the cost and benefits to transmission will be increased. If selection acts on asexual replication in this way, the parasite might then be more virulent in hosts not exposed to the intervention (figure 7.1).

Inferring selection pressures from phenotypic data, as is being attempted here, requires that the phenotypic changes resulting from the environmental changes are non-transitory. That is, the phenotypic relationships between traits in a particular

environment are assumed to be indicative of underlying genetic relationships. Phenotypic plasticity in the relationships between relevant traits or short-term acclimation (Leroi *et al.* 1994) to changing environmental conditions would render inferred selection pressures from short-term phenotypic data relatively meaningless. These problems aside, a phenotypic approach to understanding the evolution of life history traits has proved very fruitful in the past (Stearns 1992).

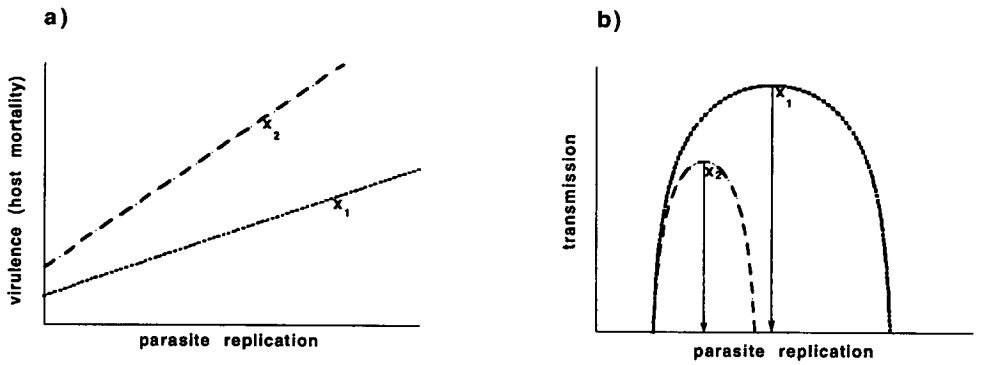


Figure 7.1: (a) Some form of anti-parasite intervention extrinsically alters the relationship between within-host parasite replication and virulence from x_2 to x_1. Potential transmission is directly proportional to parasite replication rate. (b) Actual transmission is the product of potential transmission and the 'cost of virulence'; the probability of host survival in this case. The replication rate that maximises transmission (shown by the arrows) is increased under the intervention strategy. If the intervention is very widespread and their exists genetic variation in replication rates, the change in the relationship between parasite replication and virulence caused by the intervention may select for an increase in parasite replication rate. As a result, the virulence of this parasite will be greater in individuals not exposed to the intervention.

Both genetic and phenotypic positive correlations have been found between virulence (as measured by host anaemia and weight loss) and the density of asexually replicating blood-stage parasites of the rodent malaria parasite, *Plasmodium chabaudi* (Taylor *et al.* 1998, Mackinnon & Read, submitted). Parasite densities also showed a positive genetic correlation with transmission to the mosquito vector (Taylor *et al.* 1998, Mackinnon & Read, submitted). This is consistent with the assumptions necessary for selection to act as expected in the 'adaptive trade-off models' (Levin & Pimentel 1981, Anderson & May 1982, Bremermann & Pickering 1983). Moreover, genetic variation in asexual replication rates (Mackinnon *et al.* submitted) would allow selection to act if the optimal level of replication is extrinsically altered by antimalarial intervention.

It is likely that some forms of antimalarial intervention could extrinsically alter the relationships between asexual replication, transmission and virulence, although existing data are very ambiguous. For example, although the virulence of malarial infections generally shows a positive relationship with parasite densities, some of the pathology is proximately caused by the immune system (Houba 1988). As a result, partial host immunity generally results in a decrease in virulence for a given parasite density (McGregor & Wilson 1988).

I carried out experiments to determine how immunisation (previous exposure to a malarial infection) and antimalarial prophylaxis (drugs administered before infection) affected virulence of the rodent malaria parasite *P. chabaudi* in laboratory mice. Immunity against *P. chabaudi* is in part genotype-specific (McLean *et al.* 1982, Jarra & Brown 1985, Jarra *et al.* 1986, Bates *et al.* 1988, chapter 5). Asexual densities are lower when the same genotype is used for immunisation and subsequent challenge (homologous challenge), than if the immunising and challenge genotypes differ (heterologous challenge). I wanted to determine whether the virulence of infections in immunised hosts was similarly genotype-specific. I also determined how prophylaxis

and immunisation affected the relationships between asexual replication, transmission and virulence. Any changes in these relationships might give an indication of how selection resulting from the intervention might act on parasite virulence, assuming phenotypic correlations are indicative of underlying genetic correlations. All intervention was necessarily partially protective: asexual replication was inhibited, without preventing it all together. For ethical and practical reasons, anaemia, rather than host mortality was used as the measure of virulence. *Plasmodium chabaudi* is generally non-lethal in the inbred strain of mice used in these experiments. However, in less resistant strains, host mortality is positively correlated with anaemia (Stevenson *et al.* 1982, Yap & Stevenson 1992, 1994). Moreover, anaemia is itself a severe morbidity factor of malarial infections.

7.3 MATERIALS AND METHODS

Parasitology

Chemotherapy and immunisation experiments were carried out separately. Details of methods can be found in sections 3.3 and 6.3, respectively. In summary, male C57/BL/6J mice were infected with $1x10^6$ *P. chabaudi* parasites of a single genotype (either clone CR or ER). This strain of mice is partially resistant to *P. chabaudi* infections (Stevenson *et al.* 1982). In the chemotherapy experiments, mice were administered with sub-lethal doses (to the parasite) of either chloroquine or pyrimethamine approximately 1 hour before parasite inoculation. Control mice were given distilled water or DMSO, depending on the solvent used for the drugs in each experiment. In the immunisation experiments, mice were infected with $1x10^4$ parasites (either clone CR or ER), with infections cleared by curative mefloquine treatment 4 days later. Control animals received equivalent inoculations of uninfected red blood cells (RBCs) and the same drug treatment. Mice were challenged 4 weeks after parasite clearance with the either the same (homologous challenge) or different

(heterologous challenge) clone as used for immunisation. Details of experiments can be found in tables 4.1 and 5.1.

Numbers of asexual parasites and gametocytes per RBC were counted from Giemsa-stained thin blood smears, approximately every second day. RBC densities were measured by flow cytometry every second day. Parasite densities were determined by multiplying parasites / RBC by RBC density.

Statistical analyses

Three types of measures were determined for each infection: virulence, within-host asexual replication and transmission. Virulence was estimated from both minimum and mean RBC densities (Taylor *et al.* 1998). Note that these traits are actually negatively correlated with virulence. Asexual replication was measured in 5 ways: (i) total parasites produced during the entire infection was determined by integrating under parasite density through time curves. This will be a reasonably accurate estimate, given the 24 hour asexual cycle of *P. chabaudi* (Cox 1988). (ii) peak asexual parasitaemia (asexuals / RBC), (iii) peak asexual density, (iv) rate of increasing parasitaemia, and (v) rate of increasing parasite density. These latter two measure were determined by dividing the peak asexual parasitaemia and density, respectively, by the day post-infection the peaks were reached.

Total transmission of an infection is likely to be determined by the number of gametocytes produced during the entire infection (Taylor & Read 1997). Integration under the density through time curves is a reasonable approximation of total gametocytes. The half-life of *P. chabaudi* gametocytes is less than 12 hours, and measures were taken every second day, thus very few gametocytes would survive from one day's measurement to the next.

All analyses was carried out using GLIM 4. Chemotherapy and immunisation experiments were analysed separately. Where appropriate, all measures were transformed to bring their distributions close to normal (total asexuals, peak asexual density, rate of increasing parasite density and total gametocytes were $\log_{10}$-transformed, peak asexual parasitaemia and rate of increasing parasitaemia were square-root arcsin-transformed). The effect of asexual replication on each virulence measure was determined by ANCOVA, with all the asexual replication covariates, EXPERIMENTAL BLOCK and TREATMENT (drug+, drug- in the chemotherapy experiments; non-immune, homologous and heterologous challenge in the immunisation experiments) and CLONE (CR or ER; immune experiments only) fitted as factors. CLONE was not included as a factor in the prophylaxis experiments, as the two clones were never used in the same block.

The effect of TREATMENT, BLOCK and CLONE (immune experiments only) on transmission and virulence measures, independent of relevant covariates, was determined by ANOVA. The effect of genotype-specific immunity on virulence and transmission was determined by ANOVA using only immunised animals, with IMMUNISING CLONE (ER or CR), CHALLENGE CLONE (ER or CR) and EXPERIMENT fitted as factors. Detection of a significant interaction between the immunising clone and the challenge clone is evidence for strain-specific immunity (Read & Viney 1996).

The significance of terms was determined by stepwise deletion, starting with the highest order interactions (Crawley 1993). Non-significant terms were removed from the model. Test statistics were then determined from the resulting minimal model.

7.4 RESULTS

Chemotherapy

Both mean and minimum RBC densities were greater in drugged than control animals (figure 7.2, table 7.1). Rate of increasing parasitaemia showed the best (negative) relationship with both mean and minimum RBC densities out of all the asexual replication measures, explaining 60 % of the deviance in mean RBC density (figure 7.3a, table 7.1), and 32 % of the deviance in minimum RBC density (figure 7.3b, table 7.1). None of the other asexual replication covariates explained a significant amount of additional deviance. Differences in both mean and minimum RBC densities between treatments could be explained by differences in asexual replication (figure 7.3a & b, table 7.1).

Rate of increasing parasitaemia also showed the strongest (positive) relationship with total gametocytes, explaining 50 % of the total deviance (figure 7.3c, table 7.1), which was not significantly increased by the addition of any of the other asexual replication measures ($p > 0.1$, in all cases). However, at the mean rate of increasing parasitaemia, drugged infections produced 1.9 times more gametocytes than control infections (figure 7.3c, table 7.1). As a result, total gametocytes did not significantly differ between drugged and control infections (table 7.1).

	Mean RBC density (x 10^9 / ml)	Min. RBC density (x 10^9 / ml)	Total gametocytes (x 10^6)
Mean (s.e.):			
Control	4.93 (0.22)	2.17 (0.19)	12.23 (2.00)
Drugged	5.94 (0.20)	3.04 (0.19)	8.94 (1.81)
ANOVA:			
Treatment	$F_{1,36} = 14.7^{**}$	$F_{1,39} = 9.9^{**}$	$F_{1,36} = 2.3$
Experiment	$F_{3,36} = 16.8^{**}$	$F_{3,36} = 2.3$	$F_{3,36} = 10.1^{**}$
ANCOVA:			
Asex. replication	$F_{1,36} = 46.1^{**}$	$F_{1,39} = 18.6^{**}$	$F_{1,35} = 30.13^{**}$
Treatment	$F_{1,35} = 0.01$	$F_{1,35} = 0.91$	$F_{1,35} = 5.7^{*}$
Experiment	$F_{3,35} = 10.7^{**}$	$F_{3,35} = 0.87$	$F_{3,35} = 4.1^{*}$

Table 7.1: Minimal models for the ANOVAs and ANCOVAs for the drug prophylaxis experiments. Interaction terms were removed, as all were non-significant ($p > 0.1$). * $p < 0.05$; **$p < 0.01$. Min.; minimum.

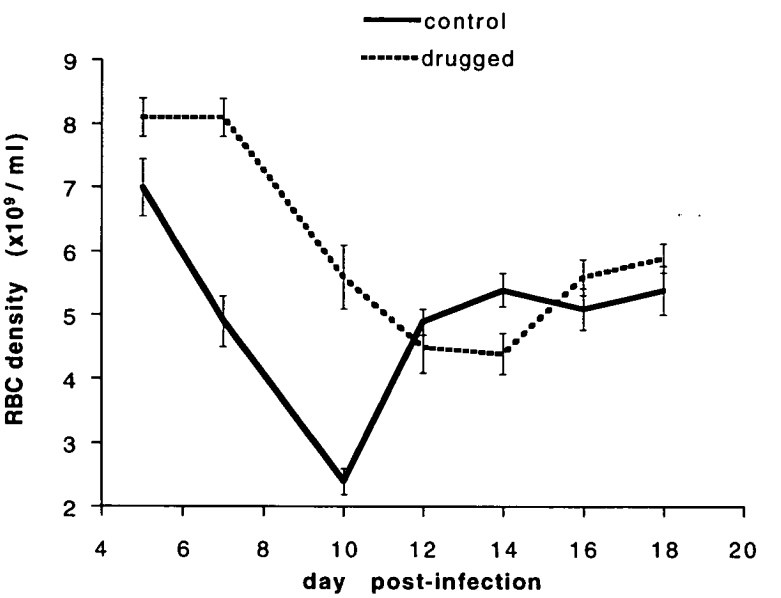


Figure 7.2: Mean (± 1 s.e.) RBC densities throughout infections of control and drugged animals

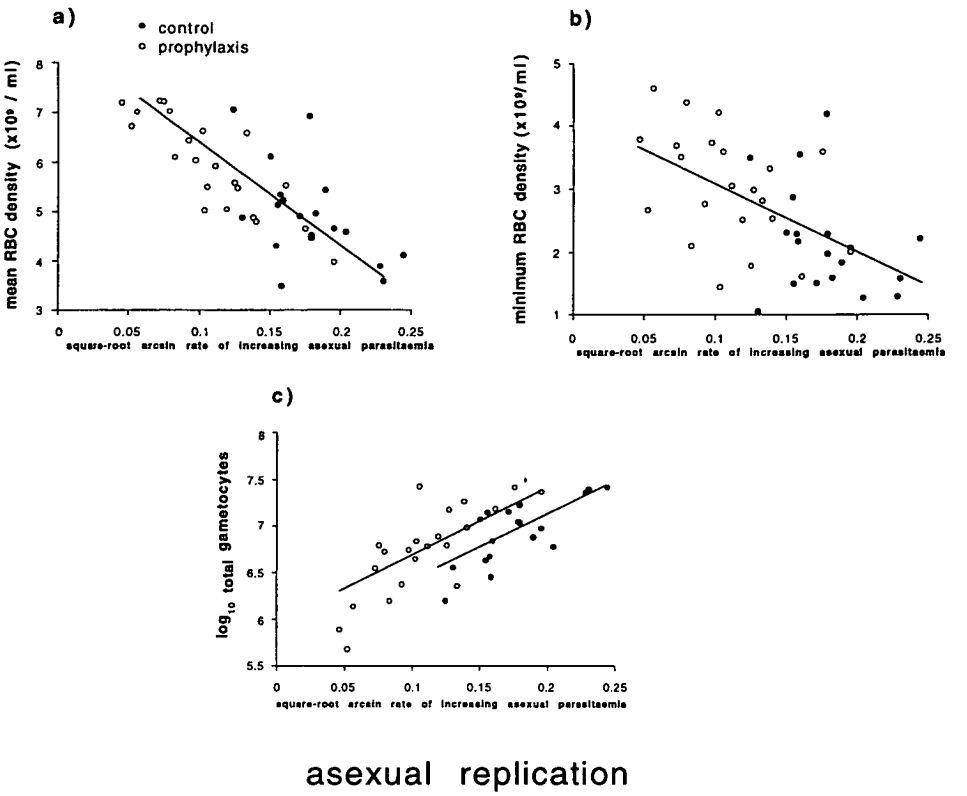


Figure 7.3: Trait relationships from chemotherapy experiments. Relationships between square-root arcsin-transformed rate of increasing asexual parasitaemia (asexual replication) and: (a) mean RBC density, (b) minimum RBC density (both measures of virulence) (c) $\log_{10}$-transformed total gametocytes (transmission). Lines show significant ($p < 0.05$) ordinary least squares regressions.

