Evolutionary Genetics of Malaria Parasites

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Declaration

I declare that the research described in this thesis is my own work, and that the thesis is my own composition

Contents

List of Tables

List of Figures

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Abstract

In evolutionary terms, malaria parasites are successful. They have infected human and other hosts since pre-history, and their populations are robust in the face of massive control programmes. Even in endemic areas, where host immune systems are continually challenged by the parasite, they cause significant morbidity and mortality. Why is it that malaria parasites still cause such problems? A popular answer to this question is that these parasites adapt rapidly to the prevailing environment imposed by their host and vector. This implies that genetic variation in the parasite is the key to their success. An alternative explanation is that persistence and variability in disease are consequences of the complex epidemiological interplay between an inefficient host immune response, host genetic variation, parasite reproductive capacity and vector density. This alternative assumes that parasite genetic variation is relatively unimportant. In this thesis, these alternatives are evaluated by examining various aspects of the evolutionary forces, consequences and constraints which govern the parasite population.

Two empirical studies on parasite virulence using the laboratory model of the rodent malaria parasite, *Plasmodium chabaudi*, in inbred mice, were conducted. In the first, the average virulence (as measured by anaemia and weight loss in the host), and transmissibility (measured by gametocyte density and infection rates in mosquitoes) in eight parasite clones obtained from the wild were measured over four replicate experiments. Large amounts of genetic (between-clone) variation in virulence and transmissibility were observed. Virulence was strongly correlated, both phenotypically and genetically, to population growth rate (parasitaemia) and less strongly correlated to transmissibility. These results provide support for the basic tenet of most evolutionary models of parasite virulence, namely, that virulence is a by-product of the parasite's need to replicate fast in order to be transmitted. In the second study, between-host selection for high and low virulence was performed within two parasite clones. Over eleven generations, all the selection lines increased in virulence and transmissibility. Thus, despite artificial between-host selection, parasite variation within clones

coupled with inadvertent within-host selection allowed the parasite to adapt to a novel host. Together, these studies show that virulence is strongly determined by parasite genetics.

A large data set on disease severity from a 3-year longitudinal field study in Sri Lanka was analysed for the effects of host genetic variability, age, immune experience, parasite species (*P. falciparum* vs. *P. vivax*), bednet use, sex and other host or environmental factors. After accounting for all other known effects, 20% of the remaining variation in disease severity could be accounted for by repeatable differences between individual hosts, and typically less than 5% was attributable to host genetics. On the other hand, there was a moderate amount of genetic variation between hosts in the number of times they became clinically ill during the fixed study period. Increases in sickness with age were partly attributable to longer times between infections, but there was no correlation between severity of symptoms and the number of previous infections in the lifetime. People sleeping under bednets rapidly lost clinical immunity and so suffered stronger symptoms when they did become infected. In general, the results highlighted the importance of short-term acquired immunity in causing large between-host variation in virulence. This is consistent with the view that parasite variability is responsible for inefficient clinical immunity to malarial disease, although this could not be examined directly and alternative explanations are possible.

Two theoretical models incorporating parasite population structure into population genetics were used to predict the probability and rate of evolution of multi-locus drug resistance. These studies show that, in addition to the recognised need to minimise drug pressure, reducing transmission rate both in the whole population and from individual drug-treated hosts will, in the large majority of cases, help retard the emergence and spread of multiple drug resistance. This exercise illustrated that population structure, through a combination of sexual recombination and structured selection pressure, can have a profound effect on the rate of parasite evolution.

All of these studies suggest that parasite genetic variation plays a key role in the evolution of the malaria host-parasite association.

Chapter 1

Introduction

1.1 Introduction

In host-parasite relationships, both parties engage in the process of finding a way to co-exist. This does not always work amicably: by definition, parasites are those symbionts which go so far as to harm their hosts. Parasites are not benign mutualists because this is not in their own interest. On the other side, hosts harm their parasites: this is also for their own sake, and is not without cost. The settling of the matter depends on the armament of genes on each side. Both organisms genetically adapt to maximise their own fitness under the prevailing selective forces imposed by parasite on host, and host on parasite. This is the modern paradigm for parasite evolution (Dawkins and Krebs, 1979; Bremermann, 1980; May and Anderson, 1983). The work described in this thesis addresses the question of whether this paradigm fits one of the most successful parasites of man - malaria. The emphasis is on genetic adaptation within the parasite rather than the host.

It is known that malaria parasites (*Plasmodium* spp.) are extraordinarily variable at the genetic and molecular level (Kemp et al., 1990). From this, it is tempting to assume that genetic variability is the key to the parasite's success because it would, in theory, enable the population to adapt rapidly under selection pressure from host immune systems or from changing environmental conditions. Variability in malaria parasites has been used to explain why it takes many exposures for people to develop effective immunity, and why people vary so much in the expression of disease symptoms, which range from mild to very severe (Day and Marsh, 1991; Mendis et al., 1991; Day et al., 1992; Marsh, 1992; Gupta et al., 1994a,b, Gupta and Day, 1994a). Three lines of evidence, reviewed later, support the view that parasite variation could be responsible for these observations. A second hypothesis is that parasite genetic variability is irrelevant to the host's immune response because immunity is against conserved molecules of the parasite, and that immunity takes a long time to build up because the parasite is weakly

immunogenic. Under this hypothesis, variation in disease severity can be attributed to variation between hosts in exposure to the parasite due to ecological factors, and hence variation in levels of clinical immunity. A third hypothesis is that the ability to reinfect and cause disease in semi-immune hosts is largely due to host variability (Marsh, 1992). Such variability could arise from genetic differences in the host's ability to mount an immune response (Hill et al., 1991; Luzzi et al., 1991; Hill et al., 1992, 1994) or to limit the growth rate of the parasite (Pasvol et al., 1977, 1978), or non-genetic differences in factors such as nutrition and behaviour (Marsh, 1992). Under these latter hypotheses, parasite variation is assumed to be unimportant to persistence and virulence of the disease and therefore not a threat to control programmes which impose selection on the parasite population. A final view is that both host and parasite variability are important in determining the dynamics and variability in malaria epidemiology. These host and parasite factors may or may not interact strongly. While the above hypotheses are not mutually exclusive, it is helpful to delineate the rôles of parasite and host genetic variation and environmental variation in understanding the variability in malarial disease observed in the field.

As will be become evident from the review below, the argument that variation is the key to the malaria parasite's success, even though plausible, is somewhat insufficient because the evidence is either indirect or retrospective. An alternative approach, and one which has recently become popular in infectious disease research, is that offered by evolutionary theory: it is believed that an understanding of how parasites evolve to become virulent may lead to better strategies for disease control (Ewald, 1980, 1983, 1994; Read et al., in press). By paying closer attention to the processes of evolution governing the malaria parasite population - namely, the sources of new variation, the manner and intensity of natural selection, and the constraints on achieving evolutionary change - it may be possible to *determine whether parasite variation is important in controlling malaria.*

If direct empirical evidence showed that parasite variation was a key factor in maintaining its current success, the benefits would be both practical and

fundamental. First, malaria control strategies might focus on this variability by using diverse drugs and vaccine components. Alternatively, the parasite's mechanisms for generating heritable diversity might themselves be targeted. Theoretical studies show that variability in parasite fitness traits can have important consequences to the control of the disease using transmission-reduction measures (Gupta et al., 1994a,b,c), or vaccines (McLean, 1995; Gupta et al., 1997). Second, by establishing that the malaria parasite is undergoing a continual process of adaptation to the host, some much-needed empirical support would be provided to underpin the large and expanding body of theory on evolution and co-evolution of parasites in general (reviewed by Bull, 1994; Read, 1994; Frank, 1996). No doubt the classic status and economic importance of the malaria parasite would strengthen the apparent value of such evidence.

Before proceeding to the work in this thesis, the foundation for the hypothesis that parasite variation is important in malaria is discussed in the following way. After establishing that the malaria parasite is 'successful', the following questions are addressed. First, "Does the malaria parasite possess the general requirements for rapid adaptation?". Second, "What is the evidence that it does adapt in the short-term?". Third, "What are the constraints on its rate of evolution?". Particular emphasis is given to the role of natural selection because, of the two requirements for rapid adaptation - renewable variation and selection - this is the component about which least is known.

1.2 Review of malaria's adaptability

In this review, malaria parasites are treated generally. Most of the data are derived from the major species of man, (*P. falciparum* and *P. vivax*), rodents (*P. chabaudi* and *P. berghei*) and monkeys (*P. knowlesi* and *P. cynomolgi*) but specific details are not given because the argument is general. More detailed reviews are given at the beginning of each chapter.

1.2.1 Malaria parasites are successful

Plasmodium species are very successful parasites: they have been associated with human populations since pre-history (Bruce-Chwatt, 1965), and over 120

species infect a wide range of other vertebrate hosts, including reptiles and birds (Bruce-Chwatt, 1985). They seem to have co-evolved with their hosts for a very long time. Many species are virulent, i.e. they cause significant damage to their hosts, particularly the major species of man, *P. falciparum* and *P. vivax*, which together are responsible for high mortality and morbidity rates, thus creating a major world health problem. Massive efforts have been made to eradicate or control the disease through vector control programmes, use of drugs, and by limiting exposure, but the parasite has managed to persist in many cases where the campaigns have either not been intense enough or have lapsed (Bruce-Chwatt, 1985). Taken together, the long history, ubiquity, virulence and persistence of malaria earns *Plasmodium* the status of being a successful parasite.

1.2.2 General requirements for adaptation

The two keys to rapid adaptation are the ability to generate new or retain genetic diversity and to respond to selection. Malaria parasites possess all of the major mechanisms for generating genetic variation. These occur in both the major stages of the life cycle – asexual and sexual - which is depicted in Figure 1.1. The opportunities for generation of diversity and for selection at each stage of the life cycle are now described.

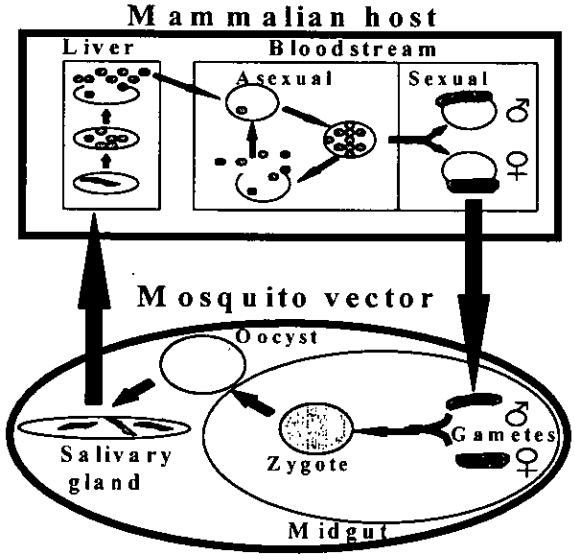


Figure 1.1. Life cycle of the malaria parasite showing the asexual stages in the host and sexual stages in the mosquito vector.

1.2.2.1 Adaptation during the asexual phase

Upon entry into the mammalian host as a haploid single or few sporozoites, the parasite undergoes rapid proliferation by mitotic replication. This occurs first in the liver, expanding from one to around 10,000-40,000 parasites in one cycle, and then in subsequent cycles in the red blood cells where in every cycle, each parasite produces 6-14 new parasites (reviewed by Cox, 1988). Such rapid population expansion allows large opportunity for the production of new mutations, which if advantageous, and if they occur early enough in the infection, have time to increase in frequency within the host. The rate of mutations in malaria parasites is unknown, however, and the genetic diversity generated by mutation during this expansion phase may range from zero to very large.

This rapid population growth is then curtailed by host immune responses which provides opportunity for selection. Though intracellular, the parasites present surface antigens on the infected cell, some of which are recognised by the immune system (reviewed by Brown et al., 1986, Anders and Brown, 1990; Holder, 1993). If the parasite is not completely cleared by the host, the parasite population can continue to cycle in size for weeks or months. A further feature of malaria parasites is that they possess the ability to switch phenotypically the surface antigens on the infected erythrocyte thus creating phenotypic variation from a genetically uniform population (Cox, 1962; Brown and Brown, 1965; Brown et al., 1968; McLean et al., 1982, 1986; Handunetti et al., 1987; Hommel et al., 1983, Gilks et al., 1990; Biggs et al., 1991; Roberts et al., 1992). This mechanism is believed to be a means by which the parasite can prolong the infection by presenting novel antigens which are not then recognised by the immune system (Brown and Brown, 1965; reviewed by Brown et al., 1986). However, more empirical and theoretical work on within-host population dynamics is required to establish how important antigenic variation is to within-host persistence as there are also plausible alternative models based on red blood cell dynamics or non-specific immunity for explaining the persistence of infections (Hellriegel, 1992; Yap and Stevenson, 1994; Gravenor et al., 1995; Hetzel and Anderson, 1996; McKenzie and Bossert, 1997).

Thus, during the asexual part of the cycle, the generation of genetic and phenotypic diversity is potentially large and the intensity of within-host selection may be strong, thereby potentially providing some basis for rapid adaptation. However, apart from evidence of many phenotypic variants arising within one infection (Brannan et al., 1994), there are no data to indicate how important the contribution of within-host genetic diversity is to adaptation.

1.2.2.2 Adaptation during the sexual phase

After the initial period of asexual replication (which varies between species), the parasite starts to produce sexual forms (gametocytes) which derive from a small proportion (<2%) of the asexual forms (Figure 1.1). These sexual forms are either male (microgametocytes) or female (macrogametocytes) and do not replicate themselves. Relative to the asexual population, their numbers are few and so their relative potential for mutation is low. On the other hand, any new mutations will have an initially higher frequency and therefore can become established more easily. Gametocytes, and the gametes they subsequently release in the mosquito midgut, seem to be the target of some immunity (reviewed by Carter et al., 1990; Carter, 1994; Sinden et al., 1996). If a mosquito bites, mixtures of parasitised cells are ingested with the blood meal into the midgut. The gametocytes are released from their cells where they differentiate into gametes and then undergo fertilisation. The ensuing meiosis appears to be conventional in that chromosomal pairing occurs, followed by formation of synaptonemal complexes (Sinden and Hartley, 1985). At these junctions, genetic recombination occurs which results in the creation of genetically mixed daughter chromosomes if the male and female gametes derive from genetically different parasites, i.e. if the parasite outcrosses (Walliker et al., 1973, 1975; Walliker et al., 1987; Ranford-Cartwright et al., 1993; Babiker et al., 1994). This generates genetic diversity which is advantageous to short-term selection and adaptation in changing environments (Maynard-Smith, 1978). At the same time, deleterious mutations are purged more efficiently and advantageous mutations are more likely to survive (reviewed in Michod and Levin, 1988) by transferring them to new genetic backgrounds, thus also furthering genetic adaptation. Within the genome,

recombination seems to occur freely on all chromosomes (Carlton et al., in press). At the population level, the frequency of outcrossing is estimated to be on average about 60-70% in endemic areas of high transmission such as Tanzania and Senegal (Hill and Babiker, 1995; Hill et al., 1995; Ntoumi et al., 1995; Babiker et al., 1997; Joshi et al., 1997), but 0-10% in low transmission areas such as New Guinea (Paul et al., 1995). These are probably underestimates because of the inability to detect all genotypes circulating in a host from point samples (Farnert et al., 1997), and because of limited numbers of alleles at the locus for which samples were genotyped.

If the gametes derive from the same parasite, i.e. the parasite inbreeds, recombination does not result in new variants. Nevertheless, in both the outcrossing and inbreeding cases, the process of chromosomal pairing and replication may allow crucial repair of DNA errors, which have accumulated during the asexual growth phase. However, the wide variation in chromosomal size maintained in nature (Corcoran et al., 1986), and the observation that meiosis involving different sized chromosomes is successfully resolved to produce viable gametes (reviewed by Walliker, 1989) may indicate that the malaria genome is quite unconstrained by the need for DNA repair, at least at the gross level.

To add to the diversity created through sexual recombination, there is some evidence that new variants of the phenotypic switching antigens are also generated during mosquito transmission (Brannan et al., 1993) although this has not been found in other studies (McLean et al., 1987).

Following meiosis, the zygote migrates to the surface of the midgut in the form of an ookinete (not shown in Figure 1.1) where it develops into an oocyst. High attrition rates between the formation of the zygote and oocyst occur (Vaughan et al., 1992) thus providing further opportunity for selection. Genetic variation among mosquitoes for oocyst number and survival has been demonstrated (Severson et al., 1995; Gorman et al., 1997) and some degree of parasite-mosquito specificity has been found (Warren and Collins, 1981) indicating that the genotype of the vector partly governs parasite fitness. Inside the oocyst, massive replication over a period of 10-14 days eventually produces around 2000 sporozoites per

oocyst thus providing another opportunity for mutation. The majority of these sporozoites migrate to the salivary glands where upon feeding on a host, around 1-1000 sporozoites are injected (Rosenberg et al., 1990; Ponnudurai et al., 1991). Probably only a few of these survive to form liver schizonts (Wéry, 1968).

Thus in the sexual part of the cycle, genetic variation can be generated through recombination at meiosis, and mutation during sporozoite production. Selection can occur through vector immunity or through host immunity carried over from the host (transmission-blocking immunity). Perhaps importantly, large stochastic processes leading to population bottlenecks also operate during this phase of the life cycle.

In summary, the parasite has incorporated into its life cycle the three major mechanisms for generating genetic diversity - sexual recombination, mutation in large populations, and, at the phenotypic level, antigenic variation. Combined with the mobility of the vector, which allows ready transfer of new genetic combinations to the wider population, these should, in principle, provide an inexhaustible supply of variation from which the parasite can adapt.

Kemp et al. (1990) reviewed the data on genetic and molecular variability in malaria parasite populations, drawing together information on protein and enzyme polymorphisms, variation in antigens, sequence variation and drug resistant mutants. The conclusion was that the amount of variation was vast, whether at the within-parasite level, the within-population level, or across-population level. This observation is entirely consistent with the many mechanisms for generating diversity built into the parasite's life cycle described above. In addition, there are many reports of parasite variation in phenotypic traits such as virulence, replication rate and transmissibility (James et al., 1932; Covell and Nicol, 1951; Schmidt, 1978; Ponnudurai et al., 1982; Graves et al., 1984; Wellems et al., 1987; Marsh, 1992), although most of these relate to differences between isolated populations which may not reflect within-population diversity, or the data were obtained *in vitro*. Variation in fitness traits has not been systematically studied, however, and so it remains to be seen whether the variation at the molecular level translates to variation in parasite fitness and hence adaptability. This is discussed in the next

section.

1.2.3 Evidence for adaptation

The fact that extensive molecular genetic variation exists in the malaria parasite population does not necessarily reflect immune or other selection for diversity. In a review of the relationship between immune protection and antigenic diversity, Brown (1991) made the following caution:

> *'It is not going too far to say that not infrequently the host-parasite relationship is assumed to conform to the convenient interpretation of the molecular data, rather than molecular data interpreted in relation to the inconvenient facts of natural host-parasite relationships'.*

Nevertheless, there are three lines of evidence which have been argued to indicate that the genetic variation observed at loci coding for antigens is maintained by positive selection for diversity. The antigens in question are those on the asexual stages of the parasites which are the main targets of antibody-mediated immunity. Attention has focused on these as potential vaccine components (reviewed by Engers and Godal, 1998).

First, some of these antigens have shown indirect evidence of selection for diversity on the basis of high ratios of nonsynonymous to synonymous mutations (Hughes, 1991; Hughes and Hughes, 1995) (but see Arnot, 1991) and by analysis of relative heterozygosity (Conway, 1997). It is unclear to over what scale of evolutionary time this diversity has been maintained, i.e. whether it is maintained within populations by strong immune selection in the majority of hosts in the short term, or whether it has accumulated over a long period of evolutionary time due to mutation, migration and perhaps weak selection (Hughes and Hughes, 1995 cf. Rich et al., 1998).

Second, most of these antigens have elicited significant antibody responses indicating that they may be targets of the immune system. This evidence is based on *in vitro* assays of immune cell stimulation and inhibition of growth and invasion of red blood cells, and *in vivo* tests of protection from disease or reinfection following vaccination. None of these have proved to be fully protective and the effects on parasite fitness (i.e. transmission) of such immune selection have not

been measured in the *in vivo* studies on epitope-specific immunity (Riley et al., 1992, 1993; Collins et al., 1994; Al-Yaman et al., 1994, 1995). Nevertheless, there have been a few examples where *in vivo* immune selection has given rise to a deletion or sequence mutation in the selected antigen thus allowing it to escape the immune system (David et al., 1985; Klotz et al., 1987; Hudson et al., 1988; Wood et al., 1989). The fate of such mutants under selection in new hosts or during mosquito transmission is not known. In general then, the effect of specific immunity on parasite fitness, especially at the population level, is not well understood. The complexity introduced by the many other immune effector mechanisms which interact with specific antibody killing also makes its contribution difficult to determine (Mendis et al., 1991; Brown, 1991). There is a also a small amount of variability in sexual stage antigens but the significance of this variability in selection against parasite transmission is not clear (Carter, 1994).

Third, much laboratory data show that immunity to malaria parasites is somewhat strain-specific (Jarra and Brown, 1985). This means that when a host is infected with one strain, it is difficult to re-infect it with the same strain, whereas it can be relatively easily infected with a genetically distinct strain. Thus it is believed that immune selection acts against one or more polymorphic antigens or other component which is specific to each strain. The phenomenon of strain-specific immunity has been used to explain why immunity to malaria is slow to acquire, only reaching protective levels after many repeated exposures when most or all of the strain-specific components has been experienced (Brown, 1991; Marsh, 1992; Gupta et al., 1994a,b). If true, this strongly supports the contention that parasite variation is a major factor driving the epidemiology of the disease, and is under continuous selection pressure. Evidence has been sought in longitudinal data for changes in antigen frequencies that would reflect such selection (Forsyth et al., 1988; Conway et al., 1992; Babiker et al., 1995). However, the stochastic nature of transmission and sampling variation in field data make a selectionist argument difficult to support (Conway, 1997). The argument in favour of dynamic parasite adaptation through strain variability would greatly benefit from direct field

evidence of frequency-dependent selection of parasites bearing different antigenic types (Dye and Targett, 1994).

Apart from antigens, there are few published examples of other genes or phenotypes under selection either in the laboratory or in the field. And yet, the two traits of most concern to us- virulence and transmissibility - are, in theory, the ones most likely to adapt under selection pressure because they relate to parasite fitness. While it could be argued that virulence is largely a function of host immune status, and therefore covered by the argument that antigens are the targets of selection, there are also likely to be other factors controlling virulence. Transmissibility has been largely ignored, particularly the amount of parasite-derived genetic variation and the selective forces which govern its evolution. Its relationship with virulence, which is essential to understanding how it evolves, has only recently received proper attention at the phenotypic level (Taylor et al., in press), but studies at the genetic level have not been conducted. Quantitative studies of the phenotypic expression of virulence and transmission may provide additional information on virulence and maintenance of parasite diversity to complement the molecular studies.

1.2.4 Constraints on adaptation

Although malaria parasites are apparently well equipped to generate genetic diversity and probably undergo selection, there are some potential constraints on their realised rate of evolution. Some of these are discussed here.

First, there is a large degree of stochasticity and bottlenecking involved in the life cycle of the parasite. Only a minute fraction of the parasites in the host are sampled by the vector, and then further narrowing of the gene pool occurs after zygote formation with typically less than three oocysts being formed per mosquito (Graves et al., 1988; Tchuinkam et al., 1993). The effects of variability in sampling and small population sizes on slowing the rate of evolution is well known in population genetics, and is potentially important in malaria parasite evolution.

Second, the malaria parasite population is strongly structured because of the limited number of parasite haplotypes per host. This restricts the amount of genetic mixing that can occur. It also influences the pattern of selection on the parasite

population. Spatial structuring (clustering) of malaria parasites is probably typical in the field (e.g. Gamage-Mendis et al., 1991) causing further limits on gene flow through the population. The effect of population structure on parasite evolution has not been well studied, but it is recognised that such structuring has important implications to the rate of adaptation (e.g. Frank, 1986).

Third, there may be conflicts between the evolutionary optima of within-host selection compared with between-host selection. For example, theory predicts that within hosts, if parasites with different haplotypes compete, the level of virulence which evolves is higher than is optimal if only one parasite haplotype is present within the host (Levin and Pimentel, 1981; Bremermann and Pickering, 1983; Frank, 1992; Antia et al., 1994). Recent studies by Taylor et al. (1997a,b, in press) have explored some aspects of within-host versus between-host fitness in malaria parasites. These studies showed that, at the phenotypic level, within-host competition clearly enhances between-host fitness, and so it seems that parasite-parasite interactions are more complex than theory assumes. This has not been studied empirically at the genetic level.

Fourth, as for all organisms, the fitness of malaria parasites is probably constrained by some costs. The most obvious example is that of a trade-off between virulence and transmission. If virulence is a function of within-host replication rate, and if high replication rate leads to higher transmission, then virulence should be increased by selection on parasite fitness. However, if virulence is too high such that hosts die, then transmission is nil. This theme is the basis for the evolutionary prediction that virulence evolves toward some optimum value which depends on the nature of the relationship between virulence and transmission (Bremermann, 1980; Levin and Pimentel, 1981; Anderson and May, 1982; Ewald, 1983; May and Anderson, 1983). A further example of a trade-off in the malaria life cycle is that between asexual and sexual reproduction: since gametocytes are derived from asexual parasites and do not replicate, then asexual production, and perhaps length of the infection, are compromised by increase in transmission. Whether these hypothesised trade-offs exist in malaria remains to be determined.

Fifth, the versatility of the immune system in recognising and remembering a vast array of parasite antigens makes the host a very difficult target for the parasite. While parasites usually have the advantage over hosts of having much shorter generations and therefore faster rates of adaptation, it is also true that the vertebrate immune system is capable of very rapid somatic evolution. This means that parasites are constantly confronted with host heterogeneity which, in effect, confounds the parasite's evolutionary progress (Lively and Apanius, 1995; Gupta and Hill, 1995).

In summary, there is a variety of evidence which supports the argument that genetic variability in the malaria parasite is crucial to its survival in the face of host immune systems which themselves have the ability to adapt rapidly to the parasite population via somatic mutation. The key question is whether this variation is the Achilles heel of the parasite: if we select the parasite using vaccines and transmission-reducing measures, will it quickly adapt to the new environment? Alternatively, the control measures may achieve their desired goal without inducing such genetic changes in the parasite population. The answer to this question cannot be found without a better understanding of the selective forces operating on the parasite population.

This thesis examines various aspects of the evolutionary forces determining malaria parasite adaptation. The first two chapters focus on genetic variation in virulence and transmissibility and the relationship between the two. The next chapter presents an analysis of disease severity data from a longitudinal field study in Sri Lanka, with emphasis on the contribution of host factors to variation in virulence. In the final two chapters, theoretical models are used to evaluate the effects of population structure and other factors on the rate of evolution of parasites under selection by the host. A trait under simple genetic control in parasites - that of drug resistance - was used for these latter studies.