Immunisation

Both mean and minimum RBC densities were higher in immunised compared with control animals (figure 7.4, table 7.2). Mean RBC densities were higher in immunised animals that were challenged with the homologous compared to the heterologous clone (figure 7.5, table 7.2, IMMUNISING CLONE by CHALLENGE CLONE interaction: $F_{1,24} = 12.57$, $p < 0.01$), although there was no difference in

minimum RBC densities (table 7.2, IMMUNISING CLONE by CHALLENGE CLONE interaction $F_{1,26} = 1.94$, $p > 0.1$). Peak asexual parasitaemia negatively correlated with mean RBC density, explaining 40 % of the deviance (figure 7.6a, table 7.2); more than the other asexual replication covariates, which did not explain a significant amount of additional deviance ($p > 0.1$, in all cases). Peak asexual parasitaemia explained 26 % of the minimum RBC density deviance (figure 7.6b, table 7.2). A significant amount (approximately 10 %) of additional minimum RBC density deviance was explained by total asexuals (table 7.2). Differences in virulence between treatments could be explained by differences in asexual replication between treatments (figure 7.6a & b, table 7.2).

Total gametocytes were higher in control compared with immunised mice (table 7.2), with no significant difference in total gametocytes between immunised animals that were challenged with the homologous or heterologous clone (IMMUNISING CLONE by CHALLENGE CLONE interaction: $F_{1,23} = 1.42$, $p < 0.1$). Total asexuals positively correlated with total gametocytes, explaining 36 % of the deviance associated with total gametocytes (figure 7.6c, table 7.2); more than the other asexual replication covariates, which did not explain a significant amount of additional deviance ($p > 0.1$, in all cases). Differences in total gametocytes between treatments could be explained by differences in asexual replication (figure 7.6c, table 7.2).

Clone differences

Mean and minimum RBC densities were slightly greater in clone CR than clone ER, although these differences were not significant (table 7.2). However, the relationship between peak asexual parasitaemia and virulence differed between clones: at the mean peak parasitaemia, clone CR had mean RBC density 5 % greater, and minimum RBC density 20 % greater, than clone ER (table 7.2). Total gametocytes did not

significantly differ between clone CR and ER, nor was there a difference between clones in the relationship between total gametocytes and total asexuals (table 7.2).

	Mean RBC density (x 10^9 / ml)	**Min. RBC density (x 10^9 / ml)**	**Total gametocytes (x 10^6)**
Mean (s.e.):			
Control	4.83 (0.13)	2.00 (0.43)	17.45 (3.43)
Hom. challenge	6.9 (0.18)	3.6 (0.24)	4.4 (1.4)
Het. challenge	6.1 (0.15)	3.2 (0.19)	4.6 (1.1)
Clone CR	6 (0.23)	3.1 (0.22)	12.8 (3.9)
Clone ER	5.8 (0.21)	2.7 (0.2)	6 (2.1)
ANOVA:			
Treatment	$F_{2,38} = 54.1$**	$F_{2,40} = 22.2$**	$F_{2,41} = 3.93$*
Clone	$F_{1,37} = 3.96$	$F_{1,40} = 7.9$**	$F_{1,39} = 1.59$
Experiment	$F_{1,38} = 1.54$	$F_{1,39} = 0.65$	$F_{1,39} = 3.94$
Treat x exp.	$F_{1,38} = 5.62$*		
ANCOVA:			
Asex. replication	$F_{1,40} = 159.4$**	$F_{1,40} = 22.3$**[1]	$F_{1,42} = 23.48$**
Treatment	$F_{2,38} = 1.65$	$F_{2,38} = 0.54$	$F_{2,38} = 0.2$
Clone	$F_{1,40} = 5.18$*	$F_{1,40} = 7.1$*	$F_{1,38} = 2.46$
Experiment	$F_{1,40} = 8.41$**	$F_{1,38} = 1.2$	$F_{1,38} = 0.4$

Table 7.2: Minimal models for the ANOVAs and ANCOVAs for the immunisation experiments. Interaction terms were removed when non-significant. * $p < 0.05$; **$p < 0.01$. Min.; minimum. [1]An additional within-host replication covariate (total asexuals) explained a significant amount of additional deviance ($F_{1,40} = 7.34$, $p < 0.01$), and is included in the model.

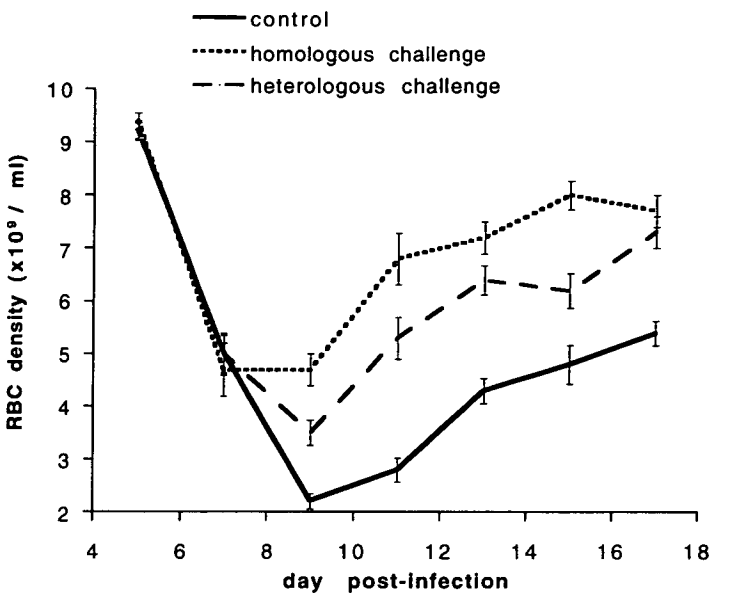


Figure 7.4: Mean (± 1 s.e.) RBC densities throughout infection of control and immunised animals. Homologous and heterologous challenge refer to whether the challenge genotype was the same or different to the immunising genotype, respectively.

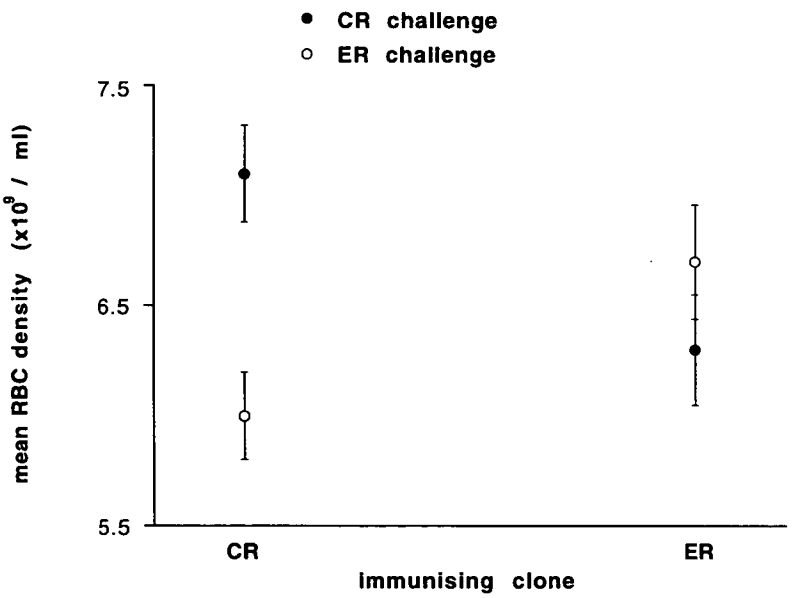


Figure 7.5: Mean (± 1 s.e.) RBC densities for immunised animals. ER and CR are different *P. chabaudi* clones.

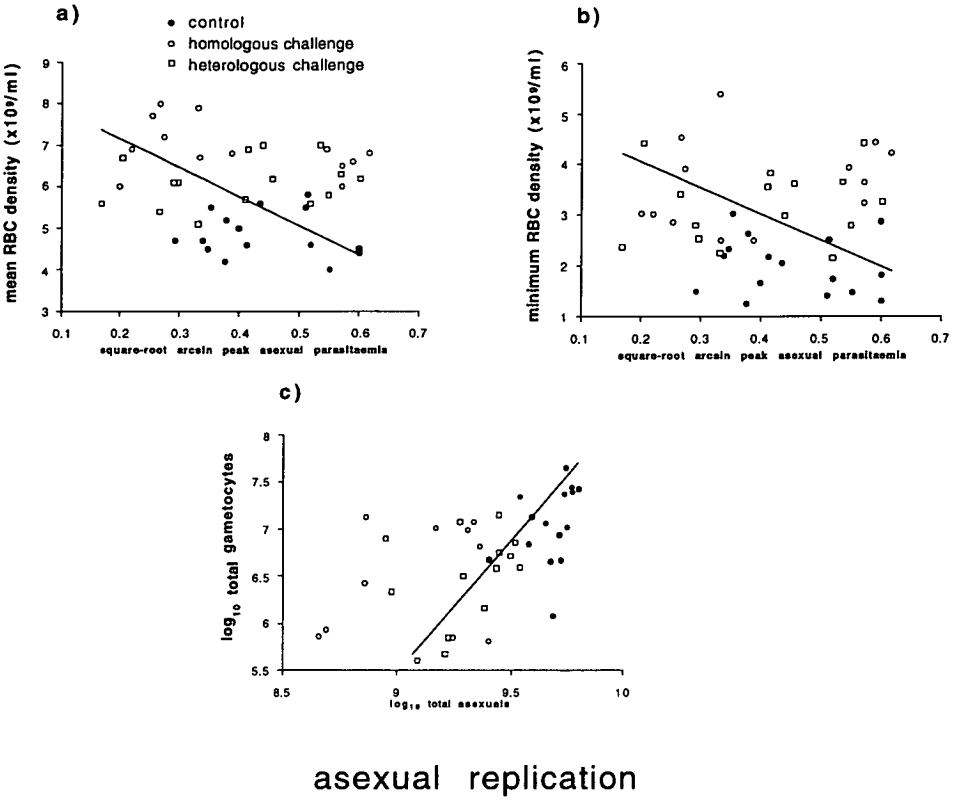


Figure 7.6: Trait relationships from immunisation experiments. Relationships between square-root arcsin-transformed peak asexual parasitaemia (asexual replication) and: (a) mean RBC density, (b) minimum RBC density (both measures of virulence). (c) Relationship between $\log_{10}$-transformed total asexuals (asexual replication) and $\log_{10}$-transformed total gametocytes. Lines show significant ($p < 0.05$) ordinary least squares regressions. Homologous and heterologous challenge refer to whether the challenge genotype was the same or different to the immunising genotype, respectively.

7.5 DISCUSSION

Both antimalarial prophylaxis and immunisation resulted in a reduction in the anaemia (virulence) caused by infections with *P. chabaudi* (table 7.1 & 7.2, figures 7.2 & 7.4). The reduction in virulence resulting from immunisation was in part genotype-specific: mean RBC densities were lower in animals following homologous, compared to

heterologous, challenge (figure 7.5), although there was no difference in minimum RBC densities. The severity of malarial infections of partially immune animals is thus likely to be dependent on whether the animal has been previously infected with the current infecting genotypes. This is consistent with data from people infected with *P. falciparum* : the probability of developing clinical symptoms is greater following infection with a novel, compared to a previously encountered, *P. falciparum* genotype (Mercereau-Puijalon 1996, Roper *et al.* 1998).

Strong positive correlations were found between asexual replication and anaemia (virulence), and between asexual replication and total numbers of gametocytes (transmission). These phenotypic data support previously reported phenotypic (Taylor *et al.* 1998, Mackinnon & Read, submitted) and, more importantly, genetic correlations (Mackinnon & Read, submitted) between these traits in *P. chabaudi.* All these data are consistent with the assumptions necessary for selection to act as expected in the 'adaptive trade-off models' (Levin & Pimemtel 1981, Anderson & May 1982, Bremermann & Pickering 1983).

Any method of intervention that reduces transmission through a reduction in asexual parasite replication will impose strong selection for compensatory increases in asexual replication. As a result, the widespread use of chemotherapy and vaccination may select for parasites that will have greater virulence in the absence of the intervention (Read 1994). This selection pressure might however have negligible effects: selection for increased asexual replication is likely to be strong in the absence of intervention, as most host populations in malaria-endemic areas have some anti-malarial immunity (McGregor & Wilson 1988). As a result, asexual replication and transmission of natural malarial infections are considerably reduced compared to infections in non-immune hosts (James *et al.* 1936, Landau & Chabaud 1994). These arguments would be irrelevant if asexual replication was limited by host mortality, as assumed by the

adaptive trade-off models of the evolution of virulence. Host mortality resulting from malarial infections is however generally relatively low (people; Targett 1992, rodents; Landau & Chabaud 1994, lizards; Schall 1996). If, as suggested, it is the host immune system that limits asexual replication, observed levels of virulence of malaria parasites are unlikely to be largely the result of parasite adaptation, as assumed by most models of the evolution of virulence (Bull 1994, Read 1994, Frank 1996, Ebert & Herre 1996, Lipsitch & Moxon 1997).

The relationship between asexual replication and virulence did not differ between control and drugged infections, or control and immunised infections (figures 7.3 & 7.6). Based solely on these relationships, there is no suggestion that long-term changes in virulence would arise as a result of these methods of intervention. In other words, both forms of intervention are unlikley to change the optimum level of asexual replication. Infections with clone ER were more virulent for a given level of asexual replication than infections with clone CR, suggesting parasite (genetic) factors beyond net asexual replication are important in determining virulence in *Plasmodium.*

No difference was found in the relationships between asexual replication and numbers of gametocytes between infections in immunised and control animals (figure 7.6c). However, for a given level of asexual replication, numbers of gametocytes were two times greater in drugged than control infections. As a result, drugged infections were able to produce the same number of gametocytes, but at lower virulence, than in control infections (table 7.1, figure 7.3c).

Gametocyte / asexual trade-offs and the evolution of virulence

What can a change in the relationship between virulence and transmission tell us about the change in the costs and benefits of virulence resulting from drug prophylaxis? Unfortunately, very little. The proportion of asexual parasites that develop into

gametocytes can be facultatively increased in response to conditions unfavourable for asexual replication (Carter & Graves 1988), including antimalarial chemotherapy (Buckling *et al.* 1997, chapters 2, 4 & 6). The reduction in virulence and asexual parasites in the absence of a significant reduction in gametocytes is likely to be the result of parasites adaptively increasing their investment into gametocytes (Buckling *et al.* 1997, chapter 2). Inferring selection pressures from a change in the relationship between asexual replication and transmission would only be meaningful if these phenotypic correlations were indicative of underlying genetic correlations. This is clearly not the case if the parasites were facultatively altering the relationship.

The existence of separate stages for within-host growth and transmission might have important implications for the evolution of virulence. Although positive correlations are found between asexual replication and transmission, there is also a direct trade-off between these traits: the production of a gametocyte, results in an asexual parasite not being produced, and vice versa. This may result in a cost to transmission of increased asexual replication, additional to other costs, such as increased host mortality. Selection for increased asexual replication (through, for example, within-host competition between genotypes (Frank 1996)) might be achieved by decreasing gametocyte production. Indeed, passaging asexually replicating parasites directly from vertebrate host to vertebrate host (in the absence of mosquito transmission) frequently results in increased asexual replication and reduced gametocyte production in a variety of species of malaria parasites (Carter & Graves 1988, Dearsley *et al.* 1990). None of the models for the evolution of virulence outlined above explicitly consider this trade-off, yet separate within-host replication and transmission stages are common amongst many parasites of medical and veterinary importance, including *Trypanosoma*, *Eimeria* and *Plasmodia.*

Unfortunately, it is impossible to determine the extent of the impact of reduced gametocyte production on asexual replication in the direct passage experiments mentioned above: increased asexual replication might have been achieved in other ways, such as increased numbers of progeny per parasite, or better immune evasion strategies. Similarly, the importance of enhanced gametocyte production on reducing asexual replication could not be addressed in the current study, as alteration in the relationships between asexual replication and gametocyte production was observed under conditions that inhibited asexual replication anyway (antimalarial prophylaxis). However, under most assumptions of a recent series of models, changing gametocyte production does have a notable impact on asexual parasite densities (Mckenzie & Bossert 1998b).