Chapter 2

Parasite genetics: relationships between virulence and transmission

2.1 Summary

The major hypothesis of why microparasites are virulent is that high within-host replication rates are necessary for successful transmission (parasite fitness), and that virulence (damage to the host) is a consequence of this rapid replication. The assumptions underlying this hypothesis were tested in the rodent malaria parasite, *P. chabaudi,* by examining the genetic relationships between various virulence and transmission traits. This was done by evaluating eight wild-caught clones for both traits and finding the correlations between the clonal averages for these traits. It was found that there was a large amount of between-clone variation in parasitaemia, loss of liveweight in the host, anaemia, gametocyte production and infectivity to mosquitoes, and these clone differences were maintained over four experimental replicates. Anaemia and liveweight loss, assumed to be indicators of virulence, were strongly phenotypically and genetically correlated to maximum parasitaemia: this supports the assumption that rapid replication rate causes more virulence. Gametocytaemia and infectivity to mosquitoes were also positively phenotypically and genetically correlated to parasitaemia (not always significantly), which supports the assumption that rapid replication leads to higher transmission. Thus the two prerequisites for the hypothesis of why parasites are virulent were justified in this experimental system for malaria parasites.

2.2 Introduction

Studies on virulence of the malaria parasite are of considerable interest because of the many human deaths that this disease causes. While there are undoubtedly host factors controlling the level of severity of the disease (reviewed

by Marsh, 1992; White and Ho, 1992), there is also evidence of variation between parasites in the probability that they kill their host (James et al., 1932; Covell and Nicol, 1951; Schmidt, 1978; Cox, 1988; Marsh, 1992). This is a puzzle: parasites which kill their host would seem to be unfit because in doing so they limit their opportunities for transmission (reproductive output). It is therefore somewhat surprising that malaria parasites, which have evolved in association with humans since pre-historic times (Bruce-Chwatt, 1965), still cause large morbidity and mortality rates in their hosts, defined here as 'virulence' This question has been generalised by evolutionary biologists: why are parasites virulent, rather than benign mutualists? Many answers to this question (most of them related) have been proposed (reviewed by Bull, 1994; Levin and Bull, 1994; Read, 1994; Ebert and Herre, 1996; Frank, 1996). The large majority of these answers are theoretical, and the assumptions and outcomes have rarely been put to empirical test. This study describes an experimental examination of the most favoured of these hypotheses using a laboratory model system for malaria parasites. Before describing these experiments, the models predicting how virulence evolves in parasites in general are reviewed, followed by an outline of how these models relate to malaria parasites.

2.3 Review of the literature

2.3.1 General models for the evolution of virulence

The favourite hypothesis of how virulence evolves is that natural selection acts on the parasite to optimise the level of its expressed virulence at some evolutionary stable, often intermediate, value (Bremermann, 1980; Levin and Pimentel, 1981; Anderson and May, 1982; Bremermann and Pickering, 1983; Ewald, 1983; May and Anderson, 1983). This is because, on the one hand, the parasite must replicate fast in the host in order to produce enough transmission stages to ensure its survival to the next generation, i.e. to maximise its reproductive fitness. A critical assumption here is that virulence, or damage to the host, is an inevitable side effect of the parasite's use of host resources for this rapid

replication. On the other hand, if the parasite extracts too many host resources, or does too much damage to host tissues in the process, then the host will die. In this case, the parasite will no longer transmit, and has reduced its reproductive fitness. Thus, if selection maximises parasite reproductive success in host-parasite systems, the number of new hosts infected, or the transmission rate of the parasite, should be optimised by natural selection. This hypothesis, termed the adaptive trade-off hypothesis, is thus based on parasite genetics, and while dependent on the host's expression of virulence, does not invoke host adaptation.

One of the predictions of the adaptive trade-off model is that the level at which virulence optimises depends on the form of the genetic trade-off between transmission and virulence. Most experimental tests of this model have related to an extension of this prediction, namely that parasites which are transmitted horizontally evolve to become more virulent than parasites which are transmitted vertically. This is because the latter rely on maintaining high reproductive fitness in their hosts so that more parasites are transmitted. This has been supported in several invertebrate host-parasite systems, including phage which parasitise bacteria (Bull et al., 1991), nematodes which parasitise fig wasps (Herre, 1993) and mites or lice which infest rock doves (Clayton and Tompkins, 1994), although counter examples are easily found (Hurst et al., 1994; Mangin et al., 1995). Another version of this idea for higher order hosts had previously been proposed by Ewald (1980) who argued that human diseases which are transmitted easily and directly (e.g. by physical contact) are less virulent than diseases in which transmission is more risky for the parasite, or which rely on host immobility, as for many vector-borne diseases. Across-species comparisons generally support this view (Ewald, 1983, 1994), though not without some controversy (Read et al., in press).

However, beyond these correlational data between virulence and mode of transmission/population structure, there are few data within species to support the argument that individual parasites face a genetic trade-off between virulence and replication. Lipsitch and Moxon (1997) have recently reviewed the literature, mostly on viruses, in which there are a limited number of examples of either simultaneous increases in virulence and transmission in passage experiments,

phenotypically higher virulence in faster replicating viruses, or phenotypic correlations between virus density and the likelihood of transmission. Of the non-virus examples given (trypanosomes, Diffley et al., 1987, *Pseudomonas* bacteria, Rahme et al., 1995; and a rodent malaria, Dearsly et al., 1990), only the first should be interpreted as indicating a genetic relationship between these traits. They also cite examples where these relationships do not exist, attributing them to a decoupling of pathogenesis and transmission either when parasites down-regulate the host immune response, or when the site of pathology is separate from the transmission cycle. Such examples fit into one of the alternatives to the trade-off hypothesis (see below). Further examples in bacteria are given by Anderson and May (1982) who cited Greenwood et al.'s (1936) data showing that positive between-clone relationships between virulence and infectivity exist in two bacteria, mouse plague (*Pasteurella muris*) and mouse typhoid (*Salmonella typhimurium*). Ebert (1994a) and Ebert and Mangin (in press) showed a positive correlation between spore load and host mortality in microsporidia infecting *Daphnia magnus*. However, these few non-virus examples are matched by as many examples (from snail-schistosome, and plant-fungal associations) which show zero correlations between these traits within species (Ebert, 1994b). Thus, in general, the evidence for *genetic* relationships between virulence and transmission in micro-organisms is weak and has been generally derived *post hoc*. Given the reliance of all these models on an intrinsic biological link between parasite replication rate, virulence and transmission, it is surprising that so few experiments have directly tested this assumption (Ebert and Herre, 1996; Lipsitch and Moxon, 1997; Read et al., in press), especially as parasite genetics is a central part of understanding many important human diseases (Ewald, 1994; Thompson and Lymbery, 1996).

Nevertheless, there is one compelling example in the myxoma virus of rabbits which has possibly motivated many more theoretical studies on the evolution of virulence. This virus, which causes mild infections in its natural host, the South American rabbit (*Sylvilagus* spp.), was released into a population of European rabbits in Australia. Within a decade its effect on host mortality had decreased from 99% to less than 30% (Fenner and Ratcliffe, 1965), though in subsequent

decades host mortality did not decline further (Fenner and Myers, 1978). Field studies (Fenner et al., 1957) and laboratory studies (Fenner et al., 1956) were able to show that this was partly due to lower transmission rates in more virulent strains, thus selecting the parasite towards more moderate levels of virulence. The rabbit host also showed a genetic increase in resistance during this period (Marshall and Fenner, 1957), i.e. selection was on both host and parasite. Similarly, in Bull et al's (1991) experiment, the host bacteria became more resistant to their parasitic phage during selection for virulence.

A discussion of the trade-off hypothesis would not be complete without mention of its rivals. Until less than two decades ago, it was generally argued that parasites evolve to become benign (Burnet and White, 1972; Alexander, 1981). Parallel with this is the idea that a minority of parasites are aberrations or mutations which cause virulence but which in the long-term do not survive because they kill their hosts or have lost the capacity to transmit (Levin and Bull, 1994). Alternatively, the parasite might in rare cases find itself in the inappropriate host tissue in which it causes unusual levels of damage (Levin and Svanborg Éden, 1990), i.e. virulence is an inappropriate expression of disease caused by a parasite which has lost its way. Finally, some parasite species are able to cause down-regulation of the immune response in order to survive and transmit: in these cases, virulence is negatively related to transmission success. Thus there are several alternatives to the trade-off hypothesis for which there are also clear examples in nature (reviewed by Lipsitch and Moxon, 1997).

Finally, an extension to the trade-off hypothesis predicts that higher levels of virulence than are optimal for individual parasites may evolve due to within-host competition between parasites with different genotypes (Levin and Pimentel, 1981; Bremermann and Pickering, 1983; Frank, 1992; Nowak and May, 1994; May and Nowak, 1995). This is because highly virulent parasites, even though they may have a disadvantage in killing their host when in single infections, also have the advantage of being able to outcompete less virulent parasites when together in the same host, thereby gaining a relative transmission advantage. Importantly, these models also predict that as the level of within-host or between-host competition

decreases, the range of virulence and transmission levels being maintained in the population also increases (Gupta et al., 1994a,b,c. Nowak and May, 1994; May and Nowak, 1995). Thus this extension, if true, helps explain why parasites are often virulent, and why parasites are so variable in their levels of virulence.

2.3.2 Evolution of virulence applied to malaria

The adaptive trade-off hypothesis, which is the one tested in this study, is now discussed in relation to malaria. The overall premise of this hypothesis is that there is a genetic link between virulence and transmission in the parasite. The mechanisms for why this relationship exists are based on the following assumptions. However, even if these were not met, establishment of a genetic link would still support the adaptive trade-off model.

Assumption 1. Higher replication rate leads to higher transmission to new hosts in the absence of host death. This first assumption is applicable to malaria because transmission stage forms are scarce (reviewed by Taylor and Read, 1997), and only a tiny fraction of all parasites are taken up in a blood meal by the mosquito vector. Therefore in simple terms, it might be expected that higher replication rates or numbers of asexual forms will give rise to more transmission stages (which are derived from asexual forms in the previous replication cycle), and therefore higher levels of transmission. In malaria, higher fitness resulting from higher transmission could include both greater success in transmitting for a single bite by a vector, or in greater numbers of transmission events in the parasite's infection period, and therefore may be a function of both intensity and persistence of gametocyte production. However, both theory (Hellriegel, 1992; Gravenor et al., 1995; Koella and Antia, 1995; Hetzel and Anderson, 1996) and data (Taylor et al., 1997a, b) show that this proposed relationship between replication rate and transmission is much more complex than stated above and therefore difficult to detect. Asexual parasite numbers are highly dynamic and transient during the infection, and in consequence, gametocyte output is also very transient. Moreover, environmental factors within the host (usually reflecting parasite stress) also

influence gametocyte output (Carter and Miller, 1979; Carter and Graves, 1988; Alano and Carter, 1990; Sinden et al., 1996; Buckling et al., 1997) but these are not yet well understood, especially in relation to the asexual dynamics. Further empirical and theoretical analysis of the relationship between these traits in malaria parasites is required before this assumption can be justified.

Assumption 2. Higher replication rate leads to higher virulence. Malaria parasites destroy their host cells at the end of every replication cycle. This causes severe anaemia, the release of parasite debris and toxins, fever, dramatic immune response, and consequent host morbidity (reviewed by White and Ho, 1992; Marsh and Snow, 1997). In general, higher levels of parasitaemia lead to more host sickness, although this relationship is often difficult to observe in the field (Greenwood et al., 1991). This relationship is blurred by the many indirect effects of the parasite which depend on host-controlled responses. The most studied host response is the release of tumour necrosis factor (TNF) from activated macrophages (Clark et al., 1981, Grau et al., 1988, Bate et al., 1989), the level of which has been shown to be related to the severity of the disease (Grau et al., 1989; Kwiatkowski et al., 1990; reviewed by Carter et al., 1997). Similarly, severe anaemia is induced not only by red blood cell destruction but by an decrease in the rate of production of these cells, and there are many other host responses which contribute to the level of illness expressed (reviewed by Marsh, 1992; White and Ho, 1992; Marsh and Snow, 1997). Furthermore, these host immune responses tend to raise the level of parasite density required to generate clinical symptoms in later infections which further confuses the relationship between parasite density and illness. Direct effects of the parasite are caused by release of parasite 'toxins' upon schizont rupture, some of them stimulants of TNF production (Kwiatkowski et al., 1989; Bate et al., 1992), and these seem to play an important part in the regulation of parasite density (Kwiatkowski, 1995). Indeed, it is the subject of continuing debate as to whether treatment of malaria should take an anti-disease or an anti-parasite approach because the relationship between parasite numbers and disease expression is very unclear. However, the mechanisms behind

the expression of virulence are not important here: the aim is to simply establish whether there is a relationship between parasite replication rates and the level of virulence which they cause when the factors which could mask such a relationship in the field are removed.

Assumption 3. High virulence leads to host death or lower parasite transmission. High virulence could curb transmission either by limiting the opportunities for transmission by killing the host (at which point mosquitoes give up biting) or by reducing the host's infectiousness to mosquitoes. The fact that high virulence kills hosts through high morbidity is true for many malaria species, including the major human species *P. falciparum* (McGregor et al., 1956; Molineaux and Gramiccia, 1980), several of the lizard malarias, including those with very ancient associations with their host (Schall, 1996), and for some rodent malaria species (Carter and Diggs, 1977). For the species used in this study, *P. chabaudi*, it is not known what proportion of its natural host population (the thicket rat, *Thamnomys rutilans*) die from their infections: in the wild, most hosts are found to be infected, and they harbour parasites at sub-clinical levels for a lifetime (Landau and Chabaud, 1965; Carter and Diggs, 1977). This has been taken to suggest that this species is not very virulent in its natural host (Landau and Chabaud, 1994), although a significant rate of mortality or morbidity could easily have been missed because such populations have not been thoroughly surveyed, and such field observations on virulence in wild-caught hosts are unreliable indicators of natural levels of virulence (Read, 1994). Infections in laboratory mice result in high levels of mortality which vary widely for different mouse strains (Stevenson et al., 1982) demonstrating that *P. chabaudi* has the capacity for high virulence. Thus it seems reasonable to assume that the level of virulence relates to the probability of host death (Brewster et al., 1990). Even if this probability is on average low in some host species, variation in host susceptibility and parasite virulence is expected to cause some mortality.