If fitness of malaria parasites positively correlates with transmission, why don't infections in immunised and control animals behave like infections in drugged animals, producing more gametocytes for a given level of asexual replication? It is possible that this is the result of within-host competition. Theory predicts that within-host competition between different conspecific genotypes results in selection for increased within-host replication (May & Anderson 1983, Frank 1992, Hellriegel 1992, Nowak & May 1994, van Baalen & Sabelis 1995, Frank 1996). Natural *P. chabaudi* infections have frequently been found to consist of more than one genotype (Carter 1978), thus selection for increased asexual replication through within-host competition may have occurred. As *P. chabaudi* does not appear to facultatively increase asexual replication rates in the presence of other genotypes (Taylor *et al.* 1998), observed levels of asexual replication are likely to be partly dependent on the frequency of mixed-genotype infections in the field. The effect of within-host selection is therefore likely to be apparent in single genotype infections in this study. Additionally, given the huge within-host population size of asexual parasites (at least 10^9 on the day of peak asexual parasite densities in untreated infections in these experiments), it is

possible that considerable genetic variation could be created during the course of a single infection. Within-host selection might therefore operate, even if infections are generally initiated by genetically homogenous parasites. Stimulating increased gametocyte production by antimalarial chemotherapy might have counteracted the effects of within-host selection.

Other possible explanations for the apparent excess of asexual replication (and virulence) of control and immunised infections need to be considered. First, our estimates of transmission may not be accurate. After clearance of a primary *P. chabaudi* infection (the infections measured in these experiments), parasites periodically increase in numbers to detectable levels (recrudescences). Ideally, these recrudescences should be considered when calculating the total transmission from a *P. chabaudi* infection, although, as gametocytes are rarely seen in recrudescences (A. Read & M. Anwar, unpublished data), they are unlikely to contribute much to the total transmission success of the parasite.

Second, observed levels of virulence may be the result of the parasites experiencing novel conditions. *P. chabaudi* may have been selected in hosts where replication rates are suppressed, resulting in excessive and maladaptive rates of asexual replication in the current experiments. Infections in the rodent host from which *P. chabaudi* was isolated, *T. rutilans*, are often chronic and life long (Landau & Boulard 1978), suggesting that replication rates will commonly be suppressed by immunity. Furthermore, by initiating infections with infected blood, as opposed to natural mosquito inoculation, the host has no time to mount an immune response before parasites start replicating in RBCs. We might therefore expect to observe adaptive relationships between virulence and transmission if asexual replication is inhibited to a greater extent than in control infections in these experiments. This appears to occur in drugged, but not in immunised, animals. Unless drugged infections approximate

natural infections better than unmanipulated infections or infections in immune hosts (which seems very unlikely) this hypothesis is unlikely to be an adequate explanation of the results.

High virulence may have direct transmission-enhancing effects. It has been suggested that host incapacitation may reduce anti-mosquito defensive behaviour, resulting in increased probability of a mosquito obtaining a blood-meal and becoming infected (Day & Edman 1983, Day *et al.* 1983, Ewald 1994). It is debatable whether incapacitation would increase the probability of a mosquito successfully obtaining a blood-meal under natural conditions. Although a sick animal cannot defend itself so well against mosquitoes (Day & Edman 1983), it would probably spend less time out of its shelter, reducing its exposure to mosquitoes.

Field Implications

If the current results apply to *P. falciparum*, the interpretations and speculations may be of relevance to the understanding of the effects of chemotherapy and vaccination on the evolution of virulence of malaria in the field. In short, the use of such intervention is unlikely to result in more virulent parasites: selection for increased transmission (and thus increased asexual replication and virulence) is likely to be very strong anyway, given the high frequency of immune and partially immune individuals. If within-host selection is an important determinant of virulence in *P. falciparum* (infections frequently consist of multiple genotypes; Babiker *et al.* 1994, Ntoumi *et al.* 1995, Paul *et al.* 1995), intervention strategies are likely to result in selection for reduced virulence. A reduction in transmission (through antimalarial intervention) will probably reduce the frequency of mixed genotype infections, decreasing within-host competition and virulence. If within-host competition, and therefore high virulence, also results from the generation of novel mutants during the course of an infection initiated by a single genotype, a reduction in within-host population size by

intervention strategies may also select for decreased virulence. Selection for changes in parasite virulence may not however have a notable impact on the virulence of malarial infections in the field. Variation in host immune status, host (genetic) resistance and the use of antimalarial intervention are likely to overshadow any genetic changes in parasite virulence.

Chapter 8

GENERAL DISCUSSION

Discussions relating to the specific studies in this thesis are given in the relevant chapters. Here I summarise the main findings of those studies and address some of more general issues and questions arising.

8.1 PRINCIPLE FINDINGS

(a) Gametocytogenesis increased as a result of chemotherapy-imposed stress. Where assessed (for chloroquine-treated infections), infectivity to the mosquito vector (*Anopheles stephensi*) was not decreased as a result of chemotherapy. Similarly, chloroquine-treatment increased five-fold the proportion *of P. falciparum* parasites that were gametocytes *in vitro*. Genetic variation in the relationship between chloroquine-imposed stress and gametocytogenesis was found in this study, although this variation was independent of drug resistance (chapters 2, 4 & 6).

(b) Chloroquine did not increase the infectivity of *P. chabaudi* gametocytes to *A. stephensi* (chapter 3).

(c) Partial immunisation from prior exposure to *P. chabaudi* parasites resulted in a three to four-fold reduction in both asexual parasites and gametocytes. This differed from the effect of chemotherapy, which resulted in no, or a disproportionately small (relative to asexuals), reduction in gametocytes. Immunity was strain-specific, with a greater reduction in parasite densities when the challenge and immunising genotypes were the same (homologous challenge), compared to when they differed (heterologous challenge). Strain-specific 'transmission-blocking' immunity was also detected, with per gametocyte infectivity reduced following homologous challenge, compared with

heterologous challenge and control infections, which did not differ in per gametocyte infectivity (chapter 5).

(d) Virulence (as measured by host anaemia) was reduced by both antimalarial drug prophylaxis and immunisation; virulence was greater following heterologous challenges compared to homologous challenges. No difference in the relationship between asexual replication and anaemia was found between control, drugged and immunised infections. The relationship between asexual replication and total gametocytes produced throughout the infection did not differ between control and immunised infections, but more gametocytes were produced for a given rate of asexual replication in prophylaxis infections (chapter 7).

8.2 *PLASMODIUM* LIFE HISTORIES

Between- and within-host selection

Between-host selection on parasites is likely to act to maximise the overall transmission of a single-genotype infection from a host (Anderson & May 1982). That patterns of gametocytogenesis in unmanipulated hosts are broadly consistent with life history theory (which assumes that selection is acting to maximise reproductive success) suggests that *P. chabaudi* life histories are in part the result of between-host selection. Increases in gametocytogenesis in response to stress are consistent with this interpretation, as are possible decreases in gametocytogenesis when conditions decrease per gametocyte infectivity. Moreover, observed levels of virulence may in part be the result of between-host selection: given the positive correlations between asexual replication, virulence and transmission, virulence appears to be in part the necessary consequence of selection acting on transmission (Anderson & May 1982).

Why don't malaria parasites produce more gametocytes, as they are clearly capable of upregulation of gametocytogenesis? Taylor & Read (1997) suggested that above a

certain gametocyte density, transmission may not be increased, as a result of mosquito mortality and the development of transmission-blocking immunity. Another important consideration is the effects of within-host selection. Within-host selection results from competition between conspecific genotypes in the host, and is predicted to result in increased within-host replication (Frank 1996). If asexual replication is limited by certain physiological and biochemical constraints, selection for increased asexual replication may result in reduced gametocytogenesis (Mckenzie & Bossert 1998b). The relative importance of altering patterns of gametocytogenesis (within realistically biological ranges) has yet to be experimentally addressed.

Selection for increased asexual replication through within-host competition might be an additional important determinant of observed levels of *Plasmodium* virulence (Frank 1996). It may also explain differences in overall rates of gametocytogenesis between infections in immunised and drugged animals. *P. chabaudi* is likely to be well adapted to immune hosts. It is possible that competitive ability (asexual replication) is more important to fitness (relative to other genotypes) than further increases in gametocyte production. On the other hand, the adaptive response to a novel and harsh stress such as sub-curative chemotherapy might be massive upregulation of gametocytogenesis.

The relative importance of between-host selection (maximising overall transmission success from a host) and within-host selection (maximising competitive ability within-hosts) on *Plasmodium* life histories has yet to be experimentally addressed. Competitive ability within-hosts is likely to be reflected in relative transmission success (Bull 1994). However, within-host selection can sometimes be so strong that a good within-host competitor is very poor at transmitting ('short-sighted' evolution; Levin & Bull 1994). Selection in mixed and single genotype environments (using the full life-cycle of the parasite) would address the importance of competition on asexual replication and gametocyte production. Comparison of the life histories of the

parasites at the start and end of complete infections would address the possibility that mutants generated during the course of an infection initiated with a single genotype result in within-host competition and selection.

Phenotypic and genetic life history changes

What is the relative importance of genetic variation in gametocyte production compared with the obvious phenotypic plasticity shown by the parasite for this trait? Appropriate selection experiments involving the complete life-cycle of the parasite could address this issue.

Sex and immune evasion

The occurrence of strain-specific asexual and transmission blocking immunity are likely to be a strong selection pressure for the generation of novel genotypes. It is possible that this is a major driving force behind the evolution and maintenance of sex in *Plasmodium* (see Gemmill *et al.* 1997). The importance of sex as an immune evasion strategy could be addressed by looking at the relative transmission success of recombinant genotypes in hosts previously exposed to one (or both) of the parental genotypes.

8.3 PROXIMATE MECHANISMS

Gametocytogenesis

What are the biochemical pathways that result in gametocytogenesis? The relevance of 'stress' proteins (such as heat shock-like proteins; Motard *et al.* 1995) might be an important area to address given that 'stress' in a variety of forms appears to increase gametocytogenesis (Carter & Graves 1988). Consideration of possible cell surface receptors that recognise specific stimuli, such as antibody, might also be a sensible approach.

Virulence

The degree of host anaemia could be largely explained by net asexual replication rates in this thesis. However, different clones resulted in different levels of anaemia for a given parasite density. What other parasite factors are responsible for the general morbidity resulting from a malarial infection? Associations have been made between certain parasite traits and specific clinical symptoms (e.g. Rowe *et al.* 1995), but what other parasite factors are responsible for the general morbidity resulting from malarial infections?

8.4 RELEVANCE TO THE FIELD

The combination of results from the studies on *P. chabaudi in vivo* and *P. falciparum in vitro* suggest that chemotherapy which acts against asexual parasites, unless used curatively, is unlikely to have a very large impact on transmission. A frightening possibility is that it may actually increase transmission under some circumstances. Although (like previous reports) we found no convincing long-term gametocyte infectivity-enhancement following chloroquine treatment, the possibility that it might occur makes the need for further work imperative. Both these issues need to be addressed in the field, although there would of course be major ethical problems if appropriately controlled experiments were carried out.

Partial immunisation greatly reduced both transmission and virulence, suggesting even relatively ineffective vaccines may be of considerable use. The finding that gametocyte infectivity-reducing immunity is also strain-specific needs to be further addressed.

REFERENCES

Agnew, P. & Koella, J. C. 1997 Virulence, parasite mode of transmission, and host flucuating asymmetry. *Proc. Roy. Soc. Lond. B* **264**, 9-15.

Alano, P. & Carter, R. 1990 Sexual differentiation in malaria parasites. *A. Rev. Microbiol.* **44**, 429-449.

Alano, P., Sivestrini, F. & Roca, L. 1996 Structure and polymorphism of the upstream region of the pfg27/25 gene, transcriptionally regulated in gametocytogenesis of *Plasmodium falciparum. Mol. Biochem. Parasitol.* **79**, 207-217.

Alles, H. K., Mendis, K. N. & Carter, R. 1998 Malaria mortality rates in South Asia and Africa: implications for malaria control. *Parasitol. today* **14**, 369-375.

Anderson, R. M. & May, R. M. 1982 Coevolution of hosts and parasites. *Parasitology* **85**, 411-426.

Anderson, R. M. & May, R. M. 1991 *Infectious Diseases of Humans. Dynamics and Control.* Oxford: Oxford University Press.

Babiker, H. A., Charlwood, J. D., Smith, T. & Walliker, D. 1994 Gene flow and cross-mating in *Plasmodium falciparum* in households in a Tanzanian village. *Parasitology* **111**, 433-442.

Baird, J. K., Jones, T. R., Purnomo, Masbar, S., Ratiwayanto, S. & Leksana, B. 1991 Evidence for specific suppression of gametocytemia by *Plasmodium falciparum* in residents of hyperendemic Irian Jaya. *Am. J. Trop. Med. Hyg.* **44**, 183-190.

Baker, D. A., Daramola, O., McCrossan, M. V., Harmer, J. & Targett, G. A. T. 1994 Subcellular location of Pfs16, a *Plasmodium falciparum* gametocyte antigen. *Parasitology* **108**, 129-137.

Bates, M. D., Newbold, C. I., Jarra, W. & Brown, K. N. 1988 Protective immunity to malaria: studies with cloned lines of plasmodium chabaudi chabaudi in CBA/Ca mice. III. Protective and suppressive responses induced by immunisation with purified antigens. *Parasite Immunol.* **10**, 1-15.

Baudoin, M. 1975 Host castration as a parasitic strategy. *Evolution* **29**, 335-352.

Bayoumi, R. A. L., Creasey, A. M., Babiker, H. A., Carlton, J. M.-R., Sultan, A. A., Satti, G., Sohal, A. K., Walliker, D., Jensen, J. B. & Arnot, D. E. 1993 Drug response and genetic characterisation of *Plasmodium falciparum* clones recently isolated from a Sudanese village. *Trans. R. Soc. Trop. Med. Hyg.* **87**, 454-458.

Beale, G. H., Carter, R. & Walliker, D. 1978 Genetics. In *Rodent Malaria.* (ed. R. Killick Kendrick and W. Peters), pp. 213-246. London: Academic Press.

Bhasin, V. K. & Trager, W. 1984 Gametocyte-forming and non-gametocyte-forming clones of *Plasmodium falciparum. Am. J. Trop. Med. Hyg.* **33**, 534-537.

Bishop, A. 1954 The effect of sulphadiazine, proguanil and 2:4-diamino-6:7-di*iso*propylpteridine upon gametocyte production in *Plasmodium gallinaceum. Parasitology* **45**, 163-185.

Bishop, A. & Birkett, B. 1948 Drug-resistance in *Plasmodium gallinaceum*, and the persistence of paludrine-resistance after mosquito transmission. *Parasitology* **39**, 126-137.

Boots, M. & Begon, M. 1993 Trade-offs with resistance to a granulosis virus in the Indian meal moth, examined by a laboratory evolution experiment. *Funct. Ecol.* **7**, 528-534.

Boyd, M. F. 1942 Criteria of immunity and susceptibility in naturally induced *vivax* malaria infections. *Am. J. Trop. Med. Hyg.* **22**, 217-226.

Bremermann, H. J. & Pickering, J. 1983 A game-theoretical model of parasite virulence. *J. Theor. Biol.* **100**, 411-426.

Brockelman, C. R. 1982 Conditions favouring gametocytogenesis in the continuous culture of *Plasmodium falciparum*. *J. Protozool.* **29**, 454-458.

Bruce, M. C., Alano, P., Duthie, S. & Carter, R. 1990 Commitment of the malaria parasite Plasmodium falciparum to sexual and asexual development. *Parasitology* **100**, 191-200.

Bruce, M. C., Carter, R. N., Nakamura, K., Aikawa, M. & Carter, R. 1994 Cellular location and temporal expression of the *Plasmodium falciparum* sexual stage antigen Pfs16. *Mol. Biochem. Parasitol.* **65**, 11-22.

Bruce-Chwatt, L. J. 1985 *Essential malariology*. London: William Heinemann.

Buckling, A. G. J., Taylor, L. H., Carlton, J. M.-R. & Read, A. F. 1997 Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy. *Proc. R. Soc. Lond. B* **264**, 553-559.

Bull, J. J. 1994 The evolution of virulence. *Evolution* **48**, 1423-1437.

Bull, J. J., Molineux, I. J. & Rice, W. R. 1991 Selection of benevolence in a host-parasite system. *Evolution* **45**, 875-882.

Burkot, T., Williams, J. L. & Schneider, I. 1984 Infectivity to mosquitos of *Plasmodium falciparum* clones grown *in vitro* from the same isolate. *Trans. Roy. Soc. Trop. Med. Hyg.* **78**, 339-341.

Butcher, G. A. 1997 Antimalarial drugs and the mosquito transmission of *Plasmodium*. *Int. J. Parasitol.* **27**, 975-987.