In the absence of significant host death, transmission could still be reduced by excessive levels of virulence. There is some evidence to support this: in *P. berghei*

it seems that inhibitory factors released at the time of crisis in the infection temporarily reduces infectivity of the gametocytes to mosquitoes (reviewed by Sinden et al., 1996). In *P. vivax*, there seems to be a temporary loss of infectivity to mosquitoes during the paroxysm periods which characterise the peak of the asexual infection (Karunaweera et al., 1992; reviewed by Carter et al., 1997). Similar studies have not been done in *P. falciparum* and *P. chabaudi* in which the bulk of gametocyte production occurs well after the peak of asexual parasitaemia. Thus it is not known whether immunity stimulated by asexual parasites contributes to a trade-off between high virulence and transmission.

Assumption 4. The relationships between replication rate, virulence and transmission have a genetic basis in the parasite. Should the above assumptions hold, this final assumption must also be true in order for the adaptive trade-off hypothesis to be explained by natural selection and evolution. In malaria, between-strain variation in virulence or parasitaemia has been inferred for *P. falciparum in vitro* and *in vivo* (James et al., 1932; Covell and Nicol, 1951; Wellems et al., 1987; Marsh, 1992; Allan et al., 1993), but not conclusively demonstrated in the field. In rodent species, variation in virulence and replication rate both between (Carter and Diggs, 1978; Cox, 1988) and within species (Alger et al., 1971; Walliker et al., 1976; Knowles and Walliker, 1980; Carlton et al., in press) have been observed. Apart from the latter study, which examined genetic variation in parasitaemia in the progeny of a cross between two clones differing in replication rate, all of these studies on within-species variation can probably be attributed to mutations arising during serial passage in the laboratory (Walliker, 1981) and therefore cannot be used to infer levels of variation in the natural population. Similarly, evidence for genetic variability in transmission has been found in the artificial laboratory environment (in *in vitro* cultures of *P. falciparum* by Ponnudurai (1982), Graves et al., (1984), and between two *P. chabaudi* clones in laboratory mice (Taylor et al., 1997a,b), but, as for virulence, conclusive evidence of genetic variation in the field is not available. Quantitative studies on the genetic relationships among these traits have not been conducted. Nevertheless, there is

one molecule which appears to link replication rate with virulence and transmission, at least in *P.falciparum* - the erythrocytic membrane protein (denoted PfEMP1) (Leech et al., 1984, Howard et al., 1988, Magowan et al., 1988). This protein binds the infected parasite cell to the host's endothelium thus allowing the parasite to sequester in the vasculature and organs in order to avoid clearance by the spleen. This may allow the parasite to achieve higher parasitaemias through reduced parasite clearance rates. It also expresses antigens which are able to switch in form during the infection thus allowing evasion of the immune system and hence persistence and higher transmission success. In addition, field studies have shown a relationship between parasite sequestering and cerebral malaria, the most virulent expression of the disease (MacPherson et al., 1985). Thus there is good reason to believe that this molecule affects virulence through its role in cytoadherence, and affects persistence through its role in antigenic variation. Its effects on parasite growth rate are unclear (Gilks et al. 1990). Yet it remains to be demonstrated in a quantitative way whether growth rate, virulence and transmission are genetically linked. This is best done in the laboratory environment where controlled experiments can expose any genetic variation and covariation in these traits. Even if such variation is small in the natural environment due to optimising selection, this does not invalidate the adaptive trade-off hypothesis.

Existence of standing variation in nature for these traits is predicted by most of the models for the evolution of virulence, the amount increasing as the level of between-parasite competition is reduced. In human malaria mixed infections are very common suggesting that competition between parasite genotypes is quite low (Day et al., 1992; Babiker et al., 1994; Ntoumi et al., 1995; Paul et al., 1995). Controlled studies of competition in mixed infections in rodent malaria show that parasites do seem to interact during their asexual growth phase, but that the competition is not severe enough for the most virulent parasite to exclude the other parasite from growing or transmitting (Taylor et al., 1997a,b, in press). It has also been argued that in natural situations competition between parasites is weak because strain-specific immunity allows antigenically distinct genotypes to co-infect hosts and transmit more or less independently, consistent with epidemiological data

(Gupta et al., 1994a,b,c). Strain-specific immunity has been demonstrated in *P. falciparum* and *P. vivax* in controlled experiments on human patients (James et al., 1932), and also in mice infected with *P. chabaudi* (Jarra and Brown, 1985). Regardless of whether these forces act to maintain variation in virulence, the high frequency and opportunity for genetic exchange among malaria parasites (reviewed by Walliker, 1989) and the generally high level of variability in neutral and antigenic loci (reviewed by Kemp et al, 1990) indicate that genetic variability for phenotypic traits such as virulence and transmission is likely to be maintained if there are many genes involved (as is likely), and if selection has a stabilising nature, as in the trade-off hypothesis.

The alternative hypotheses (not examined in the present study) for why parasites are virulent may also be relevant to malaria. Both the 'aberrant mutation' hypothesis and the 'inappropriate expression in a novel tissue environment' could be applied to the highly virulent malaria *P. falciparum.* This species causes 'cerebral malaria' in a small minority of cases, although in such cases, host mortality rates are high (Brewster et al., 1990). The symptoms of this disease are manifest in the brain where the parasites sequester in the capillaries and cause damage to the microvascular tissues (reviewed by White and Ho, 1992). Such damage may be an unfortunate side effect of the parasite's strategy for avoiding elimination by the spleen by sequestering throughout the vasculature, i.e. lodgement of parasites in the brain tissue may be an inappropriate expression of an otherwise adaptive mechanism. Alternatively, recurrent mutations which occasionally reach a significant frequency in a few hosts may explain such disastrous pathology (Roberts et al., 1993). Whether 'normal' or not, it seems that parasite molecules, including those involved with cytoadherence, tumour necrosis factor (TNF) induction and rosetting capability (Clark et al., 1989; Carlson et al., 1990; Ho et al., 1991; Allan et al., 1993; Rowe et al., 1995), are significant factors contributing to the incidence of cerebral malaria.

2.4 Aims of the present experiment

The experiment reported in this study was aimed at testing the assumptions of the adaptive trade-off hypothesis with a view to validating one of the major models for the evolution of virulence. The overall aim was to determine whether there is a genetic trade-off between virulence and transmission within a natural population of *P. chabaudi* parasites. To test this, eight parasite clones were repeatedly measured for virulence and transmission characteristics in a laboratory environment. The regression of the mean virulence for each clone on the mean transmission of each clone measured the genetic (across-clone) relationship between these traits. More specific objectives relating to the underlying assumptions of the model were to:

1. Determine whether virulence and transmission traits are genetically determined in a wild-caught population of *P. chabaudi* parasites.
2. Determine whether replication rate and virulence are positively related.
3. Determine whether high replication rate leads to higher transmission.
4. Determine whether these relationships hold at the genetic level.

2.5 Materials and Methods

2.5.1 Source of parasite clones

Clones of *P. chabaudi* were obtained from isolates which had been collected from their natural host (*Thamnomys rutilans*) from the Central African Republic in 1969 and 1970 and stored in liquid nitrogen since then. The method of cloning from isolates by dilution (Beale et al., 1978) entailed injecting 14 to 30 mice with an inoculum expected to contain an average of one parasite. An average of 9% of the injected mice became infected: assuming that the number of parasites per mouse which established an infection was distributed as a Poisson variable, it is highly probable that each clone derived from one parasite. The possibility of multiple parasites founding a clone can only be ruled out by extensive genotyping. Some of these isolates comprised more than one species of parasite, the predominant one

being *P. chabaudi*, with occasional *P. yoelii* and *P. vinckei* species. Therefore, after cloning, the species of each clone was confirmed as *P. chabaudi* by morphology (D. Walliker, R. Carter, pers. comm.). Only one clone from each isolate, which themselves had previously been shown to be genetically distinct using electrophoretic enzymes (Carter, 1978), was allowed in the experimental panel of clones to minimise the chances that the clones were genetically identical.

2.5.2 Maintenance and genotypes of mice and mosquitoes

Mice were housed at a temperature of $21(\pm 1)^0$C and fed on SDS rat and mouse maintenance diet with 0.05% para-aminobenzoic acid (PABA) added to their drinking water to assist with parasite growth.

The mice used during the cloning phase were a mixture of inbred and first generation crosses between inbred lines, i.e. mouse genotype was not kept uniform at this stage. However, there was no confounding of mouse genotype with clone. During the experimental phase, the mice used were female inbred C57BL/6J (B&K Universal, England), aged between five and eight weeks at the beginning of the infection with a maximum range in age of one week within each experimental group.

2.5.3 Passage of clones in mice

During the cloning phase it was necessary to passage the clones serially, i.e. from one mouse to another. This involved taking 5-10µl of blood from the tail of an infected mouse (usually before the peak of the infection) into 0.1ml of citrate saline (0.85% NaCl, 1.5% tri-sodium citrate) and injecting it into the peritoneal cavity of a naive mouse. The number of passages per clone was kept to a minimum to avoid somatic mutations accumulating in the parasites which may render the parasites unable to transmit (Wéry, 1968), and to minimise the amount of selection that may occur in this artificial laboratory system. One of the clones (ER) was not obtained directly from the isolates in the natural host but instead was derived from a clone which had been stored in the laboratory of C. Hamers in Belgium between 1969 and 1983 and was then transmitted through mosquitoes in this laboratory. Table 2.1 summarises the laboratory history of the clones,

including the number of asexual passages between isolation from the wild and use in these experiments.

Table 2.1. Laboratory history of the clones used in these experiments

Clone	Isolate	Species found in original isolate	Replicates in which included	No. passages until Replicates 1,2,3,4[1]
AD	2AD1	P. chabaudi + P. yoelii	1-4	6,9,7,8
AJ	AJ	P. chabaudi	1-4	6,9,7,8
AQ	2AQ	P. chabaudi + P. yoelii	1-4	7,9,9,10
AS	2AS	Unrecorded	3-4	-,- 8,9
AT	4AT	P. chabaudi	1-4	7,9,9,10
BC	1BC	P. chabaudi + P. yoelii	1-4	6,9,7,8
CW	2CW	Unrecorded[2]	1-4	6,9,6,7
ER[3]	56L	Unrecorded	3-4	5,6

1. From natural host to experiments, including pre-cloning.
2. Not recorded, but parasites derived from this isolate have been *P. chabaudi* or *P. yoelii*
3. ER was maintained by C. Hamers in Belgium from 1969 to 1983. Thereafter, it was cloned in Edinburgh, maintained for 18 serial passages, transmitted through *Anopheles stephensi* and then underwent 9 passages before being used in this experiment.

During the experimental phase, when accurate inoculum size was required, blood from the donor mouse was diluted in medium suitable for parasite maintenance (50% heat-inactivated calf serum, 50% Ringer solution (27mM KCl, 27mM $CaCl_2$, 0.15M NaCl), with 20 units of heparin/ml mouse blood) to the appropriate concentration for the inoculum size. The required volume of blood was calculated from the blood cell density and parasitaemia in donor mice calculated immediately before experimental inoculations. All injections were into the peritoneal cavity in a 0.1ml volume.

2.5.4 Mosquito maintenance and feeding on mice

Mosquitoes of the species *Anopheles stephensi* were maintained in cages in rooms with fluctuations in temperature between 24 and 28^{0}C and relative humidity between 65 and 85%. Adults were fed on glucose at a concentration of 10% with 0.05% PABA. To assess the degree of transmissibility to mosquitoes from

individual mice, 40-80 female mosquitoes aged between four and eight days were allowed to take a blood meal for 20 to 30 minutes on each mouse. During the feed, mice were anaesthetised with a 2:1 mixture of the drugs Rompun (Bayer Ltd, UK) and Vetalar (Parke-Davis Veterinary, UK) and immobilised by taping them to small cork boards. After the feed, mosquitoes which remained partially fed or unfed were discarded. Mosquitoes which fed on the same mouse were maintained in individual pots at 25^{0}C in an incubator for 8-9 days with glucose/PABA supplied. The survivors were then killed with chloroform, their midguts dissected in phosphate buffered saline (PBS, pH 7.4) using a dissecting microscope with 40-fold magnification, and the number of oocysts per gut were counted. Records were not kept on the number of mosquitoes which died between the day of feeding and the day of dissection.

2.5.5 Experimental design

Four replicates of infections were performed. In Replicates 1 and 2, six clones and an uninfected control were used, and in Replicates 1 to 4, an additional two clones and no control were used. Four mice per clone were used in Replicates 1 and 2, and two per clone in Replicates 3 and 4 giving a total of ten mice per clone. The size of inocula were 10^6, 10^4, 10^5 and 10^5 in Replicates 1 to 4, respectively. Parasites for inoculating mice in Replicates 1 and 3 were obtained from frozen stocks which had been serially passaged once or twice. Parasites for inoculating mice in Replicates 2 and 4 were obtained on after serial passage 1-3 times from single mice in Replicates 1 and 3, respectively. For each replicate, clone-groups were inoculated in random order.

At two or three day intervals until the infections had almost expired, measurements of asexual parasitaemia, liveweight loss, anaemia, gametocytaemia and infectivity to mosquitoes were made on each mouse. The methods of measurement are given in more detail later. The sampling regime is shown in Table 2.2. Emphasis was on measurement of virulence in Replicates 1 and 2, and measurement of transmission in Replicates 3 and 4. All measurements were taken between 9.00 a.m. and 1.00 p.m., except for infectivity to mosquitoes where feeds

were conducted between 7.00 p.m. and 8.00 p.m.. The order in which the clone-groups were measured was randomised each day.