Cadigan, F. C. & Chaicumpa, V. 1969 *Plasmodium falciparum* in the white-handed gibbon: protection afforded by previous infection with homologous and heterologous strains obtained in Thailand. *Mil. Med.* **134**, 1135-1139.

Cambie, G., Verdier, F., Gaudebout, C., Clavier, F. & Ginsburg, H. 1994 The pharmacokinetics of chloroquine in healthy and *Plasmodium chabaudi*-infected mice: implications for chronotherapy. *Parasite* **1**, 219-226.

Carter, R. 1978 Studies on enzyme variation in the murine malaria parasites *Plasmodium berghei, P. yoelii, P. vinckei* and *P. chabaudi* by starch gel electrophoresis. *Parasitology* **76**, 241-267.

Carter, R. & Diggs, C. L. 1977 *Plasmodia* of rodents. In *Parasitic Protozoa*. (ed. J. P. Kreier), pp. 359-465. New York: Academic Press.

Carter, R. & Graves, P. M. 1988 Gametocytes. In *Malaria. Principles and Practice of Malariology*. (ed. W. H. Wernsdorfer and I. McGregor), pp. 253-305. Edinburgh: Churchill Livingstone.

Carter, R., Graves, P. M., Keister, D. B. & Quakyi, I. A. 1990 Properties of epitopes of Pfs48/45, a target of transmission-blocking monoclonal antibodies, on gametes of different isolates of *Plasmodium falciparum*. *Parasite Immunol.* **12**, 587-603.

Carter, R. & Miller, L. H. 1979 Evidence for environmental modulation of gametocytogenesis in *Plasmodium falciparum* in continuous culture. *Bull. Wld Hlth Org.* **57** (Suppl. 1), S37-S52.

Carter, R., Wijesekera, S. K., Karunaweera, N. D. & Mendis, K. N. 1997 Mediators and mechanisms associated with paroxysm in *Plasmodium vivax* malaria. *J. Pharm. Pharmacol.* **49** (Suppl. 2), S35-S41.

Choi, H. W., Breman, J. G., Teutsch, S. M., Liu, S., Hightower, A. W. & Sexton, J. D. 1995 The effectiveness of insecticide-impregnated bed nets in reducing cases of malaria infection: a meta-analysis of published results. *Am. J. Trop. Med. Hyg.* **52**, 377-382.

Chutmongkonkul, M., Maier, W. A. & Seitz, H. M. 1992 *Plasmodium falciparum*: effect of chloroquine, halofantrine and pyrimethamine on the infectivity of gametocytes for *Anopheles stephensi* mosquitoes. *Ann. Trop. Med. Parasitol.* **86**, 103-110.

Cohen, D. 1971 Maximising final yield when growth is limited by time or by limiting resources. *J. theor. Biol.* **33**, 299-307.

Collins, F. H., Sakai, R. K., Vernick, K. D., Paskewitz, S., Seeley, D. C., Miller, L. H., Collins, W. E., Campbell, C. C. & Gwadz, R. W. 1986 Genetic selecton of a *Plasmodium*-refractory strain of the malaria vector Anopheles gambiae. *Science, Wash.* **234**, 607-610.

Conder, G. A. & Campbell, W. C. 1995 Chemotherapy of nematode infections of veterinary importance, with special reference to drug resistance. *Adv. Parasitol.* **35**, 1-84.

Cornelissen, A. W. & Walliker, D. 1985 Gametocyte development of *Plasmodium chabaudi* in mice and rats: evidence for host induction of gametocytogenesis. *Z Parasitenkd* **71**, 297-303.

Cox, F. E. G. 1988 Major animal models in malaria research: rodent. In *Malaria. Principles and Practice of Malariology.* (ed. W. H. Wernsdorfer and I. McGregor), pp. 1503-1543. Edinburgh: Churchill Livingstone.

Crawley, M. 1993 *GLIM for Ecologists.* Oxford: Blackwell Scientific.

Crowl, T. A. & Covich, A. P. 1990 Predator-induced life history variation in a freshwater snail. *Science, Wash.* **247**, 949-951.

Day, J. F., Ebert, K. M. & Edman, J. D. 1983 Feeding patterns of mosquitoes (Diptera: Culicidae) simultaneously exposed to malarious and healthy mice, including a method for separating blood meals from conspecific hosts. *J. Med. Entomol.* **20**, 120-127.

Day, J. F. & Edman, J. D. 1983 Malaria renders mice susceptible to mosquito feeding when gametocytes are most infective. *J. Parasitol.* **69**, 163-170.

Dearsly, A. L., Sinden, R. E. & Self, I. A. 1990 Sexual development in malarial parasites - gametocyte production, fertility and infectivity to the mosquito vector. *Parasitology* **100**, 359-368.

Desjardins, R. E., Doberstyn, E. B. & Wernsdorfer, W. H. 1988 The treatment and prophylaxis of malaria. In *Malaria. Principles and Practice of Malariology.* (ed. W. H. Wernsdorfer and I. McGregor), pp. 827-863. Edinburgh: Churchill livingstone.

Desjardins, R. E. & Trenholme, G. M. 1984 Antimalarial chemotherapy. In *Parasitic diseases 2: The chemotherapy.* (ed. J. M. Mansfield), pp. New York, Basel: Marcel Dekker Inc.

Ebert, D. & Herre, E. A. 1996 The evolution of parasitic diseases. *Parasitol. Today* **12**, 96-101.

Ewald, P. W. 1994 *Evolution of Infectious Diseases.* Oxford: Oxford University Press.

Findlay, G. M., Maegraith, B. G. & Holden, J. R. 1946 Investigations in the chemotherapy of malaria in West Africa. V. Sulphonamide compounds. *Ann. Trop. Med. Parasitol.* **40**, 358-367.

Foo, A., Carter, R., Lambros, C., Graves, P. M., Quakyi, I. A., Targett, G. A. T., Ponnundurai, T. & Lewis, G. 1991 Conserved and variant epitopes of target antigens of transmission-blocking antibodies among isolates of *P. falciparum* from Malasia. *Am. J. Trop. Med. Hyg.* **44**, 623-631.

Fox, R. I. & Kang, H.-I. 1993 Mechanism of action of antimalarial drugs: inhibition of antigen processing and presentation. *Lupus* **2** (Suppl. 1), S9-S12.

Foy, H. & Kondi, A. 1952 Effect of daraprim on the gametocytes of *Plasmodium falciparum. Trans. R. Soc. Trop. Med. Hyg.* **46**, 370-371.

Frank, S. A. 1992 A kin selection model for the evolution of virulence. *Proc. Roy. Soc. B* **250**, 195-197.

Frank, S. A. 1996 Models of parasite virulence. *Q. Rev. Biol.* **71**, 37-78.

Gadgil, M. & Bossert, W. H. 1970 Life historical consequences of natural selection. *Am. Nat.* **104**, 1-24.

Garnham, P. C. C. 1988 Malaria parasites of man: life-cycles and morphology. In *Malaria. Principles amd practices of malariology.* (ed. W. H. Wernsdorfer and I. McGregor), pp. 61-96. Edinburgh: Churchill Livingstone.

Gautret, P. F., Miltgen, F., Gantier, J.-C., Chabaud, A. G. & Landau, I. 1997 Enhanced gametocyte formation by *Plasmodium chabaudi* in immature erythrocytes: patterns of production and infectivity to mosquitoes. *J. Parasitol.* **82**, 900-906.

Gemmill, A., Viney, M. E. & Read, A. F. 1997 Host immune status determines sexuality in a parasitic nematode. *Evolution* **51**, 393-401.

Githeko, A. K., Brandling-Bennett, A. D., Beier, M., Atieli, F., Owaga, M. & Collins, F. H. 1992 The reservoir of *Plasmodium falciparum* malaria in a holoendemic area of western Kenya. *Trans. R. Soc. Trop. Med. Hyg.* **86**, 355-358.

Gluzman, I. Y., Schlesinger, P. H. & Krogstad, D. J. 1987 Inoculum effect with chloroquine and *Plasmodium falciparum*. *Antimic. Ag. Chemo.* **31**, 32-36.

Goodier, M. R. & Targett, G. A. T. 1997 Polyclonal T-cell responses to Plasmodium falciparum gametocytes in malaria non-exposed donors. *Parasite Immunol.* **19**, 419-425.

Gotthard, K. & Nylin, S. 1995 Adaptive plasticity and plasticity as an adaptation: a selective review of plasticity in animal morphology and life history. *Oikos* **74**, 3-17.

Gould, S. J. & Lewontin, R. C. 1979 The spandrels of San Marco and the Panglossian paradigm: a critique of the adaptationist programme. *Proc. Roy. Soc. Lond. B* **205**, 581-598.

Gramiccia, G. & Beales, P. F. 1988 The recent history of malaria control and eradication. In *Malaria. Priciples and practices of malariology.* (ed. W. Wernsdorfer and I. McGregor), pp. 1335-1378. Edinburgh: Churchill Livingstone.

Graves, P. M., Carter, R., Burkot, T. R., Quakyi, I. A. & Kumar, N. 1988 Antibodies to *Plasmodium falciparum* gamete surface antigens in Papua New Guinea sera. *Parasite. Immunol.* **10**, 209-218.

Graves, P. M., Carter, R. & McNeill, K. M. 1984 Gametocyte production in cloned lines of *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* **33**, 1045-1050.

Graves, P. M., Doubrovsky, A. & Beckers, P. 1991 Antibody responses to *Plasmodium falciparum* gametocyte antigens during and after malaria attacks in schoolchildren from Madang, Papua New Guinea. *Parasite. Immunol.* **13**, 291-299.

Greenwood, B. M. 1997 What can be expected from malaria vaccines? *Ann. Trop. Med. Parasitol.* (Suppl.) **91**, S9-S13.

Gupta, S., Hill, A. V. S., Kwiatkowski, D., Greenwood, A. M., Greenwood, B. M. & Day, K. P. 1994a Parasite virulence and disease patterns in *P. falciparum* malaria. *Proc. Natl. Acad. Sci. USA* **91**, 3715-3719.

Gupta, S., Maiden, M., Feavers, I., Nee, S., May, R. M. & Anderson, R. M. 1996 The maintenance of strain structure in populations of recombining infectious agents. *Nature Med.* **2**, 437-442.

Gupta, S., Swinton, J. & Anderson, R. M. 1994b Theoretical studies of the effects of heterogeneity in the parasite population on the transmission dynamics of malaria. *Proc.R. Soc. Lond. B* **256**, 231-238.

Hadley, T. J., Klotz, F. W. & Miller, L. H. 1986 Invasion of erythrocytes by malaria parasites - a cellular and biochemical overview. *A. Rev. Microbiol.* **40**, 451-477.

Hamidi, A. N. 1977 The effect of chloroquine on the infectivity of rodent malaria parasites to *Anopheles stephensi*. *Iranian J. Publ. Hlth* **6**, 6-11.

Handunnetti, S. M., Gunewardena, D. M., Pathirana, P. P. S. L., Ekanayake, K., Weerasinghe, S. & Mendis, K. N. 1996 Features of recrudescent chloroquine-resistant *Plasmodium falciparum* infections confer a survival advantage on parasites and have implications for disease control. *Trans. R. Soc. Trop. Med. Hyg.* **90**, 563-567.

Healer, J., Hopcroft, P., Haley, S., Carter, R. & Riley, E. 1997 Complement-mediated lysis of *Plasmodium falciparum* gametes by malaria-immune human sera is associated with antibodies to the gamete surface antigen Pfs 230. *Parasite Immunol.* **65**, 3017-3023.

Hellriegel, B. 1992 Modelling the immune response to malaria with ecological concepts: short-term behaviour against long-term equilibrium. *Proc. R. Soc. Lond. B* **250**, 249-256.

Hogh, B., Gamage-Mendis, A., Butcher, G. A., Thompson, R., Begtrup, K., Mendis, C., Enosse, S. M., Dgedge, M., Barreto, J., Eling, W. & Sinden, R. E. 1998 The differing impact of chloroquine and pyrimethamine/sulfadoxine upon the infectivity of malaria species to the mosquito vector. *Am. J. Trop. Med. Hyg.* **58**, 176-182.

Hogh, B., Thompson, R., Hetzel, C., Fleck, S. L., Kruse, N. A., Jones, I., Dgedge, M., Barreto, J. & Sinden, R. E. 1995 Specific and nonspecific responses to *Plasmodium falciparum* blood-stage parasites and observations on the gametocytemia in schoolchildren living in a malaria-endemic area of Mozambique. *Am. J. Trop. Med. Hyg.* **52**, 50-59.

Houba, V. 1988 Specific immunity: immunopathology and imunosuppression. In *Malaria. Principles and practices of malariology.* (ed. W. H. Wernsdorfer and I. McGregor), pp. 621-638. Edinburgh: Churchill Livingstone.

Ichimori, K., Curtis, C. F. & Targett, G. A. T. 1990 The effects of chloroquine on the infectivity of chloroquine-sensitive and -resistant populations of *Plasmodium yoelii nigeriensis* to mosquitoes. *Parasitology* **100**, 377-381.

Inselberg, J. 1983 Gametocyte formation by the progeny of single *Plasmodium falciparum* schizonts. *J. Parasitol.* **69**, 584-591.

James, S. P., Nicol, W. D. & Shute, P. G. 1936 Clinical and Parasitological Observations of Induced Malaria. *Proc. R. Soc. Med.* **29**, 879-893.

Jarra, W. & Brown, K. N. 1985 protective immunity to malaria: studies with cloned lines of *Plasmodium chabaudi* and *P. berghei* in CBA/Ca mice. I. The effectiveness and inter- and intra-species specificity of immunity induced by infection. *Parasite Immunol.* **7**, 595-606.

Jarra, W. & Brown, K. N. 1989 Protective immunity to malaria: studies with cloned lines of rodent malaria in CBA/Ca mice. IV. The specificity of mechanisms resulting in crisis and resolution of the primary acute phase parasitaemia of *Plasmodium chabaudi chabaudi* and *P. yoellii yoellii. Parasite immunol.* **11**, 1-13.

Jarra, W., Hills, L. A., March, J. C. & Brown, K. N. 1986 Protective immunity to malaria. Studies with cloned lines of *Plasmodium chabaudi chabaudi* and *P. berghei* in CBA/Ca mice. II. The effectiveness and inter- or intra-species specificity of the passive transfer of immunity with serum. *Parasite Immunol.* **8**, 239-254.

Jeffery, G. M. 1958 Infectivity to mosquitoes of *Plasmodium vivax* following treatment with chloroquine and other antimalarials. *Am. J. Trop. Med. Hyg.* **7**, 207-211.

Jeffery, G. M. 1966 Epidemiological significance of repeated infections with homologous and heterologous strains of *Plasmodium. Bull. Wld. Hlth Org.* **35**, 873-882.

Jeffery, G. M., Young, M. D. & Eyles, D. E. 1956 The treatment of *Plasmodium falciparum* infection with chloroquine, with a note on infectivity to mosquitoes of primaquine- and pyrimethamine cases. *Am. J. Hyg.* **64**, 1-11.