2.5.6 Measures of virulence

As a measure of parasite replication rate, the proportion of red blood cells (rbc) infected by asexual parasites – parasitaemia - was measured during the infection. This was done by staining thin blood smears on glass slides with a 10% Giemsa solution for 30-40 minutes and then counting the number of infected and uninfected cells. On average, approximately 500 cells were counted and these were obtained from 2 to 3 separate fields of vision on the microscope using a magnification factor of 800.

Loss of body weight of the mouse during the critical phase of infection was considered to be a likely indicator of the damage to host fitness caused by the parasite, i.e. an indicator of virulence. Therefore, mice were weighed to an accuracy of 0.01g at two to four-day intervals at the beginning and end of infection, and daily during the peak of the asexual infection.

Anaemia – another measure of virulence - was assessed by measuring red blood cell density every two to four days. To do this, 2µl of blood from the tail of the mouse was taken by capillary action into micropipettes, diluted by a factor of 50,000 or 100,000 in Isoton solution and then counted for red blood cells in a 0.5ml sample by flow cytometry (Coulter Electronics). Readings were then converted to red blood cells per ml (rbc/ml) of mouse blood using a standardisation curve.

2.5.7 Measures of transmission

The density of pre-transmission stages of the parasites (gametocytes) was obtained by counting the number of red blood cells containing mature gametocytes (distinguishable from asexual parasites by their morphology and especially pigment as detected by polarised light) in the same thin blood smears used for counting asexual parasites. Between 30 to 100 fields were scanned for each measurement, and the average number of cells per field estimated from the counts of asexual parasites.

Infectivity to mosquitoes was assessed by dissecting typically 15-25 mosquitoes from each pot, i.e. from each mouse-feed. Feeds took place on Days 12 to 15 to obtain a measure of total infectivity in the lifetime of an infection. In Replicates 1 and 2, each mouse was subjected to mosquito feeding only once (two mice per clone on Days 12 and 13 in Replicate 1 and Days 14 and 15 in Replicate 2), and in Replicates 3 and 4, each mouse was used twice (one mouse per clone per day on Days 12, 13, 14 or 15).

Table 2.2. Days on which measurements were taken in each replicate of the experiment

	Weight				Blood cell density				Asexual parasites				Gametocytes				Infectivity to mosquitoes			
Replicate	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Inoculum	10^6	10^4	10^5	10^5																
No. mice per clone	4	4	2	2																
No. clones	6	6	8	8																
Day																				
0	*	*			*	*														
1			*																	
2	*			*																
3		*																		
4	*																			
5			*	*	*															
6		*				*				*										
7	*	*	*	*	*		*	*	*		*	*								
8	*	*																		
9	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*					
10	*	*	*																	
11	*	*	*	*		*	*	*		*	*	*		*	*	*				
12	*	*	*	*	*				*		*	*	*		*	*	*		*	*
13		*	*	*		*	*	*			*	*			*	*	*		*	*
14	*	*	*	*	*				*	*	*	*	*	*	*	*		*	*	*
15		*	*	*			*	*	*			*	*		*	*		*	*	*
16	*	*		*		*				*	*	*		*	*	*				
17				*				*			*	*			*	*				
18	*	*			*	*			*	*		*	*	*						
19											*	*			*	*				
20																				
21		*																		
22	*																			
23		*				*														

2.5.8 Statistical analysis

2.5.8.1 Trait definitions

The traits analysed fell into two categories - virulence traits (parasitaemia, liveweight, anaemia) and transmission traits (gametocytaemia, infectivity to mosquitoes). (Note that parasitaemia is not a measure of virulence *per se* but for convenience is included among the 'virulence traits' for the remainder of the discussion). Within each category, there were two types of traits - the maximum value (or minimum for weight and red blood cell density) of the trait during the infection, and the day on which these maxima/minima occurred, i.e. the timing of the infection pattern. Thus the data analysed comprised only one measurement per mouse for each trait and so the analysis did not involve repeated measures. Because some traits were measured two or more days apart it was probable that in some mice the peak value was not observed: this would cause some inaccuracy in measuring both the levels and timing of the peaks.

Before statistical analysis, the data were transformed to bring the distributions close to normal so that the statistical assumptions of the analyses could be justified. This was not done for liveweight, nor for infectivity to mosquitoes which was analysed taking into account the binomial error structure of the data. It was necessary to apply two transformations to maximum gametocytaemia because the first transformation (arcsine square-root) failed to normalise the data. Trait definitions, abbreviations and their transformations are given in Table 2.3.

2.5.8.2 Experimental replicate and clone effects

A linear model with factors for experimental replicate, clone and number of passages of the clone since isolation from the natural host (Table 2.1) was fitted to the data using least-squares techniques using PROC GLM of the statistical package SAS (SAS, 1990). For analyses of minimum liveweight and minimum blood cell density, a covariate for the average of two liveweights or two blood densities measured early in the infection (Day 0-6) was also fitted to adjust for the mouse's initial values of these variables. Significance levels for each effect were found

Table 2.3 Definitions, transformations and summary statistics of traits used in the statistical analysis

Variable (y)	Definition and units of measurement	Transformation of variable y	[1]No. of records	[1]Mean	[1]Standard deviation
Virulence					
Maximum parasitaemia (%)	Maximum proportion of cells infected with asexual parasites (no. per 100 rbc)	Arcsine$\sqrt{(y/100)}$	73	21.2	10.9
Day of max. parasitaemia	Day of above	None		9.8	2.0
Minimum liveweight (g)	Minimum liveweight reached by mouse around the peak of infection (g)	None	79	18.0	2.2
Day of minimum weight	Day of above	None		10.8	2.4
Minimum blood density (10^9 x rbc/ml)	Minimum rbc density around peak of infection i.e. maximum anaemia	$Log_{10}(y)$-9	79	1.54	1.11
Day of min. rbc density	Day of above	None		11.0	2.2
Parasitaemia at max. gametocytaemia (%)	Parasitaemia on the day of maximum gametocytaemia	$Log_{10}(y)$	71	3.8	6.4
Transmission					
Maximum gametocytaemia (no./1000rbc)	Maximum proportion of cells infected with gametocytes	Log_{10} (Arcsine$\sqrt{(y/10)}$)	71	0.62	0.82
Day of maximum gametocytaemia	Day of above	None		13.5	2.1
Total gametocytaemia	Proportion of cells infected with gametocytes, summed over the whole period of infection (no./1000 rbc)	$Log_{10}(y)$	62	1.63	2.32
Total no. gametocytes ($x10^6$/ml)	Absolute no. of gametocytes produced during the whole infection (calculated from gametocytaemia and blood density)	$Log_{10}(y)$	60	5.62	7.29
Infectivity (%)	Proportion of mosquitoes infected, adjusted for day of feed and then averaged for each mouse.	Logit	30	17.1	23.4

1. From untransformed data over all replicates and clones, excluding control mice

by F-tests: least-squares means of clone and replicate effects were also computed. A clone x replicate interaction was not fitted for these traits because two of the eight clones were not measured in Replicates 1 and 2.

Infectivity to mosquitoes was analysed by fitting a model with factors for experimental replicate, clone, day of feed (Day 12, 13, 14 or 15), number of previous passages of the clone and all two-way interactions (where significant) to data which comprised the number of mosquitoes infected as a proportion of the number of mosquitoes infected for each mouse on each day. This was done using the PROC GENMOD procedure (SAS, 1990) which accounts for the binomial error structure of the data by transforming it to the logit scale. Clone was then dropped out of the model and the residuals from this analysis (on the logit scale) were then averaged for each mouse (to obtain a single data point per mouse, as for other traits): these means on the transformed scale were used for the remaining analyses of between-clone variation.

2.5.8.3 Repeatabilities and correlations

For each trait, estimates of the total phenotypic variance (σ_P^2), between-clone variance (σ_C^2) and within-clone variance (σ_E^2) were estimated by maximum likelihood using the PROC MIXED procedure of SAS (SAS, 1990) by fitting the same models described above, except that clone was fitted as a random effect rather than as a fixed effect. For infectivity data, the residuals on the logit scale were analysed. The proportion of the total variance attributable to between-clone variance measures the repeatability (t) of the trait. Here, repeatability reflects the correlation between repeated measures of a clone both within an experimental replicate and across experimental replicates established by asexual passage, i.e. with no sexual recombination.

Estimates of phenotypic correlations (r_P), genetic correlations (r_G) and residual (within-clone) correlations (r_E) among the traits were also obtained by performing bivariate analysis of variance fitting replicate as a fixed effect and clone as a random effect using PROC GLM and the MANOVA and RANDOM options of

SAS (SAS, 1990). As a check, approximate estimates of genetic correlations were also obtained by computing correlations between the clone least-squares means.

Phenotypic correlations were tested for being significantly different from zero using Pearson's (1938) distribution of the correlation coefficient in small samples. Approximate standard errors of t were obtained using the formula given in Falconer and Mackay (1996), viz.;

$$\sigma_t = \sqrt{\frac{2[1+(n-1)t]^2(1-t)^2}{n(n-1)(N-1)}}$$

where n is the number of mice per clone and N is the number of clones. Approximate standard errors for genetic correlations were obtained using the formula of Robertson (1959), viz:

$$\sigma_{r_G} = \frac{1-r_G^2}{\sqrt{2}}\sqrt{\frac{\sigma_{t_X}\sigma_{t_Y}}{t_X t_Y}}$$

where X and Y denote the two traits in question. Using these standard errors, approximate confidence intervals using the t-test were constructed to determine whether estimates of t and r_G were significantly different from zero. Likelihood ratio tests were also performed to test whether t estimates were different from zero. This required computing the likelihood of the data given the estimate and the likelihood of the data given that t was zero. The natural logarithm of this likelihood ratio is assumed to be distributed as half a chi-squared variable with one degree of freedom under the assumption of large sample size. Sample size was small in this study and so these should be treated as minimum confidence limits. The variance component estimation programme of Meyer (1991) was used for this purpose.

2.6 Results

Across all experiments, 88 mice were measured for their infection patterns of which 8 were uninfected controls. One mouse in the BC clone group failed to develop an infection and so its data were excluded from the analysis. Ten of the infected mice died during the course of the experiment, although all of these deaths occurred under anaesthetic during the mosquito feeds late in the infection and therefore were not directly due to excessive virulence. An analysis of variance showed that the mice which died under anaesthetic had significantly higher ($P<0.05$) parasitaemias on the day before death (days 12-19), but not significantly higher weight loss, anaemia or parasitaemia earlier in the infection. This indicates that uncontrolled parasitaemia correlates with the probability of host death.

A total of 1213 mosquitoes were dissected from a total of 59 feeds on 31 different mice giving an average of 20.1 mosquitoes per feed. No mosquitoes became infected in Replicates 1 and 2. This was probably due to the omission of PABA from the glucose preparation on which mosquitoes were fed.

To illustrate the typical infection patterns and variation between mice, the data on parasitaemia, weight, red blood cell density and gametocytaemia during the infection are shown in Figure 2.1 for four mice infected with the AJ clone in Replicate 2. Distributions of the data before and after transformation are shown in Figure 2.2. Means and standard deviations of the traits over all experimental replicates and clones are shown in Table 2.2. Typically, maximum parasitaemias of 21% were reached on Days 8-10, followed by rapid weight loss and anaemia the next two days, after which a peak of gametocytes was reached between Days 12 and 16. When gametocytes reached a peak, asexual parasitaemias were around 4%.

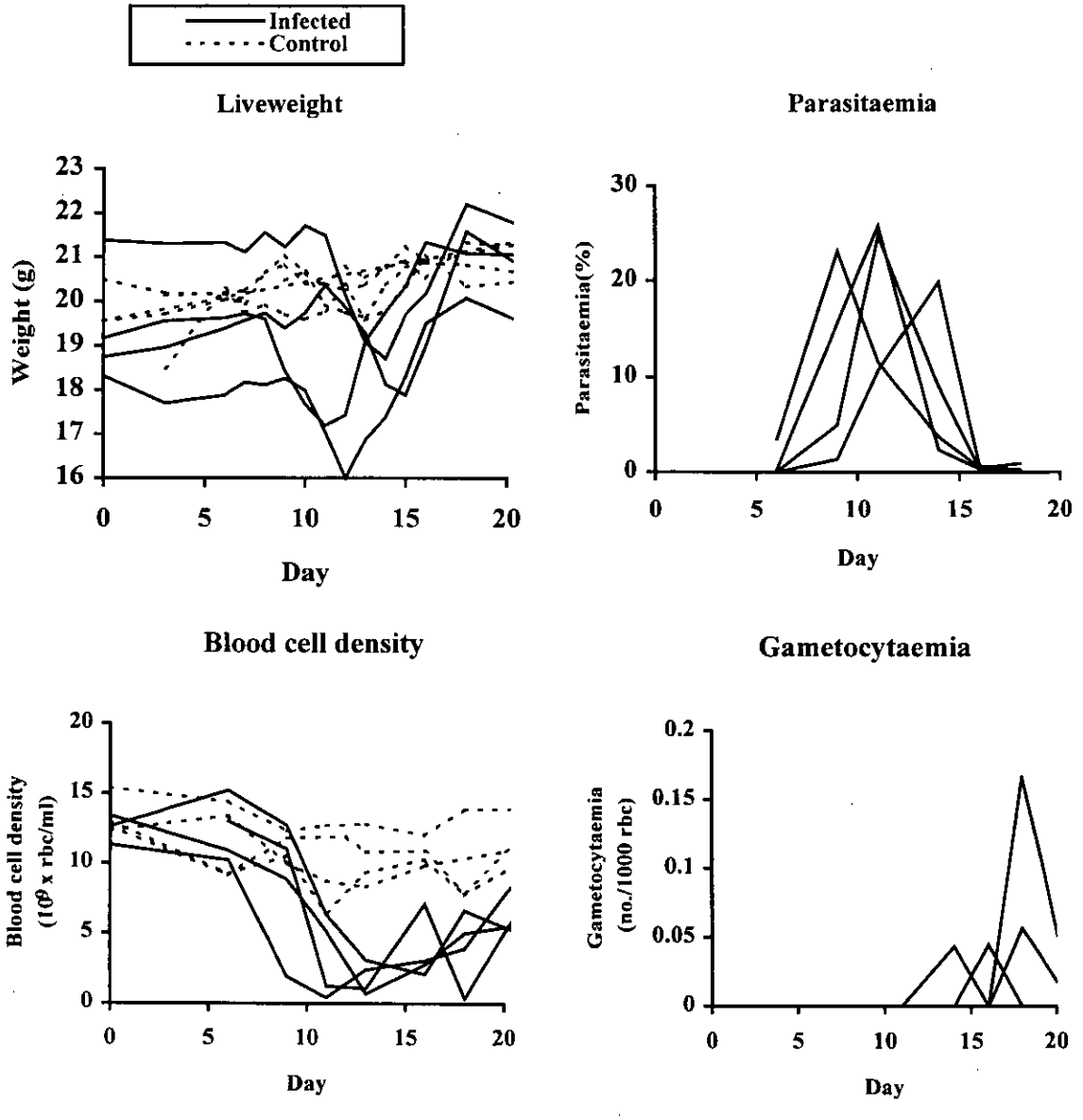


Figure 2.1. Infection patterns for individual mice infected with clone AJ in Replicate 2.