Jensen, J. B. 1979 Observations on gametogenesis of *Plasmodium falciparum* in continuous culture. *J. Protozool.* **26**, 129-132.
Kaushal, D. C., Carter, R., Miller, L. H. & Krishna, G. 1980 Gametocytogenesis by malaria parasites in continuous culture. *Nature, Lond.* **286**, 490-492.
King, D. & Roughgarden, J. 1982 Graded allocation between vegetative growth for annual plants in growing seasons of random length. *Theor. Pop. Biol.* **22**, 1-16.
Klein, T. A., Tada, M. S., Lima, J. B. P. & Katsuragawa, T. H. 1991 Infection of *Anopheles darlingi* fed on patients infected with *Plasmodium vivax* before and during treatment with chloroquine in Costa Marques, Brazil. *Am. J. Trop. Med. Hyg.* **45**, 471-478.
Koella, J. C. 1994 Linking evolutionary ecology with epidemiology. *Rev. Sui. Zoo.* **101**, 865-874.
Koella, J. C. 1998 Costs and benefits of resistance against antimalarial drugs. *Parasitol. Today* **14**, 360-364.
Koella, J. C. & Antia, R. 1995 Optimal pattern of replication and transmission for parasites with two stages in their life cycle. *Theor. Pop. Biol.* **47**, 277-291.
Koella, J. C., Mshinda, H., Teuscher, T., Sublet, A., Betschart, B. & Tanner, M. 1990 Patterns of chloroquine usage. *Bull. Soc. Franc. Parasitol.* **8** (suppl. 1), S460.
Kozlowski, J. & Uchmanski, J. 1987 Optimal individual growth and reproduction in perennial species with indeterminate growth. *Evol. Ecol.* **1**, 214-230.
Kraaijeveld, A. R. & Godfray, H. C. J. 1997 Trade-off between parasitoid resistance and larval competition ability in *Drosophila Melanogaster. Nature, Lond.* **389**, 278-280.
Lafferty, K. D. 1993 The marine snail, *Cerithidia californica*, matures at smaller sizes when parasitism is high. *Oikos* **68**, 3-11.
Landau, I. & Boulard, Y. 1978 Life cycles and morphology. In *Rodent malaria.* (ed. R. Killick-Kendrick and W. Peters), pp. 53-84. London: Academic Press.
Landau, I. & Chabaud, A. 1994 *Plasmodium* species infecting *Thamnomys rutilans:* a zoological study. *Adv. Parasitol.* **33**, 49-90.
Langhorne, J. 1989 The role of CD4+ T-cells in the immune response of *Plasmodium chabaudi. Parasitol. Today* **5**, 362-364.
Larder, B. A. & Kemp, S. D. 1989 Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). *Science, Wash.* **246**, 1155-1158.
Leroi, A. M., Bennett, A. F. & Lenski, R. E. 1994 Temperature acclimation and competitive fitness: An experimental test of the beneficial acclimation assumption. *Proc. Natl. Acad. Sci. USA* **91**, 1917-1921.
Levin, B. R. & Bull, J. J. 1994 Short-sighted evolution and the virulence of pathogenic microbes. *Tr. Microbiol.* **2**, 76-81.
Levin, S. & Pimentel, D. 1981 Selection of intermediate rates of increase in host-parasite systems. *Am. Nat.* **117**, 308-315.
Lines, J. D., Wilkes, T. J. & Lyimo, E. O. 1991 Human malaria infectiousness measured by age-specific sporozoite rates in *Anopheles gambiae* in Tanzania. *Parasitology* **102**, 167-77.
Lingnau, A., Margos, G., Maier, W. A. & Seitz, H. M. 1993 The effects of hormones on the gametocytogenesis of *Plasmodium falciparum in vitro. Appl. Parasitol* **34**, 153-160.
Lipsitch, M. & Moxon, E. R. 1997 Virulence and transmissability of pathogens: what is the relationship? *Tr. Microbiol.* **5**, 31-36.
Mackerras, M. J. & Ercole, Q. N. 1948 Observations on the action of paludrine on malarial parasites. *Trans. R. Soc. Trop. Med. Hyg.* **41**, 365-376.

Mackerras, M. J. & Ercole, Q. N. 1949a Observations on the action of quinine, atebrin and plasmoquine on the gametocytes of *Plasmodium falciparum*. *Trans. R. Soc. Trop. Med. Hyg.* **42**, 455-463.

Mackerras, M. J. & Ercole, Q. N. 1949b Some observations on the action of quinine, atebrin and plasmoquine on *Plasmodium vivax*. *Trans. R. Soc. Trop. Med. Hyg.* **42**, 443-454.

Mackinnon, M. J. & Hastings, I. M. 1998 The evolution of multiple drug resistance in malaria parasites. *Trans. R. Soc. Trop. Med. Hyg.* **92**, 188-195.

Mackinnon, M. J. & Read, A. F. Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*. Submitted to *Evolution*

Mann, V. H., Law, M. H., Watters, D. & Saul, A. 1996 The effects of Bistratene A on the development of *Plasmodium falciparum* in culture. *Int. J. Parasitol.* **26**, 117-121.

Marwoto, H. A., Susetyono, Sulaksono, S. E., Ompusunggu, S. & Suwarni 1986 Gametocyte production in patients of *falciparum* malaria treated with Fansidar. *Bul. Penelit. Kesehat.* **14**, 5-7.

Maswoswe, S. M., Peters, W. & Warhurst, D. C. 1985 Corticosteroid stimulation of the growth of *Plasmodium falciparum* gametocytes *in vitro*. *Ann. Trop. Med. Parasitol.* **79**, 607-616.

May, R. M. & Anderson, R. 1983 Epidemiology and genetics in the coevolution of parasites and hosts. *Proc. Roy. Soc. Lond. B* **219**, 281-313.

McBride, J. S. & Heidrich, H.-G. 1987 Fragments of the polymorphic M_r 185000 glycoprotein from the surface of isolated *Plasmodium falciparum* merozoites form an antigenic complex. *Mol. Biochem. Parasitol.* **23**, 71-84.

McCarthy, V. C. & Clyde, D. F. 1973 Influence of sulfalene upon gametocytogenesis of *Plasmodium falciparum* and subsequent infection patterns in *Anopheles stephensi*. *Exp. Parasitol.* **33**, 73-78.

McGregor, I. A. & Wilson, R. J. M. 1988 Specific immunity: acquired in man. In *Malaria. Principles and practice of malariology*. (ed. W. H. Wernsdorfer and I. McGregor), pp. 559-620. Edinburgh: Churchill Livingstone.

McKenzie, F. E. & Bossert, W. H. 1998a The optimal production of gametocytes by *Plasmodium falciparum*. *J. Theor. Biol.* **193**, 419-428.

McKenzie, F. E. & Bossert, W. H. 1998b A target for intervention in *Plasmodium falciparum* infections. *Am. J. Trop. Med. Hyg.* **58**, 763-767.

McLean, A. 1986 Genetic studies on antigens of the rodent malaria parasite *Plasmodium chabaudi*. University of Edinburgh.

McLean, A. P., Lainson, F. A., Sharkey, A. M. & Walliker, D. 1991 Genetic studies on a major merozoite surface antigen of the malaria parasite of rodents, *Plasmodium chabaudi*. *Parasite immunol.* **13**, 369-378.

McLean, S. A., Pearson, C. D. & Phillips, R. S. 1982 *Plasmodium chabaudi*: antigenic variation during recrudescent parasitaemias in mice. *Exp. Parasitol* **54**, 296-304.

Mendis, K. N., Munesinghe, Y. D., de Silva, Y. N., Keragalla, I. & Carter, R. 1987 Malaria transmission-blocking immunity induced by natural infections of *Plasmodium vivax* in humans. *Infect. Immun.* **55**, 369-372.

Mercereau-Puijalon, O. 1996 Revisiting host/parasite interactions: molecular analysis of parasites collected during longitudinal and cross-sectional surveys in humans. *Par. Immun.* **18**, 173-180.

Metselaar, D. 1960 Relative increase in the prevalence of *Plasmodium falciparum* some years after the beginning of a house spraying campaign in Netherlands New Guinea. *Trans. R. Soc. Trop. Med. Hyg.* **54**, 523-528.

Miller, M. 1958 Observations on the natural history of malaria in the semi-resistant West African. *Trans. R. Soc. Trop. Med. Hyg.* **52**, 152-168.

Minchella, D. J. & LoVerde, P. T. 1981 A cost of increased early reproductive effort in the snail *Biomphilaria glabrata*. *Am. Nat.* **118**, 876-881.

Molineaux, L. & Gramiccia, G. 1980 *The Garki Project. Research on the epidemiology and control of malaria in the Sudan Savanna of West Africa.* Geneva: World Health Organisation.

Mons, B. 1985 Induction of sexual differentiation in malaria. *Parasitol. Today* **1**, 87-89.

Motard, A., Marussig, M., Renia, L., Baccam, D., Landau, I., Mattei, D., Targett, G. A. T. & Mazier, D. 1995 Immunisation with the malaria heat shock like protein hsp70-1 enhances transmission to the mosquito. *Int. Immunol.* **7**, 147-150.

Mulder, B., Tchuinkam, T., Dechering, K., Verhave, J. P., Carnevale, P., Meuwissen, J. H. & Robert, V. 1994 Malaria transmission-blocking activity in experimental infections of *Anopheles gambiae* from naturally infected *Plasmodium falciparum* gametocyte carriers. *Trans. R. Soc. Trop. Med. Hyg.* **88**, 121-125.

Nowak, M. & May, R. M. 1994 Superinfection and the evolution of parasite virulence. *Proc. Roy. Soc. B* **255**, 81-89.

Ntoumi, F., Contamin, H., Rogier, C., Bonnefoy, S., Trape, J.-F. & Mercereau-Puijalon, O. 1995 Age-dependent carriage of multiple *Plasmodium falciparum* merozoite surface antigen-2 alleles in asymptomatic malaria infections. *Am. J. Trop. Med. Hyg.* **52**, 81-88.

Ono, T., Nakai, T. & Nakabayashi, T. 1986 Induction of gametocytogenesis in *Plasmodium falciparum* by the culture supernatant of hybridoma cells producing anti-*P. falciparum* antibody. *Biken J.* **29**, 77-81.

Ono, T., Ohnishi, Y., Nagamune, K. & Kano, M. 1993 Gametocytogenesis induction by Berenil in cultured *Plasmodium falciparum. Exp. Parasitol.* **77**, 74-78.

Pant, C. P. 1988 Malaria vector control: imagociding. In *Malaria. Principles and practices of malariology.* (ed. W. Wernsdorfer and I. McGregor), pp. 1173-1212. Edinburgh: Churchill Livingstone.

Paul, R. E. L., Packer, M. J., Walmsley, M., Lagog, M., Ranford-Cartwright, L. C., Paru, R. & Day, K. P. 1995 Mating patterns in malaria parasite populations of Papua New Guinea. *Science, Wash.* **269**, 1709-1711.

Peters, W. 1987 *Chemotherapy and drug resistance in malaria.* London: Academic Press.

Phillips, R. S., Brannan, L. R., Balmer, P. & Neuville, P. 1997 Antigenic variation during malaria infection-the contribution from the murine parasite *Plasmodium chabaudi. Parasite Immunol.* **19**, 427-434.

Ponnudurai, T., Lensen, A. H., Van Gemert, G. J., Bensink, M. P., Bolmer, M. & Meuwissen, J. H. 1989 Infectivity of cultured *Plasmodium falciparum* gametocytes to mosquitoes. *Parasitology* **98**, 165-173.

Ponnudurai, T., Meuwissen, J. H. E. T., Leeuwenberg, A. D. E. M., Verhave, J. P. & Lensen, A. H. W. 1982 The production of mature gametocytes of *Plasmodium falciparum* in continuous cultures of different isolates infective to mosquitos. *Trans. Roy. Soc. Trop. Med. Hyg.* **76**, 242-250.

Ponnundurai, T., Leeuwenberg, A. D. E. M. & Meuwissen, J. H. E. T. 1981 Chloroquine sensitivity of isolates of *Plasmodium falciparum* adapted to *in vitro* culture. *Trop. Geo. Med.* **33**, 50-54.

Ramakrishnan, S. P., Young, M. D., Jeffery, G. M., Burgess, R. W. & McLendon, S. B. 1952 The effect of single and multiple doses of paludrine on *Plasmodium falciparum. Am. J. Hyg.* **55**, 239-245.

Ramkaran, A. E. & Peters, W. 1969 Infectivity of chloroquine resistant *Plasmodium berghei* to *Anopheles stephens*i enhanced by chloroquine. *Nature, Lond.* **223**, 635-636.

Read, A. F. 1994 The evolution of virulence. *Tr. Microbiol.* **2**, 73-76.

Read, A. F. & Viney, M. E. 1996 Helminth Immunogenetics: why bother. *Parasitol. Today* **12**, 337-343.

Reuner, B., Vassella, E., Yutzy, B. & Boshart, M. 1997 Cell density triggers slender to stumpy differentiation of *Trypanasoma brucei* bloodstream forms in culture. *Mol. Biochem. Parasitol.* **90**, 269-280.

Reznick, D. A., Bryga, H. & Endler, J. A. 1990 Experimentally induced life history evolution in a natural population. *Nature, Lond.* **346**, 357-359.

Rice, W. R. & Gaines, S. D. 1994 Extending nondirectional heterogeneity tests to evaluate simply ordered alternative hypotheses. *Proc. Natl. Acad. Sci. USA* **91**, 225-226.

Robert, V., Molez, J.-F. & Trape, J.-F. 1996 Gametocytes, chloroquine pressure, and the relative parasite survival advantage of resistant strains of *falciparum* malaria in West Africa. *Am. J. Trop. Med. Hyg.* **55**, 350-351.

Roberts, D. J., Craig, A. G., Berendt, A. R., Pinches, R., Nash, G., Marsh, K. & Newbold, C. I. 1992 Rapid switching to multiple antigenic and adhesive phenotypes in malaria. *Nature, Lond.* **357**, 689-692.

Roeffen, W., Mulder, B., Teelen, K., Bolmer, M., Eling, W., Targett, G. A. T., Beckers, P. J. A. & Sauerwein, R. 1996 Association between anti-Pfs48/45 and *P. falciparum* transmission-blocking activity in sera from Cameroon. *Parasite Immunol.* **18**, 103-109.

Roff, D. A. 1992 *The Evolution of Life Histories. Theory and Analysis*. New York: Chapman and Hall.

Roper, C., Richardson, W., Elhassan, I. M., Giha, H., Hviid, L., Satti, G. M. H., Theander, T. G. & Arnot, D. E. 1998 Seasonal changes in the *Plasmodium falciparum* population in individuals and their relationship to clinical malaria: a longitudinal study in a Sudanese village. *Parasitology* **116**, 501-510.

Rosario, V. E., Vaughn, J. A., Murphy, M., Harrod, V. & Coleman, R. 1988 Effect of chloroquine on the sporogonic cycle of *Plasmodium falciparum* and *Plasmodium berghei* in *Anopheline* mosquitoes. *Acta Leid.* **57**, 53-60.

Rowe, A., Obeiro, J. & Newbold, C. I. 1995 *Plasmodium falciparum* rosetting is associated with malaria severity in Kenya. *Infect. Imm.* **63**, 2323-2326.

Sadun, E. H., Hickman, R. L., Wellde, B. T., Moon, A. P. & Udeozo, I. O. K. 1966 Active and passive immunisation of chimpanzees infected with West African and Southeast Asian strains of *Plasmodium falciparum*. *Mil. Med.* **131**, 1250-1262.

Schall, J. J. 1996 Malarial parasites of lizards: diversity and ecology. *Adv. Parasitol.* **37**, 255-333.

Seed, J. R. & Sechelski, J. B. 1989 Mechanism of long slender (LS) to short stumpy (SS) transformation in the African Trypanosomes. *J. Protozool.* **36**, 572-577.

Shine, R. 1978 Propagule size and parental care: the 'safe harbour' hypothesis. *J. Theor. Biol.* **75**, 417-424.

Shute, P. G. & Maryon, M. 1951 A study of gametocytes in a West African strain of *Plasmodium falciparum*. *Trans. R. Soc. Trop. Med. Hyg.* **44**, 421-438.

Sinden, R. E. 1983 Sexual development of malarial parasites. *Adv. Parasitol.* **22**, 153-216.

Sinden, R. E. 1991 Asexual blood stages of malaria modulate gametocyte infectivity to the mosquito vector-possible implications for control strategies. *Parasitology* **2**, 191-196.

Sinden, R. E. 1997 Antimalarial transmission-blocking vaccines. *Ann. Trop. Med. Parasitol.* **91** (Suppl.), S69-S84.

Sinden, R. E., Butcher, G. A., Billker, O. & Fleck, S. L. 1996 Regulation of infectivity of *Plasmodium* to the mosquito vector. *Adv. Parasitol.* **38**, 53-117.