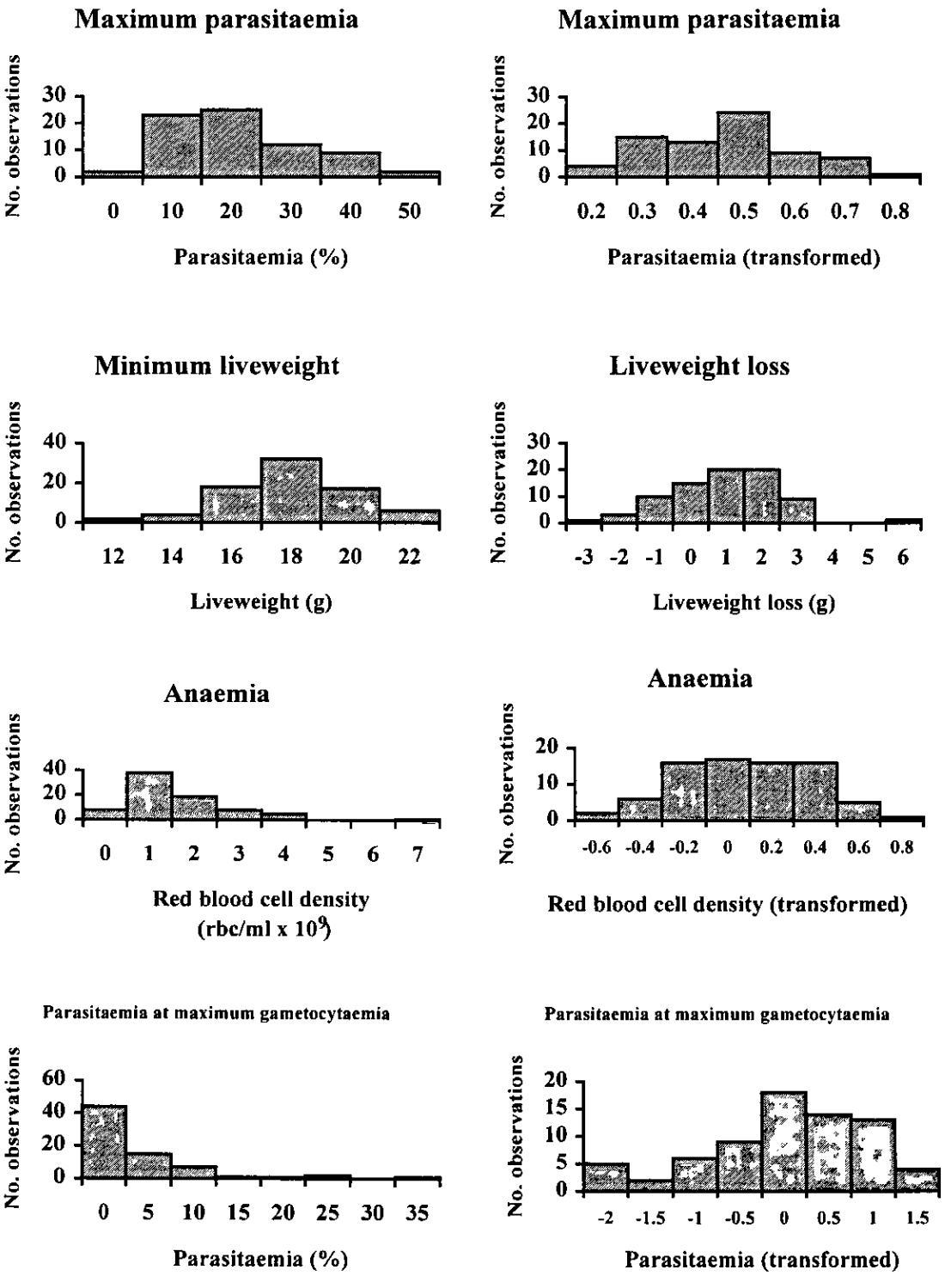


Figure 2.2. Distributions of raw data for virulence and transmission traits before (left) and after (right) transformation. (Continued overleaf).

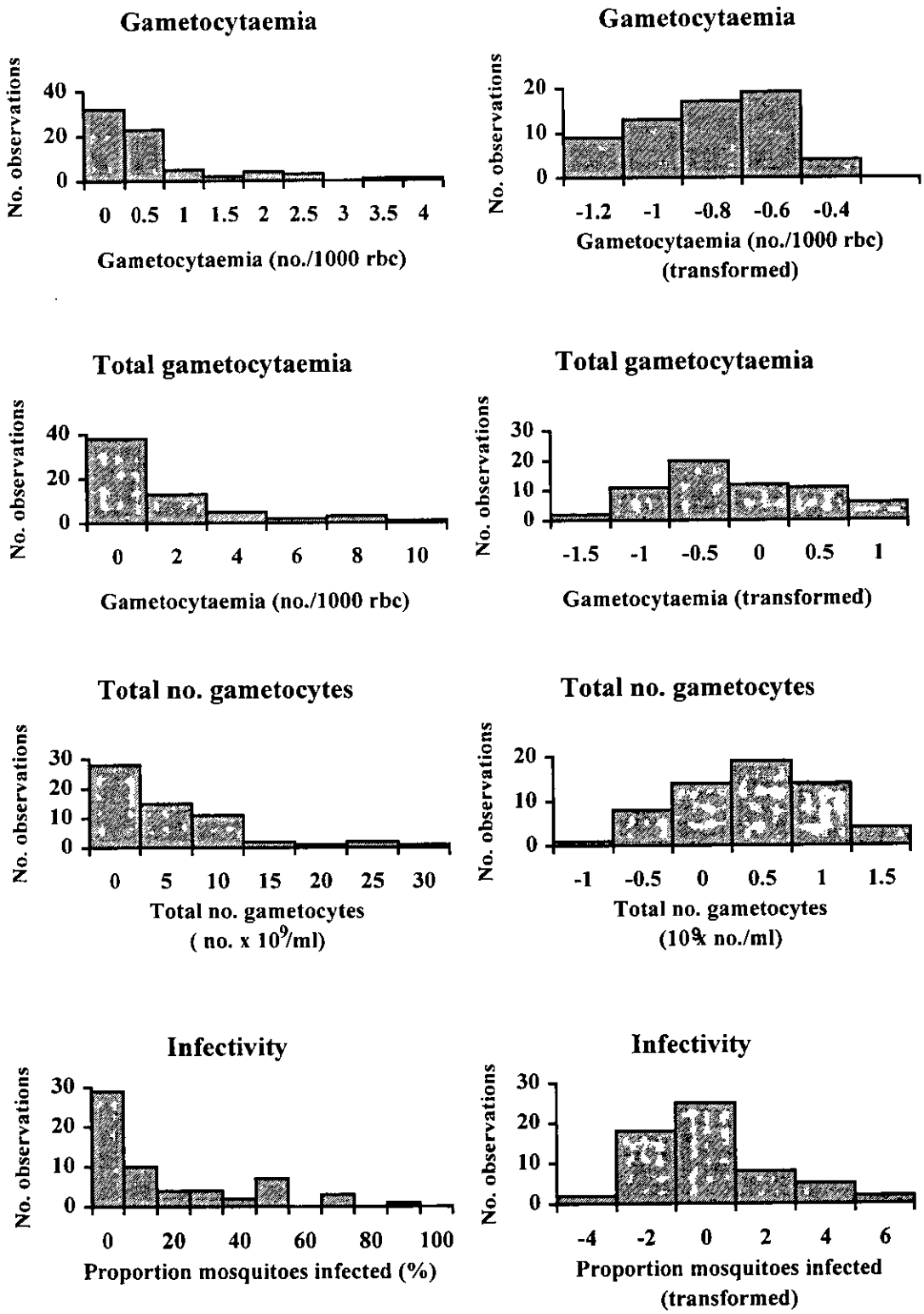


Figure 2.2. Distributions of raw data for virulence and transmission traits before (left) and after (right) transformation. (Infectivity data are for individual feeds with two feeds per mouse). (Continued from previous page).

2.6.1 Effects of experimental replicate and inoculum size

There was no significant effect ($P > 0.05$) of the number of previous passages of the clone on any of the traits and so this factor was dropped from the model for the remaining analyses.

Figure 2.3 shows liveweights, parasitaemia and gametocytaemia over the course of the infection averaged for each replicate. From these it can be seen that a 10-fold reduction in the size of the inoculum resulted in a 1-2 day delay in the course of the infection. It appears from this figure that peak parasitaemias occurred on days 7, 9 and 11 for inocula of 10^6, 10^5 and 10^4 respectively, followed by minimum weights one day later, peak anaemia (minimum blood cell density) two days later, and peak gametocytaemia on Days 12, 13 and 14. However, these estimates of timing effects are biased by day of sampling which differed between replicates, and are inaccurate because most measurements were taken two days apart. Least-squares estimates of replicate effects (Table 2.4) bear this out because they show that the replicates were not consistent in the average delay between peak parasitaemia and peak gametocytaemia. Even though comparisons between inoculum size were not contemporaneous, the analysis of replicate effects does show a clear and significant delay in the infection as inoculum size is reduced (Table 2.4) suggesting that higher establishment rates of the parasite led to more rapid onset of symptoms.

The other main result from the comparison between replicates is that there were no significant differences in weight loss, anaemia or total number of gametocytes, but there were significant differences between replicates in the level of parasitaemia and gametocytaemia. These differences did not rank consistently with inoculum size and therefore may be an artefact of the incomplete sampling regime (i.e. peaks may have been missed) or some other unexplained condition affecting parasite growth, e.g. the concentration of PABA supplied in the water.

In summary, the replicate comparisons suggested that larger inocula led to earlier infections but this did not lead to higher virulence or transmission.

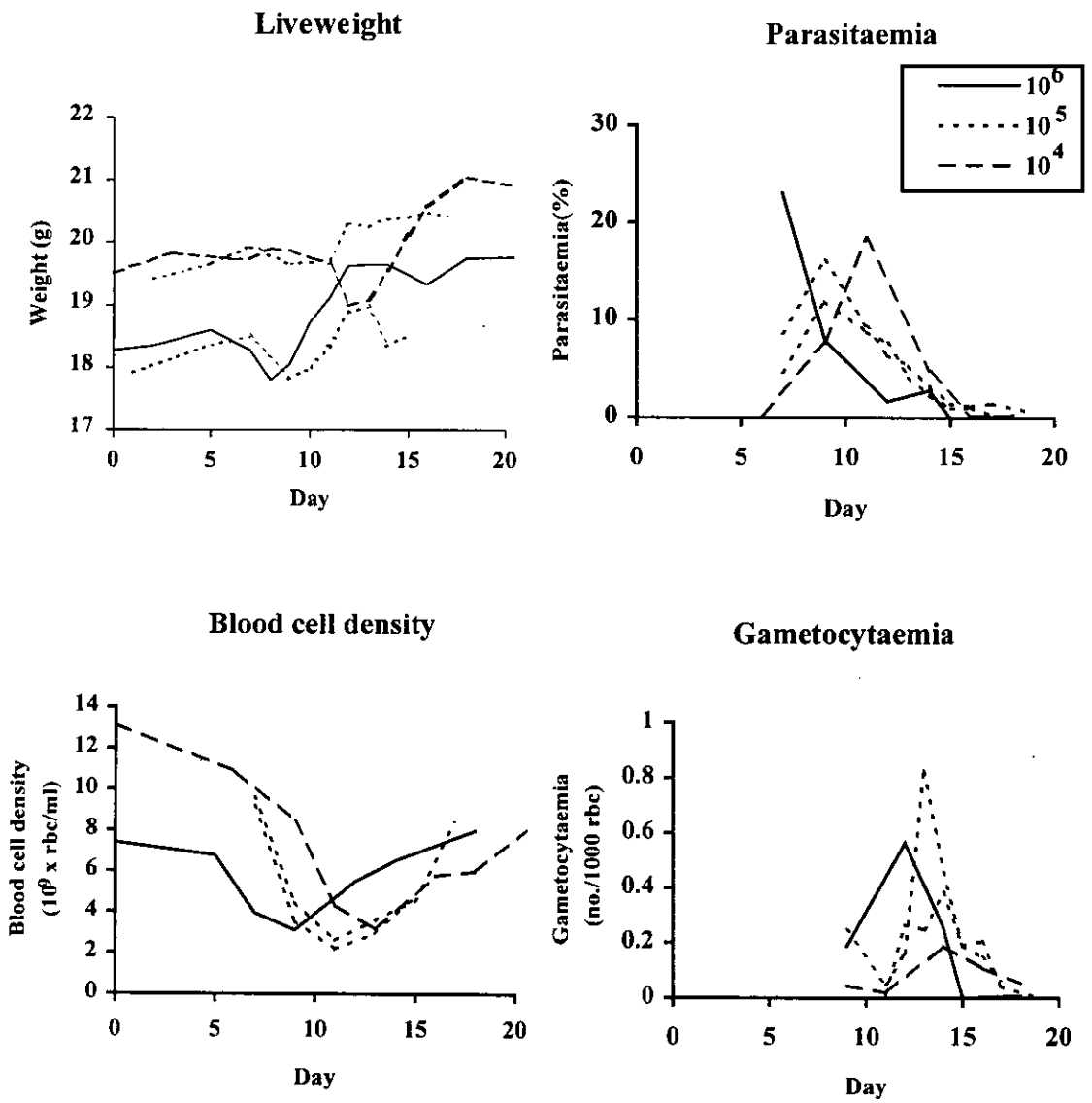


Figure 2.3. Mean infection patterns for four experimental replicates started with inocula of either 10^4, 10^5 or 10^6 parasites per mouse.