Smalley, M. E. 1976 *Plasmodium falciparum* gametocytogenesis *in vitro*. *Nature, Lond.* **264**, 271-272.
Smalley, M. E. & Brown, J. 1981 *Plasmodium falciparum* gametocytogenesis stimulated by lymphocytes and serum from infected Gambian children. *Trans. R. Soc. Trop. Med. Hyg.* **75**, 316-317.
Smalley, M. E., Brown, J. & Bassett, N. M. 1981 The rate of production of *Plasmodium falciparum* gametocytes during natural infections. *Trans. R. Soc. Trop. Med. Hyg.* **75**, 318-319.
Smalley, M. E. & Sinden, R. E. 1977 *Plasmodium falciparum* gametocytes: their longevity and infectivity. *Parasitology* **74**, 1-8.
Snow, R. W. & Marsh, K. 1995 Will reducing *Plasmodium falciparum* transmission alter malaria mortality among African children? *Parasitol. Today* **11**, 188-190.
Sokal, R. R. & Rohlf, F. J. 1995 *Biometry*. New York: W. H. Freeman.
Stanley, H. A., Howard, R. F. & Reese, R. T. 1985 Recognition of a M_r 56000 glycoprotein on the surface of *Plasmodium falciparum* merozoites by mouse monoclonal antibodies. *J. Immunol.* **134**, 3439-3444.
Stearns, S. C. 1992 *Evolution of Life Histories*. Oxford: Oxford University Press.
Stevenson, M. M., Lyanga, J. J. & Skamene, E. 1982 Murine malaria: genetic control of resistance to *Plasmodium chabaudi*. *Infect. Immun.* **38**, 80-88.
Strickland, G. T., Khaliq, A. A., Sarwar, M., Hassan, H., Pervez, M. & Fox, E. 1986 Effects of Fansidar on chloroquine-resistant *Plasmodium falciparum* in Pakistan. *Am. J. Trop. Med. Hyg.* **35**, 61-65.
Targett, G. A. T. 1992a Drug use and the immune response. *Parasitology* (Suppl.) **105**, S61-S70.
Targett, G. A. T. 1992b Virulence and the immune response in malaria. *Mem. Inst. Oswaldo Cruz* **87** (Suppl. 5), S137-S144.
Targett, G. A. T., Harte, P. G., Eida, S., Rogers, N. C. & Ong, C. S. L. 1990 *P. falciparum* sexual stage antigens: immunogenicity and cell-mediated responses. *Immunol. Letters* **25**, 77-82.
Taylor, L. H. 1997 Epidemiological and Evolutionary Consequences of Mixed-Genotype Infections of Malaria Parasites. University of Edinburgh.
Taylor, L. H., Mackinnon, M. J. & Read, A. F. 1998 Virulence of mixed-clone and single-clone infections of the rodent malaria *Plasmodium chabaudi*. *Evolution* **52**, 583-591.
Taylor, L. H. & Read, A. F. 1997 Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitol. Today* **13**, 135-140.
Taylor, L. H., Walliker, D. & Read, A. F. 1997 Mixed-genotype infections of the rodent malaria *Plasmodium chabaudi* are more infectious to mosquitoes than single-genotype infections. *Parasitology* **115**, 121-132.
Taylor-Robinson, A. W. 1995 Regulation of immunity to malaria: valuable lessons learned from murine models. *Parasitol. Today* **11**, 334-341.
Tchuinkam, T., Mulder, B., Dechering, K., Stoffels, H., Verhave, J. P., Cot, M., Carnevale, P., Meuwissen, J. H. & Robert, V. 1993 Experimental infections of *Anopheles gambiae* with *Plasmodium falciparum* of naturally infected gametocyte carriers in Cameroon: factors influencing the infectivity to mosquitoes. *Trop. Med. Parasitol.* **44**, 271-276.
Tin, F. & Nyunt-Hlaing 1984 Comparative drug trial of sulfadoxine/pyrimethamine and a sulfalene/pyrimethamine combination against *Plasmodium falciparum* infections in semi-immune populations of Burma. *S.E. As. J. Trop. Med. Publ. Hlth* **15**, 238-248.
Trager, W. & Gill, G. S. 1989 *Plasmodium falciparum* gametocyte formation in vitro: Its stimulation by Phorbol Diesters and by 8-Bromo Cyclic Adenosine Monophosphate. *J. Protozool.* **36**, 451-454.
Trager, W. & Jensen, J. B. 1976 Human malaria parasites in continuous culture. *Science, Wash.* **193**, 673-675.

Trape, J. F. & Rogier, C. 1996 Combating malaria morbidity and mortality by reducing transmission. *Parasitol. Today* **12**, 236-240.
Trussell, G. C. 1996 Phenotypic plasticity in an intertidal snail: the role of a common crab predator. *Evolution* **50**, 448-454.
Turner, C. M. R. 1997 Trypanosomes with multicoloured coats. *Parasitol. Today* **13**, 247-248.
van Baalen, M. & Sabelis, M. W. 1995 The dynamics of multiple infection and the evolution of virulence. *Am. Nat.* **146**, 881-910.
Walliker, D., Quakyi, I. A., Wellems, T. E., McCutchan, T. F., Szarfman, A., London, W. T., Corcoran, L. M., Burkot, T. R. & Carter, R. 1987 Genetic analysis of the human malaria parasite *Plasmodium falciparum*. *Science, Wash.* **236**, 1661-1666.
Wernsdorfer, W. H. 1994 Epidemiology of drug resistance in malaria. *Acta Trop.* **56**, 143-156.
Wery, M. 1968 Studies on the sporogony of rodent malaria parasites. *Ann. Soc. Belge. Med. Trop.* **48**, 1-138.
Westley, L. C. 1993 The effect of inflorescence bud removal on tuber production in *Helianthus tuberosus* L. (Asteraceae). *Ecology* **74**, 2136-2144.
Wiliamson, K. C. & Kaslow, D. C. 1993 Strain polymorphism of *Plasmodium falciparum* transmission-blocking target antigen Pfs230. *Mol. Biochem. Parasitol.* **62**, 125-127.
Wilkinson, R. N., Noeypatimanondh, S. & Gould, D. J. 1976 Infectivity of *falciparum* malaria patients for *Anopheline* mosquitoes before and after chloroquine treatment. *Trans. R. Soc. Trop. Med. Hyg.* **70**, 306-307.
Williams, G. C. 1966a *Adaptation and Natural Selection.* Princeton: Princeton University Press.
Williams, G. C. 1966b Natural selection, the cost of reproduction, and a refinement of Lack's priciple. *Am. Nat.* **100**, 687-690.
Wilson, K. & Grenfell, B. T. 1997 Generalised linear modelling for parasitologists. *Parasitol. Today* **13**, 33-38.
Yan, G., Severson, D. W. & Christensen, B. M. 1997 Costs and benefits of mosquito refractoriness to malaria parasites: implications for genetic variability of mosquitoes and genetic control of malaria. *Evolution* **51**, 441-450.
Yap, G. S. & Stevenson, M. M. 1992 *Plasmodium chabaudi* AS: Erythropoietic responses during infection in resistant and susceptible mice. *Exp. Parasitol.* **75**, 340-352.
Yap, G. S. & Stevenson, M. M. 1994 Blood transfusion alters the course and outcome of Plasmodium chabaudi AS infection in mice. *Infect. Immun.* **62**, 3761-3765.

APPENDIX

Publications arising from this thesis

Chapter 2 of this thesis is published - a copy of the paper is enclosed:

Buckling, A. G. J., Taylor, L. H., Carlton, J. M.-R. & Read, A. F. 1997 Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy. *Proc. R. Soc. Lond. B* **264**, 553-559.

Chapter 6 has been accepted for publication in *Parasitology*.

Chapter 3 has been accepted for publication in *International Journal for Parasitology*.

Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy

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SUMMARY

Both theory and data suggest that malaria parasites divert resources from within-host replication to the production of transmission stages (gametocytes) when conditions deteriorate. Increased investment into transmission stages should therefore follow subcurative treatment with antimalarial drugs, but relevant clinical studies necessarily lack adequate control groups. We therefore carried out controlled experiments to test this hypothesis, using a rodent malaria (*Plasmodium chabaudi*) model. Infections treated with a subcurative dose of the antimalarial chloroquine showed an earlier peak and a greater rate of gametocyte production relative to untreated controls. These alterations led to correlated changes in infectivity to mosquitoes, with the consequence that chloroquine treatment had no effect on the proportion of mosquitoes infected. Treatment of human malaria commonly does not result in complete parasite clearance. If surviving parasites produce compensatory increases in their rate of gametocyte production similar to those reported here, such treatment may have minimal effect on decreasing, and may actually increase, transmission. Importantly, if increased investment in transmission is a generalized stress response, the effect might be observed following a variety of antimalarial treatments, including other drugs and potential vaccines. Similar parasite life history counter-adaptations to intervention strategies are likely to occur in many disease-causing organisms.

1. INTRODUCTION

Strategies of resource allocation that maximize fitness can differ between benign and stressful environments. Consequently, natural selection often favours phenotypic alteration of reproductive effort in response to stress (Minchella & LoVerde 1981; Crowl & Covich 1990; Roff 1992; Stearns 1992). In disease-causing organisms, such adaptive alterations could render intervention strategies against parasitic diseases less effective than anticipated. During the course of an infection, malaria parasites (*Plasmodium* spp.) are capable of modulating the proportion of replicating parasites (asexuals) that develop into non-replicating transmission stages (gametocytes). If this modulation is stress-induced, medical interventions, such as chemotherapy, could lead to greater investment in gametocyte production, thus offsetting much of the transmission-reducing benefits of killing parasites. Here we test this idea experimentally using a rodent malaria–mouse model.

Extrapolating from metazoan life history studies, there are two reasons to expect that malaria parasites will switch investment from asexuals to gametocytes when conditions deteriorate. First, increased reproductive effort should occur following cues associated with decreased probability of future survival or reproduction. For example, enhanced egg production has been demonstrated in snails following exposure to castrating trematode infections (the 'fecundity compensation' hypothesis; Minchella & LoVerde 1981). Increased investment by malaria parasites into gametocytes should therefore occur in response to environmental cues associated with the decline in future transmission potential, such as clearance of the infection or the onset of transmission-blocking immunity (Koella & Antia 1995; Taylor & Read 1997). Second, if conditions change such that one life history stage becomes relatively more vulnerable than another, increased investment into the least vulnerable stage is predicted (the 'safe harbour' hypothesis; Shine 1978). In the Jerusalem artichoke (*Helianthus tuberosus* L.), for example, inhibition of sexual reproduction by removing flowers results in increased investment in asexual reproduction via tuber development (Westley 1993). If conditions become less favourable for asexuals relative to gametocytes (for example as stage-specific immunity develops), increased investment into transmission is again predicted.

The environmental cues that stimulate gametocyte production (gametocytogenesis) are poorly understood (Sinden 1983; Carter & Graves 1988; Alano & Carter 1990), but there is some evidence that the rate of gametocyte production increases in response to conditions unfavourable for asexual replication. *P. falciparum* (the most common and virulent human malaria parasite) produces more gametocytes *in vitro* when there is a high density of parasitized red blood cells

(Carter & Miller 1979; Brockelman 1982; Bruce *et al.* 1990). By definition, antimalarial drugs acting against blood-stage parasites impose considerable stress, and some also have a greater inhibitory effect on asexual parasites than on gametocytes. Increased gametocytogenesis following treatment that greatly reduces parasite numbers is therefore to be expected. However, here the evidence is more ambiguous.

Early *in vivo* clinical trials with human *Plasmodium* frequently reported more gametocytes following treatment with drugs inhibiting folate metabolism and hence DNA synthesis (e.g. paludrine, pyrimethamine and the sulphonamides; Findlay *et al.* 1946; Mackerras & Ercole 1948; Shute & Maryon 1951; Foy & Kondi 1952; Ramakrishnan *et al.* 1952; McCarthy & Clyde 1973). More recently, increased gametocyte production has also been reported in *P. falciparum* infections following treatment with Fansidar, a synergistic combination of pyrimethamine and sulphadoxine (Tin & Nyunt-Hlaing 1984; Marwoto *et al.* 1986). However, these increases occurred more rapidly than the 8–10 day maturation period of *P. falciparum* gametocytes (Smalley 1976; Jensen 1979), and other studies using Fansidar with longer follow-up periods do not support the hypothesis (Strickland *et al.* 1986; Hogh *et al.* 1995). With other blood-stage antimalarials, such as chloroquine or quinine, most clinical studies have found no increases in gametocytogenesis following subcurative treatment (e.g. Mackerras & Ercole 1949*a*; Jeffery *et al.* 1956; Jeffery 1958; Hogh *et al.* 1995). These data have given rise to the conventional wisdom that only drugs which inhibit DNA synthesis are capable of inducing increased gametocytogenesis (Carter & Graves 1988; Alano & Carter 1990), even though there may be exceptions (Mackerras & Ercole 1949*b*).

But all these data have a common problem: chemotherapy may have been used when increases in gametocyte production would have occurred anyway. This is particularly likely if the conditions stimulating both gametocytogenesis and symptoms (and hence treatment) approximately coincide, as would be the case if, for example, high parasite densities or host stress were a common trigger. Understandably, no studies included appropriate untreated control infections, so that the effect of subcurative antimalarial chemotherapy on gametocyte production has yet to be resolved.

If enhanced gametocytogenesis can occur following subcurative treatment, reductions in infectivity might not be nearly as great as reductions in morbidity. This may have implications for malaria control and epidemiology, particularly if increased gametocytogenesis is a generalized stress response stimulated by a variety of treatments. We therefore carried out controlled experiments using a *P. chabaudi*–mouse model to test the life history prediction of increased gametocytogenesis following subcurative chloroquine chemotherapy, and to determine any impact on subsequent infectivity to a mosquito vector. Chloroquine (CQ) was used because of its wide availability in malaria-endemic areas and the general view that its use does not result in increased gametocytogenesis (Carter & Graves 1988; Alano & Carter 1990). In addition, it only affects asexual parasites and immature gametocytes (Smalley & Sinden 1977), so that increased gametocytogenesis might be expected on account of both the fecundity compensation and safe-harbour hypotheses.

2. MATERIALS AND METHODS

(*a*) *Parasites and hosts*

Male C57/BL/6J mice (Harlan-Olac, England) aged 8–14 weeks were infected with 1×10^6 red blood cells infected with a CQ-naive *P. chabaudi* clone (either CR or ER, from the WHO Registry of Standard Malaria Parasites maintained at the University of Edinburgh, UK) in a 0.1 ml intraperitoneal inoculum of 50% Ringer's solution (27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl), 45% heat-inactivated calf serum and 5% 200 units ml^{-1} heparin solution. Mice, age-matched within experiments, were housed in cages of 2–4 animals at a temperature of 25 ± 1 °C with a 0700 to 1900 hours light cycle, and provided with 41B rat and mouse maintenance diet (Harlan-Teklad, England) and water containing 0.05% pABA, *ad libitum*.

(*b*) *CQ preparation and administration*

CQ solution was prepared from a stock of 40 mg ml^{-1} CQ sulphate (Nivaquine™) diluted in distilled water and administered orally using a lubricated catheter in approximately 0.1 ml doses of 12 mg kg^{-1} of mouse weight in all cases. Preliminary experiments revealed this dose to be subcurative. Control mice were given 0.1 ml distilled water. All treatment took place between 1600 and 1700 hours.

(*c*) *Parasite counts*

From day 4 post-infection (p.i.), daily thin blood smears from the tail vein were Giemsa-stained and asexual parasites counted per 1.5×10^3 red blood cells (RBCs). At low asexual densities, parasites were counted per 10^4 RBCs. Mature gametocytes were counted per 1.25×10^4 RBCs. Parasite densities were calculated from RBC densities, measured by flow cytometry (Coulter Electronics™) every second day, multiplied by parasites per RBC.

(*d*) *Mosquito feeds and dissections*

Mice were anaesthetized by an intramuscular injection of 0.5 ml per 20 g mice 3:2:1 distilled water: Vetalar™: Rompun™, and placed onto pots covered with nylon mesh containing about 40 4–5 day-old female *Anopheles stephensi*, which had been starved for the previous 24 h. Mosquitoes

Table 1. *Details of experiments*

experiment	clone used	day p.i. of CQ treatment	no. of mice infected: CQ	no. of mice infected: control
1	CR	6	6	5
2	CR	5	6	6
3	CR	4	5	
		5	6	6
		6	5	
4	ER	5	6	6

p.i., post-infection; CQ, chloroquine.