Table 2.4 Effects of initial inoculum size (replicate least-squares means[1]) on virulence and transmissibility traits

	Replicate				Signif.	[2]s.e.
Inoculum size (parasites per mouse)	10^6	10^5	10^5	10^4		
Replicate no.	1	3	4	2		
<u>Virulence</u>						
Max. parasitaemia						
- value (%)	22.0	20.1	15.0	19.5	n.s.	1.8
- day	7.7	10.1	10.4	11.2	***	0.4
Min. weight						
- value (g)	18.1	18.1	18.4	17.5	n.s.	0.4
- day	8.7	11.1	10.8	12.7	***	0.5
Min. blood density						
- value (rbc x 10^9/ml)	1.37	1.02	1.67	1.14	n.s.	0.3
- day	9.2	11.4	10.9	13.1	***	0.5
Parasitaemia at max. gametocytaemia	0.8	2.6	2.1	0.4	*	0.4
<u>Transmission</u>						
Max. gametocytaemia						
- value(no./1000 rbc)	0.33	0.70	0.29	0.19	**	0.3
- day	12.2	13.1	13.2	14.9	***	0.4
Total gametocytaemia						
- value (no./1000 rbc)	0.83	1.26	0.51	0.44	*	0.8
Total no. gametocytes						
- value (no. x 10^6/ml)	3.78	4.60	1.78	2.14	n.s.	1.7
Infectivity						
- value(%)	-	-	19.9	16.0	n.s.	7.0

1. Estimates have been back-transformed to the original scale.
2. Standard errors of the replicate means, back-transformed.

2.6.2 Clone effects on virulence and transmission traits

Significant differences between clones were found for all traits relating to levels of parasite virulence and transmission except for infectivity (Table 2.5). For example, some clones (CW and AS) were very avirulent having 8% peak parasitaemia, causing no liveweight loss, and transmitting to 1-3% of mosquitoes, and some clones were very virulent (AT, BC and ER) reaching greater than 24% parasitaemia, causing 15% liveweight loss and transmitting to 12-28% of mosquitoes. Clones of medium virulence were also found (AD, AJ and AQ). Except for infectivity, repeatabilities for these traits were moderate (between 30 and 45%, Table 2.6) thus indicating that these were stable and repeatable characteristics of the clones when measured across different experimental replicates and mice. There was no variation in the level of asexual parasitaemia at the time of peak gametocytaemia: the mean for this trait was low (4%), however, and so any between-clone differences would have been difficult to detect. The repeatability for infectivity was low ($t=0.09$) because three of the clones with low infectivity in Replicate 3 had the highest infectivity in Replicate 4 thus causing a significant clone by replicate interaction ($P<0.01$) due to a change in ranking of the clones across the replicates (Table 2.5): this generated a between-replicate correlation (repeatability) of -0.35. However, within Replicates 3 and 4, the repeatability of the clones were 0.65 and 0.88, respectively.

In contrast to the genetic variation in peak values reached during the infection, there was no between-clone variation in the timing of the infection (Tables 2.5 and 2.6) suggesting that timing is more related to stochastic events such as number of parasites in the initial inoculum which become established rather than to the intrinsic rate of growth of the parasite. Alternatively, the inaccuracy of measuring timing traits may be responsible for the large amount of within-clone variation relative to between-clone variation. Repeatabilities for timing traits were all less than 9% (Table 2.6). Correlations with other traits were therefore not estimated.

Table 2.5 Effects (least-squares means[1]) of clone on virulence and transmissibility traits

Trait	Clone									[2]s.e.	[3]Signif
	AD	AJ	AQ	AS	AT	BC	CW	ER	Control		
Virulence											
Max. parasitaemia											
- value (%)	19.3	19.2	23.3	8.4	27.5	27.6	8.6	24.0	-	5.2	**
- day	10.4	9.4	9.7	10.2	8.8	9.8	10.2	9.5	-	0.8	n.s.
Min. weight											
- value (g)	18.2	17.8	18.6	19.5	16.9	16.9	19.4	16.8	20.1	0.7	***
- day	10.9	10.9	11.0	10.3	10.0	10.9	10.8	11.3	-	1.1	n.s.
Min. blood density											
- value (rbc x 10^9/ml)	1.68	0.98	1.19	2.79	0.77	1.03	2.07	0.78	10.53	0.58	***
- day	11.7	10.8	10.8	11.5	10.0	11.1	11.3	12.0	-	0.9	n.s.
Parasitaemia at max. gametocytaemia	1.6	1.0	2.2	0.5	0.9	1.6	0.6	2.9	-		n.s.
Transmission											
Max. gametocytaemia											
- value(no./1000 rbc)	0.40	0.14	1.17	0.23	0.29	0.26	0.18	0.81	-	0.46	***
- day	13.5	14.4	13.2	13.8	14.7	13.1	13.2	13.9		0.9	n.s.
Total gametocytaemia											
- value (no./1000 rbc)	0.58	0.22	3.37	0.44	0.64	0.40	0.36	3.01	-	1.30	***
Total no. gametocytes											
- value (no./10^6 rbc)	2.31	0.84	9.52	1.85	3.21	1.94	1.48	13.96	-	2.24	***
Infectivity											
- value (%) Overall	22.2	12.6	29.2	5.4	18.0	18.4	7.5	24.6	-	13.4	n.s.
Rep. 3	18.6	24.8	58.7	13.6	36.0	15.1	10.2	4.1			
Rep. 4	25.7	1.1	12.3	0.0	6.4	55.2	2.6	39.6			

1. Estimates have been back-transformed to the original scale.
2. Average standard errors of clone means, back-transformed.
3. Controls excluded from data for these significance tests.

Table 2.6. Estimates of repeatability and variance components for virulence and transmissibility traits

Trait	[1]Variance component			t	[2]s.e. of t	[3]-2lnLR
	σ_C^2	σ_E^2	σ_P^2			
Virulence						
Max. parasitaemia	0.009	0.011	0.020	.45	.16	11.3***
Day of max. parasitaemia	0.118	1.891	2.008	.06	.09	0.8
Min. weight	1.001	1.297	2.298	.44	.16	9.6**
Day of min. weight	0.000	3.288	3.288	.00	.08	0.0
Min. blood density	0.0278	0.0634	0.0915	.30	.15	5.2*
Day of min. blood density	0.010	2.464	2.564	.04	.07	0.2
Parasitaemia at maximum gametocytaemia	.000	0.686	0.686	.00	.07	0.0
Transmission						
Max. gametocytaemia	0.00075	0.00156	0.00023	.32	.15	5.9*
Day of max. gametocytaemia	0.284	2.980	3.264	.09	.08	0.2
Total gametocytaemia	0.149	0.240	0.389	.38	.16	8.1**
Total no. gametocytes	0.126	0.228	0.354	.36	.16	6.2*
Infectivity	0.389	3.833	4.221	.09	.16	1.5

1. Estimates are on the transformed scale. σ_C^2, between-clone variance; σ_P^2, phenotypic variance; σ_E^2, residual variance; t, ratio of between-clone variance to phenotypic variance.
2. Approximate standard errors of t.
3. Twice the log likelihood ratio for testing the null hypothesis that t=0. Significance tests are based on the chi-squared distribution with one degree of freedom

2.6.2.1 Phenotypic and genetic relationships among traits

Phenotypic, genetic and residual correlations among traits are shown in Table 2.7 and some key relationships are illustrated in Figures 2.4 and 2.5. In these figures, data are on the untransformed scale to illustrate the actual levels of variation and covariation observed.

There was a strong relationship between peak parasitaemia early in the infection and the two virulence measures, weight loss and anaemia (Table 2.7,

Figure 2.4a). This confirms the first of the assumptions of the trade-off hypothesis, namely that virulence is a consequence of fast early parasite replication rate. These relationships held at both the genetic and non-genetic levels (i.e. across clones, and within mice of the same clone). Correlations among transmission traits were also strong, especially at the genetic level, thus validating the use of pre-transmission forms (gametocytes) in the host as indicators of actual transmission to the vector (Table 2.7, Figure 2.4b). This was despite a low repeatability of infectivity across replicates. Parasitaemia at the time of peak gametocytaemia was strongly genetically correlated to transmission traits indicating that persistence of the infection was a key factor in transmission output from the infection.

Correlations between early parasitaemia and transmission traits were positive, especially at the genetic level, thus supporting the second of the assumptions of the trade-off model which is that higher replication rates are linked to higher levels of transmission. These correlations were significant only for lifetime transmission to mosquitoes ($P < .05$): the lack of significance for the positive correlations between peak parasitaemia and gametocyte production was probably due to a lack of statistical power, although it should be remembered that the significance tests are very approximate. Correlations between gametocyte production and the traits reflecting virulence - weight loss and anaemia – were low indicating that gametocyte production is not directly influenced by the amount of damage incurred to the host. Instead, the asexual parasitaemia at the time of peak gametocyte production was moderately related to gametocyte output suggesting that transmission success depends on the persistence of the infection after the crisis period. The high genetic correlations underlying this relationship are somewhat unreliable because the amount of between-clone variance in this trait was very low (Table 2.6).

In summary, the phenotypic and genetic correlations suggest that parasites with a genetic ability to replicate faster in the asexual phase are genetically more able to transmit at higher levels, but cause higher levels of virulence as a consequence.

Table 2.7. Phenotypic, genetic and residual correlations among virulence and transmissibility traits

Trait	Trait no.	[1]Phenotypic correlations 1	2	3	4	5	6	7	8
Virulence									
Max. parasitaemia	1	1	-.71***	-.60***	.17	.18	.21	.22	.44*
Min. weight	2		1	.72***	-.28*	-.11	-.06	-.12	-.18
Min. blood density	3			1	-.19	-.10	-.06	-.02	.23
Parasitaemia at max. gam.	4				1	.36**	.28*	.20	.32
Transmission									
Max. gametocytaemia	5					1	.95***	.89***	.63***
Total gametocytaemia	6						1	.94***	.74**
Total no. Gametocytes	7							1	.77***
Infectivity	8								1
		Residual correlations 1	2	3	4	5	6	7	8
Virulence									
Max. parasitaemia	1	1	-.53	-.40	.00	.00	.00	.08	.17
Min. weight	2		1	.61	-.29	-.16	-.16	-.18	.20
Min. blood density	3			1	-.17	-.09	-.08	-.06	.60
Parasitaemia at max. gam.	4				1	.30	.09	.20	.15
Transmission									
Max. gametocytaemia	5					1	.93	.86	.46
Total gametocytaemia	6						1	.90	.58
Total no. Gametocytes	7							1	.59
Infectivity	8								1
		Genetic correlations [2](s.e.) 1	2	3	4	5	6	7	8
Virulence									
Max. parasitaemia	1	1	-.91*** (.04)	-.88*** (.06)	.69 (.48)	.45 (.24)	.38 (.24)	.38 (.24)	.78** (.28)
Min. weight	2		1	.90*** (.07)	-.40 (.53)	-.03 (.30)	.06 (.28)	-.03 (.29)	-.70* (.37)
Min. blood density	3			1	-.32 (.96)	-.12 (.36)	-.02 (.33)	.02 (.34)	-.40 (.74)
Parasitaemia at max. gam.	4				1	.79 (.40)	.75 (.46)	.72 (.52)	.53 (1.55)
Transmission									
Max. gametocytaemia	5					1	.97*** (.05)	.93*** (.05)	.83** (.27)
Total gametocytaemia	6						1	.98*** (.01)	.86** (.26)
Total no. Gametocytes	7							1	.91*** (.17)
Infectivity	8								1

1. Significance tests for phenotypic correlations being different from zero are based on Pearson's statistic for correlations in small samples.
2. Standard errors of genetic correlations are calculated from Robertson's (1959) approximation. Approximate significance levels for testing that correlations are different from zero are based on a two-tailed t-test.