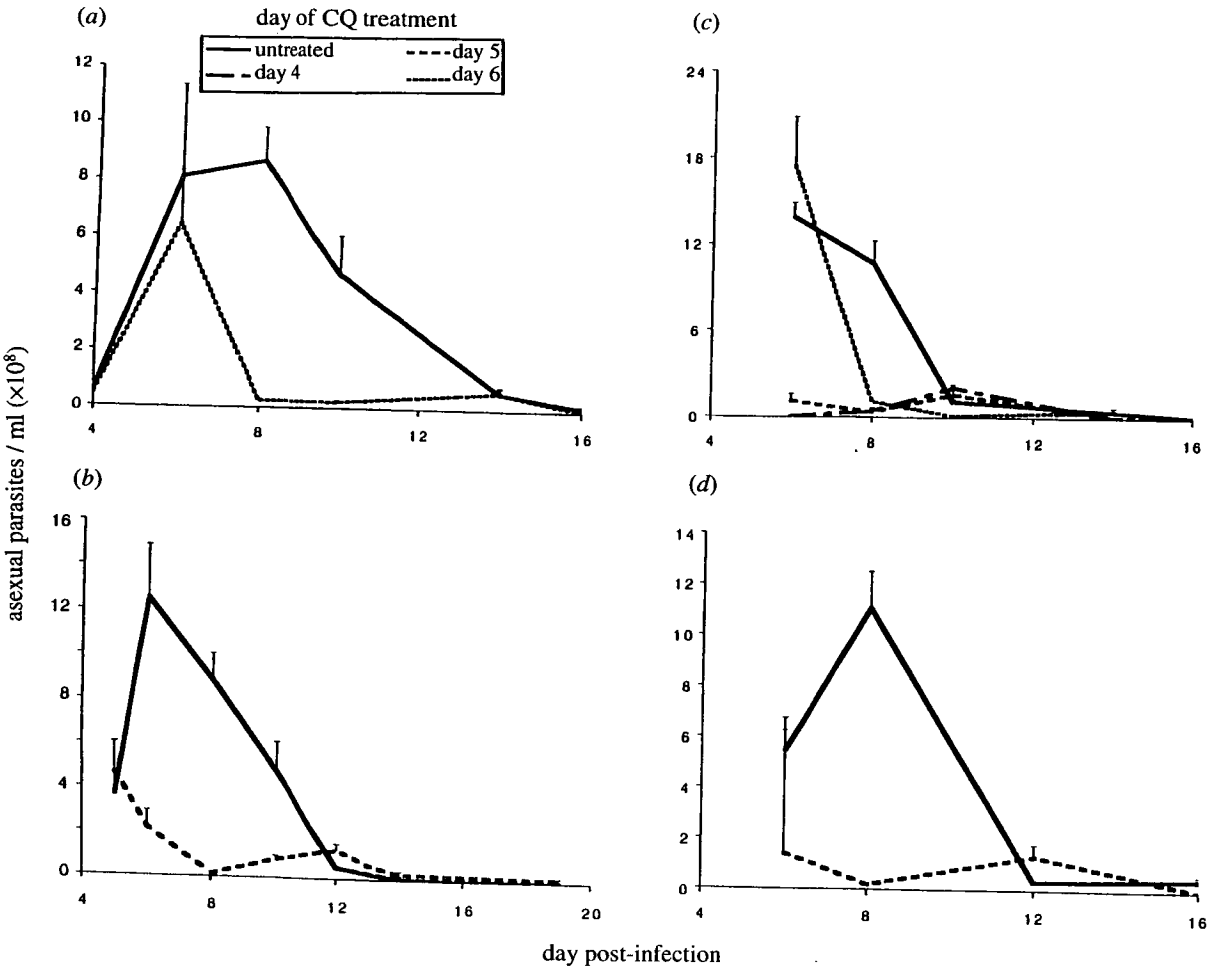

Figure 1. Mean asexual parasite densities (+1 s.e.) during infections for experiments 1–4 (*a–d* respectively). CQ, chloroquine.

were left to feed in the dark for 30 min, between 1845 and 1945 hours. They were subsequently maintained at 25–30 °C, 70–80% humidity, with a 12-h light cycle, and provided with 5% glucose, 0.05% pABA solution *ad libitum*. After 8–9 d, approximately 25 mosquitoes per mouse were dissected to determine the presence of oocysts on midguts.

(*e*) *Experiments*

Details of individual experiments are shown in table 1. In experiments 1, 2 and 4, infectivity was assayed by exposing half the mice in each experimental group to mosquitoes on day 12 p.i., and the other half on day 14 p.i. Preliminary experiments and other data (Taylor *et al.* 1997) showed that infectivity peaks during this period.

(*f*) *Statistical analysis*

Comparisons of control and CQ-treated infections were made using univariate analyses of the following summary measures for each infection:

(1) *Total asexuals.* This was estimated from parasite densities between days 8 and 16 p.i. Parasite densities prior to day 8 were not used because it was unclear whether parasites in the CQ-treated groups were dead or alive at the time of the smear, and by day 18 p.i. parasite densities were at very low levels. Estimates of total parasites between these days were obtained by integrating under the parasite density through time curves for each infection. This is a reasonably accurate measure of total numbers of asexuals (between days 8 and 16 p.i.) because the asexual cycle of *P. chabaudi* is known to be 24 h (reviewed in Cox 1988).

(2) *Total gametocytes.* Gametocytes of *P. chabaudi* take approximately 2 d to mature (A. G. J. Buckling *et al.*, unpublished data; Gautret *et al.* 1997). An index of the total number of gametocytes produced by asexuals between eight and 16 days p.i. was therefore obtained by integrating under the gametocyte density through time curves between days 10 and 18 p.i. This measure is not a direct estimate of total gametocyte numbers because gametocyte longevity is uncertain, though the data presented below suggest few survive longer than 24 h. However, the sum of the gametocyte densities on each day is likely to be well correlated with total transmission probability: gametocyte densities at any point in time correlate with both the proportion of mosquitoes infected and oocyst densities within mosquitoes (Taylor & Read 1997).

(3) *Index of gametocytogenesis (IG).* This was calculated as the ratio of total gametocytes (2) to total parasites [(1)+(2)] for each mouse.

(4) *Day of peak gametocyte density.*

(5) *The proportion of mosquitoes infected.*

Prior to analysis, estimates of total asexuals and total gametocytes were $\log_{10}$-transformed and indices of gametocytogenesis (IG) square-root arcsin-transformed. All analyses were carried out using generalized linear models (GLIM; Crawley 1993). Starting with the highest order interactions, all factors (experiment, treatment (CQ+, CQ−) and, where relevant, day of CQ treatment or day of mosquito feed) and their interactions were individually removed in turn from the

maximal model. Non-significant factors were removed and test statistics obtained from the resulting minimal model. A binomial error structure, applying Williams's correction for overdispersion, was used for the logistic regression of infection probability (Crawley 1993).

3. RESULTS

Total asexuals and total gametocytes did not differ between experiments ($F_{3,52} = 0.67$, $p = 0.58$; $F_{3,52} = 0.44$, $p = 0.73$, respectively). CQ treatment, however, had a significant effect, reducing asexuals to 20% of controls and gametocytes to 50% (figure 1, $F_{1,52} = 114.40$, $p < 0.0001$; figure 2, $F_{1,52} = 7.10$, $p = 0.01$, respectively). The effect of CQ did not differ between experiments (treatment by experiment interactions: $F_{3,49} = 1.26$, $p = 0.3$; $F_{3,49} = 1.87$, $p = 0.15$, asexuals and gametocytes, respectively).

The index of gametocytogenesis (IG) was about 2.5 times greater in CQ-treated infections than in control infections ($F_{1,52} = 16.00$, $p < 0.0001$). There were no differences between experiments, nor did treatment effects differ between experiments ($F_{3,52} = 0.76$, $p = 0.52$; $F_{3,49} = 1.54$, $p = 0.22$, respectively). Figure 3 shows the relationship between total gametocytes and total asexuals for all infections.

Peak gametocyte densities occurred approximately 2 d earlier in CQ-treated infections than in control infections for the experiments using CR (experiments 1–3: $F_{1,39} = 54.86$, $p < 0.0001$; figures 2*a–c*) and 3 d earlier in the experiment using ER (experiment 4: $F_{1,10} = 19.89$, $p = 0.001$; figure 2*d*). For experiments 1–3, the effect of treatment on timing of peak gametocyte densities did not differ between experiments (treatment by experiment interaction: $F_{2,39} =$

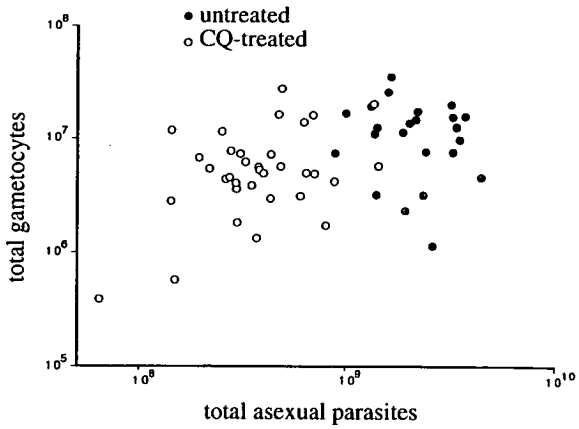

Figure 3. Total asexuals and total gametocytes (between days 8 and 16, and 10 and 18 p.i., respectively) for chloroquine-treated and untreated infections in experiments 1–4. Mean (±s.e) index of gametocytogenesis (IG): CQ-treated = 0.0180±0.0026; control = 0.0066±0.0012.

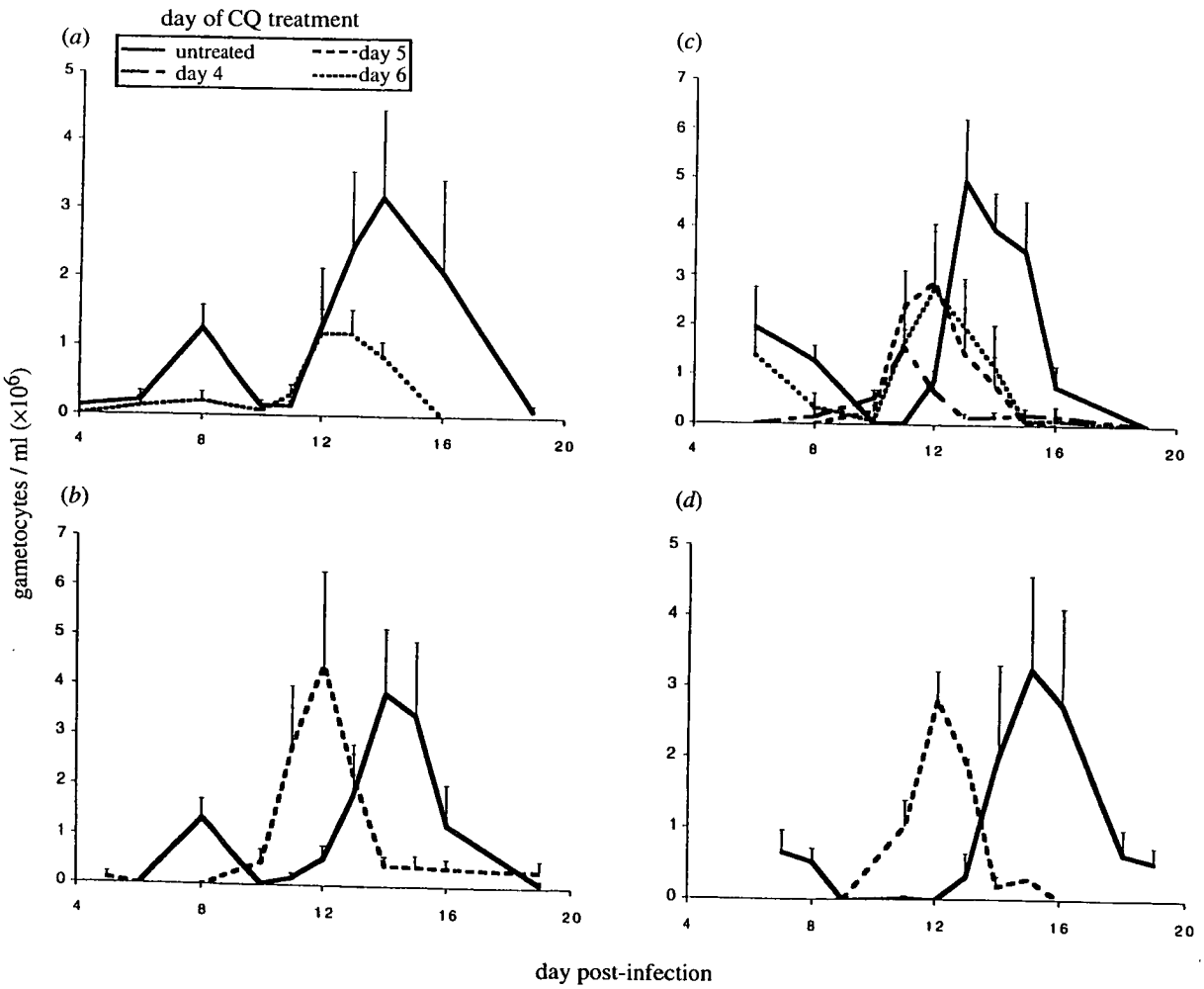

Figure 2. Mean gametocyte densities (+1 s.e.) during infections for experiments 1–4 (*a–d* respectively). CQ, chloroquine.

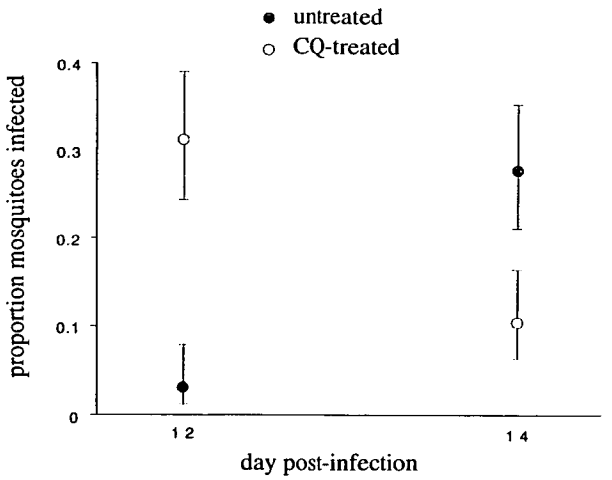

Figure 4. Mean proportions of mosquitoes infected (±1 s.e.) for chloroquine-treated and untreated mice on days 12 and 14 post-infection.

0.15, $p = 0.86$), but the timing of peak gametocyte densities differed between experiments ($F_{2,39} = 4.46$, $p = 0.02$). This was probably due to differences in timing of CQ treatment between experiments; there was no significant difference between experiments when CQ-treated groups were separated on the basis of specific day of treatment ($p > 0.25$ for controls and days 5 and 6 p.i. CQ treatment). Interestingly, there was a significant positive relationship between the day of CQ treatment and the day of peak gametocyte density for days 4, 5 and 6 p.i. CQ-treated groups in experiments 1–3 (ordered heterogeneity test (Rice & Gaines 1994): $r_s P_c = 0.96, p < 0.01$).

There was no difference in total gametocytes between days 4, 5 and 6 p.i. CQ-treated groups in experiment 3 ($F_{2,13} = 0.20$, $p = 0.83$). IG, however, significantly differed between the groups ($F_{2,13} = 4.38$, $p = 0.035$); pairwise comparisons revealed the IG to be greater in day 6 p.i. CQ-treated infections than in those treated on day 4 p.i., but there were no other significant differences (Scheffe analysis $p = 0.04$, $p > 0.2$ for both other comparisons).

Proportions of mosquitoes infected were greater in CQ-treated mice than control mice on day 12 p.i. and vice versa on day 14 p.i. (figure 4; day by treatment interaction: $\chi^2_1 = 15.74$, $p < 0.001$). There were no significant differences in overall proportion of mosquitoes infected between experiments, day and treatment, and neither treatment nor day effects, and their interaction, differed between experiments ($p > 0.2$ in all cases).

4. DISCUSSION

Infections treated with CQ produced significantly fewer asexuals and gametocytes than untreated infections, but for a given number of asexuals, the number of gametocytes was about 2.5 times greater in CQ-treated infections. This is most likely the result of increased gametocytogenesis following subcurative CQ treatment, consistent with our hypothesis.

Changes in the relative mortality rates of gametocytes and asexuals cannot adequately explain the results. First, stage-specific mortality caused by CQ cannot be involved. The half-life of whole blood CQ-concentration in mice heavily infected with *P. chabaudi* (21–25% parasitized RBCs) is in the order of 7 h (Cambie *et al.* 1994). Parasite numbers were first assayed at least 2 d after treatment, when the CQ level must have been less than 0.5 mg kg^{-1}; preliminary experiments revealed twice this concentration to have no noticeable effect on parasite numbers or infection dynamics. Moreover, virtually all the gametocytes counted must have been produced from post-treatment asexuals: any long-lived gametocytes present during the period of CQ activity would have made up only a tiny fraction of the gametocytes used to estimate subsequent gametocytogenesis because they were at such low densities when infections were drug-treated (figure 2). Second, differential mortality resulting from differences in immune response between the groups not only requires a gametocyte-specific clearance mechanism, for which there is currently no evidence (Taylor & Read 1997), but also one that is suppressed by CQ.

The best explanation for the relative increase in numbers of gametocytes following CQ treatment is therefore increased gametocytogenesis, as predicted by both the safe-harbour and the fecundity compensation hypotheses. Nevertheless, the delay between CQ treatment (hence peak asexual density) and gametocyte production (figures 1 and 2) is not entirely consistent with these adaptive hypotheses. Increased gametocytogenesis in response to stress might be expected to be immediate, resulting in mature gametocytes 2–3 days later. Instead, we observed a delay of about a week. However, it is striking that in control infections there is also a delay of approximately a week between peak asexual density and peak gametocyte density. An intriguing possibility, consistent with the hypotheses, is that a sudden drop in parasite numbers might be acting as the cue for delayed gametocytogenesis in both CQ-treated and control mice. In untreated infections, such a strategy would have the consequence of minimizing production of gametocytes during crisis, an immune-mediated response that down-regulates parasite numbers (Jarra & Brown 1989) and during which gametocyte infectivity is greatly suppressed (Wery 1968). It is feasible that CQ treatment results in an artificially early occurrence of the same cue that stimulates this crisis-avoiding delay.

Gametocytogenesis frequently follows a substantial reduction in overall parasite numbers (Sinden 1983; Carter & Graves 1988; Alano & Carter 1990; Sinden *et al.* 1996; figures 1 and 2), suggesting a positive relationship between gametocytogenesis and factors that correlate with parasite destruction. Such factors might explain how gametocytogenesis was triggered in control and CQ-treated infections despite radically different parasite dynamics (figures 1 and 2) and host condition in the two groups. Increased relative gametocytogenesis following CQ treatment might be explained by the resulting short-term very high rate of parasite destruction. This relationship would also explain why gametocytogenesis was greater in mice treated with CQ on day 6 p.i. compared with day 4 p.i.

The later treatment inevitably resulted in greater parasite death, both because by that stage there were more for CQ to kill (figure 1*c*), and because of enhanced immune activity as a result of greater exposure, in terms of both time and numbers, to parasite antigens.

Whatever the mechanism involved, these results clearly demonstrate increased gametocytogenesis following subcurative CQ treatment. As far as we are aware, this is the first fully controlled demonstration that subcurative treatment with an antimalarial drug can increase gametocytogenesis in *Plasmodium*, and thus alter patterns of infectiousness. The dose of CQ used in this study is equivalent to half the commonly recommended dose for treatment of people with *P. falciparum* (25 mg kg^{-1} over 3 d; Desjardins *et al.* 1988). Because treatment often terminates following clinical improvement, and drug-resistant parasites are common, subcurative treatment frequently occurs (Wernsdorfer 1994). The results are therefore potentially relevant to the treatment of *Plasmodium* with CQ in the field. First, even large reductions in the numbers of asexual parasites may have a much less dramatic effect on infectivity because of compensatory investment into gametocytes. It is notable that in our transmission experiments, the proportion of mosquitoes infected was similar from treated and untreated infections (figure 4). Second, transmission between hosts might actually be faster where subcurative treatment is common, because of the earlier timing of peak infectivity (figure 4). Transmission may be further enhanced by the general reduction in host immunity because of decreased exposure to the parasite (Graves *et al.* 1988) and by the increased infectiousness of gametocytes apparently induced by very low level CQ treatment (Ramkaran & Peters 1969; Wilkinson *et al.* 1976; Ichimori *et al.* 1990). Finally, if the effect is a generalized stress response, it might be observed following treatment with most blood-stage antimalarials and even potential vaccines.

Here we have only considered phenotypic modifications of gametocytogenesis. Consistent differences in gametocyte production between isolates of *P. falciparum in vitro* (Burkot *et al.* 1984; Graves *et al.* 1984) imply natural genetic variation in gametocytogenesis on which drug-imposed selection could act to generate long-term changes in resource allocation between asexuals and gametocytes. Parasites will probably increase their fitness under drug pressure by producing gametocytes in greater quantities earlier in the infections. Lines *et al.* (1991) suggested that just such adaptation may underlie increases in malaria transmission in Tanzania following long-term CQ use. This is supported by a recent study in Sri Lanka that showed that infections of CQ-resistant parasites are more likely to be gametocyte-positive than infections of sensitive parasites (Handunnetti *et al.* 1996); under drug pressure, adaptive life history changes are likely to evolve in parallel with the development of drug resistance. Both short- and long-term parasite life history changes in response to intervention strategies are to be similarly expected in many disease-causing organisms.

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REFERENCES

Alano, P. & Carter, R. 1990 Sexual differentiation in malaria parasites. *A. Rev. Microbiol.* **44**, 429–449.

Brockelman, C. R. 1982 Conditions favouring gametocytogenesis in the continuous culture of *Plasmodium falciparum*. *J. Protozool.* **29**, 454–458.

Bruce, M. C., Alano, P. & Carter, R. 1990 Commitment of the malaria parasite *Plasmodium falciparum* to sexual and asexual development. *Parasitology* **100**, 191–200.

Burkot, T., Williams, J. L. & Schneider, I. 1984 Infectivity to mosquitoes of *Plasmodium falciparum* clones grown *in vitro* from the same isolate. *Trans. R. Soc. Trop. Med. Hyg.* **78**, 339–341.

Cambie, G., Verdier, F., Gaudebout, C., Clavier, F. & Ginsburg, H. 1994 The pharmacokinetics of chloroquine in healthy and *Plasmodium chabaudi*-infected mice: implications for chronotherapy. *Parasite* **1**, 219–226.

Carter, R. & Graves, P. M. 1988 Gametocytes. In *Malaria. Principles and practice of malariology* (ed. W. H. Wernsdorfer & I. McGregor), pp. 253–306. Edinburgh: Churchill Livingstone.

Carter, R. & Miller, L. H. 1979 Evidence for environmental modulation of gametocytogenesis in *Plasmodium falciparum* in continuous culture. *Bull. Wld Hlth Org.* **57** (Suppl. 1), 37–52.

Cox, F. E. G. 1988 Major animal models in malaria research: rodent. In *Malaria. Principles and practice of malariology* (ed. W. H. Wernsdorfer & I. McGregor), pp. 1503–1543. Edinburgh: Churchill Livingstone.

Crawley, M. 1993 *GLIM for ecologists*. Oxford: Blackwell Scientific.

Crowl, T. A. & Covich, A. P. 1990 Predator-induced life-history variation in a freshwater snail. *Science, Wash.* **247**, 949–951.

Desjardins, R. E., Doberstyn, E. B. & Wernsdorfer, W. H. 1988 The treatment and prophylaxis of malaria. In *Malaria. Principles and practice of malariology* (ed. W. H. Wernsdorfer & I. McGregor), pp. 827–864. Edinburgh: Churchill Livingstone.

Findlay, G. M., Maegraith, B. G. & Holden, J. R. 1946 Investigations in the chemotherapy of malaria in West Africa. V. Sulphonamide compounds. *Ann. Trop. Med. Parasitol.* **40**, 358–367.

Foy, H. & Kondi, A. 1952 Effect of daraprim on the gametocytes of *Plasmodium falciparum*. *Trans. R. Soc. Trop. Med. Hyg.* **46**, 370–71.

Gautret, P., Miltgen, F., Gantier, J.-C., Chabaud, A. G. & Landau, I. 1997 Enhanced gametocyte formation by *Plasmodium chabaudi* in immature erythrocytes: patterns of production, sequestration and infectivity to mosquitoes. *J. Parasitol.* (In the press.)

Graves, P. M., Burkot, T. R., Carter, R. *et al.* 1988 Measurement of malarial infectivity of human populations in the Madang area, Papua New Guinea. *Parasitology* **96**, 251–263.

Graves, P. M., Carter, R. & McNeill, K. M. 1984 Gametocyte production in cloned lines of *Plasmodium falciparum*. *Am. J. Trop. Med. Hyg.* **33**, 1045–1050.

Handunnetti, S. M., Gunewardena, D. M., Pathirana, P. P. S. L., Ekanayake, K., Weerasinghe, S. & Mendis,

K. N. 1996 Features of recrudescent chloroquine-resistant *Plasmodium falciparum* infections confer a survival advantage on parasites and have implications for disease control. *Trans. R. Soc. Trop. Med. Hyg.* **90**, 563–567.

Hogh, B., Thompson, R., Hetzel, C. *et al.* 1995 Specific and non-specific responses to *Plasmodium falciparum* blood-stage parasites and observations on the gametocytaemia in schoolchildren living in a malaria-endemic area of Mozambique. *Am. J. Trop. Med. Hyg.* **52**, 50–59.

Ichimori, K., Curtis, C. F. & Targett, G. A. T. 1990 The effects of chloroquine on the infectivity of chloroquine-sensitive and -resistant populations of *Plasmodium yoelii nigeriensis* to mosquitoes. *Parasitology* **100**, 377–381.

Jarra, W. & Brown, K. N. 1989 Protective immunity to malaria: studies with cloned lines of rodent malaria in CBA/Ca mice. IV. The specificity of mechanisms resulting in crisis and resolution of the primary acute phase parasitaemia of *Plasmodium chabaudi chabaudi* and *P. yoelii yoelii*. *Parasite Immunol.* **11**, 1–13.

Jeffery, G. M. 1958 Infectivity to mosquitoes of *Plasmodium vivax* following treatment with chloroquine and other antimalarials. *Am. J. Trop. Med. Hyg.* **7**, 207–211.

Jeffery, G. M., Young, M. D. & Eyles, D. E. 1956 The treatment of *Plasmodium falciparum* infection with chloroquine, with a note on infectivity to mosquitoes of primaquine- and pyrimethamine-treated cases. *Am. J. Hyg.* **64**, 1–11.

Jensen, J. B. 1979 Observations on gametogenesis of *Plasmodium falciparum* in continuous culture. *J. Protozool.* **26**, 129–132.

Koella, J. C. & Antia, R. 1995 Optimal pattern of replication and transmission for parasites with two stages in their life cycle. *Theor. Pop. Biol.* **47**, 277–291.

Lines, J. D., Wilkes, T. J. & Lyimo, E. O. 1991 Human malaria infectiousness measured by age-specific sporozoite rates in *Anopheles gambiae* in Tanzania. *Parasitology* **102**, 167–177.

Mackerras, M. J. &. Ercole, Q. N. 1948 Observations on the action of paludrine on malarial parasites. *Trans. R. Soc. Trop. Med. Hyg.* **41**, 365–376.

Mackerras, M. J. & Ercole, Q. N. 1949*a* Observations on the action of quinine, atebrin and plasmoquine on the gametocytes of *Plasmodium falciparum*. *Trans. R. Soc. Trop. Med. Hyg.* **42**, 455–463.

Mackerras, M. J. & Ercole, Q. N. 1949*b* Some observations on the action of quinine, atebrin and plasmoquine on *Plasmodium vivax*. *Trans. R. Soc. Trop. Med. Hyg.* **42**, 443–454.

Marwoto, H. A., Susetyono, Sulaksono, S. E., Ompusunggu, S. & Suwarni 1986 Gametocyte production in patients of *falciparum* malaria treated with Fansidar. *Bul. Penelit. Kesehat.* **14**, 5–7.

McCarthy, V. C. & Clyde, D. F. 1973 Influence of sulfalene upon gametocytogenesis of *Plasmodium falciparum* and subsequent infection patterns in *Anopheles stephensi*. *Exp. Parasitol.* **33**, 73–78.

Minchella, D. J. & LoVerde, P. T. 1981 A cost of increased early reproductive effort in the snail *Biomphilaria glabrata*. *Am. Nat.* **118**, 876–881.

Ramakrishnan, S. P., Young, M. D., Jeffery, G. M., Burgess, R. W. & McLendon, S. B. 1952 The effect of single and multiple doses of paludrine on *Plasmodium falciparum*. *Am. J. Hyg.* **55**, 239–245.

Ramkaran, A. E. & Peters, W. 1969 Infectivity of chloroquine-resistant *Plasmodium berghei* to *Anopheles stephensi* enhanced by chloroquine. *Nature, Lond.* **223**, 635–636.

Rice, W. R. & Gaines, S. D. 1994 Extending non-directional heterogeneity tests to evaluate simply ordered alternative hypotheses. *Proc. Natn. Acad. Sci. USA* **91**, 225–226.

Roff, D. A. 1992 *The evolution of life histories. Theory and analysis.* New York: Chapman & Hall.

Shine, R. 1978 Propagule size and parental care: the 'safe harbor' hypothesis. *J. Theor. Biol.* **75**, 417–424.

Shute, P. G. & Maryon, M. 1951 A study of gametocytes in a West African strain of *Plasmodium falciparum*. *Trans. R. Soc. Trop. Med. Hyg.* **44**, 421–438.

Sinden, R. E. 1983 Sexual development of malarial parasites. *Adv. Parasitol.* **22**, 153–216.

Sinden, R. E., Butcher, G. A., Billker, O. & Fleck, S. L. 1996 Regulation of infectivity of *Plasmodium* to the mosquito vector. *Adv. Parasitol.* **38**, 53–117.

Smalley, M. E. 1976 *Plasmodium falciparum* gametocytogenesis *in vitro*. *Nature, Lond.* **264**, 271–272.

Smalley, M. E. & Sinden, R. E. 1977 *Plasmodium falciparum* gametocytes: their longevity and infectivity. *Parasitology* **74**, 1–8.

Stearns, S. C. 1992 *Evolution of life histories.* Oxford University Press.

Strickland, G. T., Khaliq, A. A., Sarwar, M., Hassan, H., Pervez, M. & Fox, E. 1986 Effects of Fansidar on chloroquine-resistant *Plasmodium falciparum* in Pakistan. *Am. J. Trop. Med. Hyg.* **35**, 61–65.

Taylor, L. H. & Read, A. F. 1997 Why so few transmission stages? Reproductive restraint by malaria parasites. *Parasitol. Today.* (In the press.)

Taylor, L. H., Walliker, D. & Read, A. F. 1997 Mixed-genotype infections of the rodent malaria *Plasmodium chabaudi* are more infectious to mosquitoes than single-genotype infections. *Parasitology.* (In the press.)

Tin, F. & Nyunt-Hlaing 1984 Comparative drug trial of sulfadoxine/pyrimethamine and a sulfalene/pyrimethamine combination against *Plasmodium falciparum* infections in semi-immune populations of Burma. *S.E. As. J. Trop. Med. Pub. Hlth* **15**, 238–248.

Wernsdorfer, W. H. 1994 Epidemiology of drug resistance in malaria. *Acta Trop.* **56**, 143–156.

Wery, M. 1968 Studies on the sporogony of rodent malaria parasites. *Ann. Soc. Belge. Med. Trop.* **48**, 1–138.

Westley, L. C. 1993 The effect of inflorescence bud removal on tuber production in *Helianthus tuberosus* L. (*Asteraceae*). *Ecology* **74**, 2136–2144.

Wilkinson, R. N., Noeypatimanondh, S. & Gould, D. J. 1976 Infectivity of *falciparum* malaria patients for Anopheline mosquitoes before and after chloroquine treatment. *Trans. R. Soc. Trop. Med. Hyg.* **70**, 306–307.

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