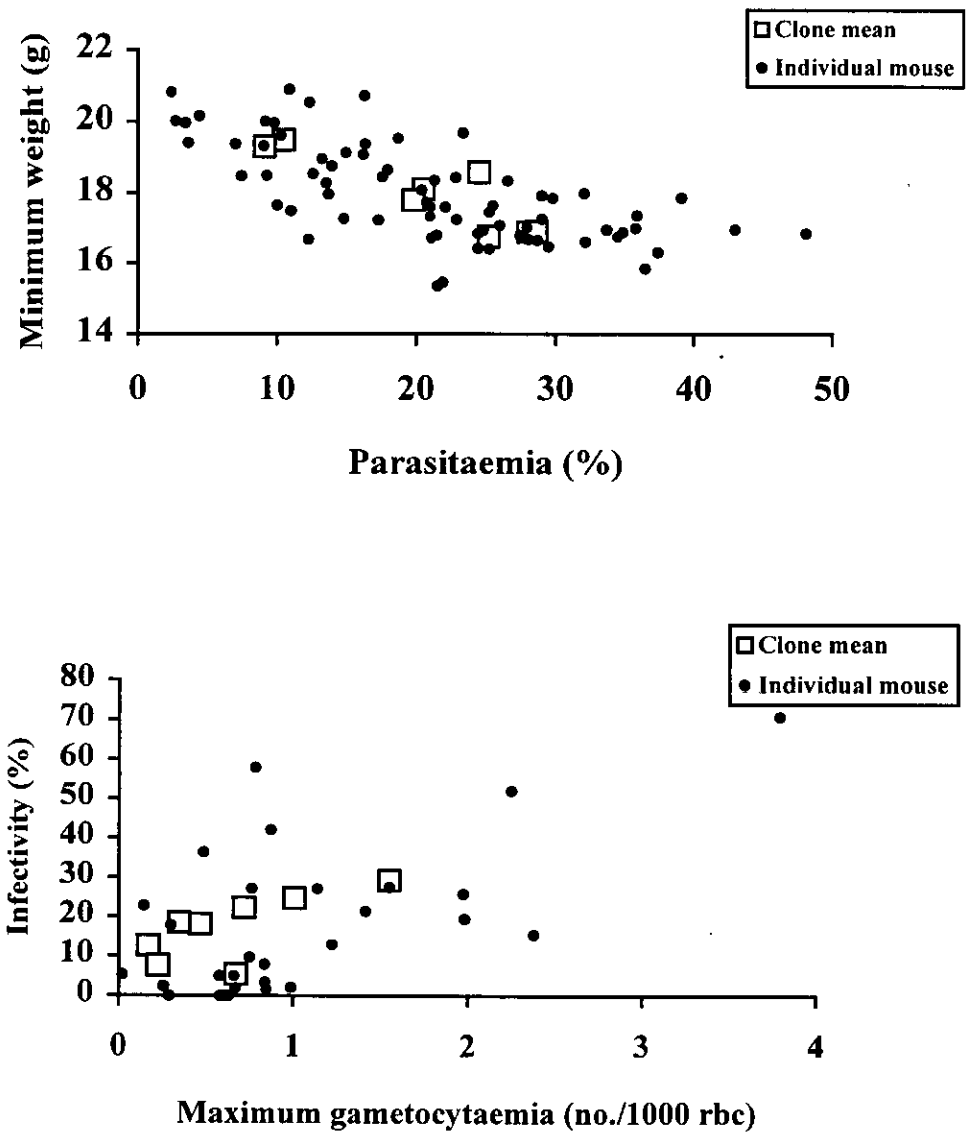


Figure 2.4 Phenotypic (circles) and genetic (squares) relationships between minimum liveweight as a measure of virulence and parasitaemia as a measure of replication rate (upper), and infectivity and gametocytes (lower).

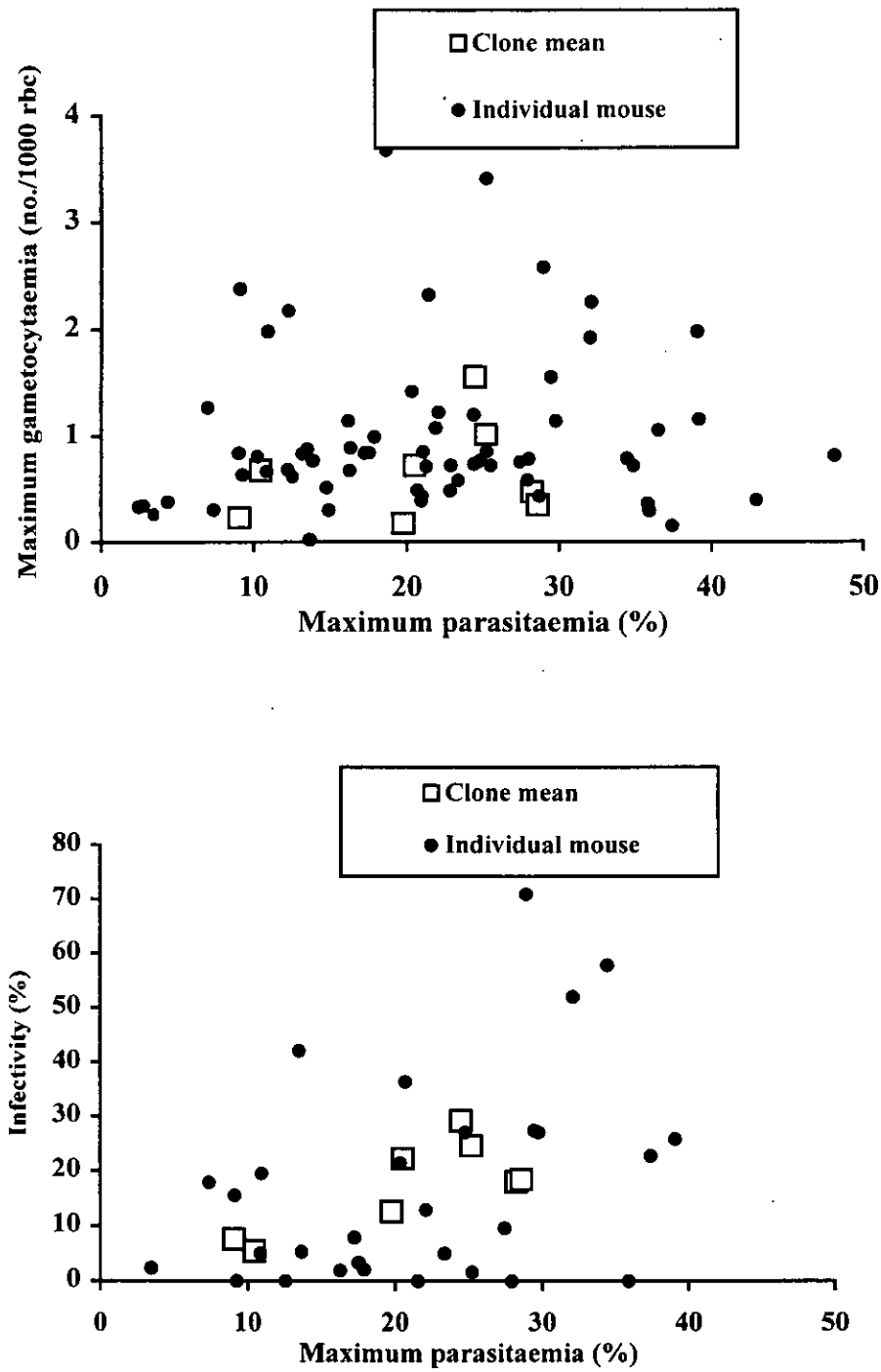


Figure 2.5. Phenotypic (circles) and genetic (squares) relationships between two measures of transmission (gametocytes - upper, infectivity - lower) and replication rate.

2.7 Discussion

This study has shown that *P. chabaudi* parasites show substantial levels of genetic variation in virulence and transmission traits in the laboratory mouse and that the relationships between these two traits seem to be integrally linked by genetic factors within the parasite. These observations support one arm of the trade-off hypothesis which is that virulence evolves to higher levels because of the parasite's need to grow fast in order to reproduce more. The two fundamental assumptions in this hypothesis - that virulence is a by-product of rapid asexual replication, and that faster replication results in higher transmission – were explicitly tested and found to be supported by the data. This is an important finding as it validates the trade-off hypothesis for one of the most important microparasites of man, and other relevant data are extremely scarce for parasites of vertebrate hosts. The implications for malaria are that selection pressure imposed on parasite replication rate (e.g. through use of asexual stage vaccines) is expected to produce a correlated increase in virulence to the point which optimises parasite fitness. The effectiveness of such artificial selection in the field situation, where natural selection through acquired immunity in the host and by vectors (either directly by the mosquito itself, or indirectly by the density of the mosquito population), will already be operating, is difficult to ascertain, and such predictions from the laboratory must be treated with caution. Nevertheless, during the era of malaria therapy for neurosyphilitics, during which natural transfer of parasites by mosquito feeding and inoculation was regularly used, it was observed that the Madagascar strain increased in virulence with a concomitant increase in gametocyte production (James et al., 1936). James et al. (1932) also concluded that virulence of a strain was directly related to its asexual parasite growth rate. The findings of the present study are concordant with these early observations from a semi-natural environment, obtained from much accumulated experience with a diverse range of parasite strains.

The other arm of the hypothesis - that parasites also need to limit this growth to avoid killing the host and hence its opportunities for reproduction – was not able

to be tested properly because there was low host mortality in these experiments. However, the few host deaths that did occur appeared to be associated with higher parasitaemias, and so it is expected that if higher levels of virulence had been reached, as occurs in other strains of laboratory mice (Stevenson et al., 1982), there would have been more host mortality. The phenomenon of cerebral malaria may be an example of this: if parasitaemia is too high, or if there is excessive cytoadherence of the parasite to achieve maintenance of asexual parasite populations, the result can be blockage of capillaries in the brain which produces this lethal form of the disease.

The question arises as to why higher replication rates lead to higher transmission. It is known that gametocytes are the products of a small proportion ($<1\%$) of schizonts grown from asexual parasites. Gametocytes do not replicate and take about two days to mature, thus their numbers should relate directly to the ability of the asexual population to grow. In this study, and in others (Taylor, 1997b, in press), the analysis failed to find a clear positive relationship between asexual parasite numbers at the peak of infection and gametocyte numbers after crisis. Given that there is a huge decrease in numbers of asexual parasites during crisis, and that the majority of gametocytes are formed when asexual parasitaemias are still low, the lack of a significant relationship between these traits is perhaps expected. However, the number of asexual parasites at the time of peak gametocytaemia was more strongly related to gametocyte numbers, and so it is suggested that transmission does depend on the maintenance of asexual parasite populations, especially later in the infection. This ability to maintain population numbers (of either asexuals or gametocytes) may have a number of components which can be broadly divided into those which relate to either birth or death rate of parasites. In the birth rate category, factors which could contribute to more rapid replication are higher numbers of merozoites per schizont (for which there is considerable genetic variation between species (Cox, 1988)), higher conversion rates of asexual parasites into gametocytes, or more successful invasion rates of merozoites into uninfected cells (e.g. could it be that rosetting, which is the surrounding of an infected cell by many uninfected cells and a determinant of

virulence (Carlson et al., 1990; Rowe et al., 1995), is a mechanism for increasing the success of merozoite invasion?). Death rates may be determined by the ability to cytoadhere and sequester and therefore avoid killing by the spleen, by efficacy of immune evasion through switching surface antigens, or by immune killing through non-specific mechanisms. While many details are known about some of these processes, the relative rôle of each in sustaining parasite populations within the host are undefined. Empirical studies of specific aspects of within-host parasite population dynamics are required to establish whether, for example, the degree of sequestration, or the ability to phenotypically switch antigens, are crucial determinants of parasite survival, as is commonly thought. In particular, such experiments should monitor the infections for a longer period than was done in this study to determine the impact of these factors on total transmission during the parasite's lifetime.

Alternatively, there may be a less functional link between asexual parasite numbers and transmission than discussed above. For example, conversion rates may be so plastic that gametocyte numbers are not able to be predicted by asexual numbers. In this case, for there to be a genetic link between parasitaemia and transmission, there would have to be a third extrinsic factor which influenced both traits. One such possibility is that immune killing may be effective against both asexual parasites and gametocytes: this appears to be realistic since PfEMP1 is expressed on the surface of cells containing gametocytes as well as those infected by asexual parasites (Hayward, 1997). Another possibility is that host-derived factors induced against asexual parasites cause a decrease in infectivity to mosquitoes with no reduction in parasite numbers. This appears to be true for *P. berghei* and *P. vivax* where temporary decreases in infectivity to mosquitoes are observed during crisis (reviewed by Sinden et al., 1996; Carter et al., 1997).

The variation between clones has so far been interpreted to be genetic in origin. However, the possibility must also be considered that some environmental factor caused a correlation between mice infected with the same clone and therefore a higher relative amount of between-clone variation to within-clone variation. Two potential sources of covariance are envisaged here. One is due to injecting mice

with the same clone from just one syringe, i.e. mice were not infected independently within each experimental replicate. They were also housed in the same box which may have caused a correlation due to a common environment. However, the fact that the clones were repeatable *over* experimental replicates as well as within replicates suggests that this was not an important influence. The second potential source of covariation is the transfer of some unidentified factor (e.g. in the parasite's cytoplasm, or another pathogen in the blood) during serial passage. If such an agent had been acquired before the experiment (e.g. during the cloning process), it may have generated clone differences which were then maintained during the course of the experiment. It is difficult to rule out such a possibility. If it was true, then the phenotypic correlations would be valid, but the genetic correlations would not be due to parasite genetics, but instead due to common environmental influences generated by the concomitant organism.

The parasite clones used in these experiments were expected to consist of one genotype only. However, the possibility that there was more than one genotype in the cloned line of parasites could not be ruled out. If, in fact, mixtures of genotypes were present in some of the clones, the genetic correlations might be biased as upwards it is known that mixed genotype infections cause an increase in virulence (Taylor et al., 1998) and in transmission (Taylor et al., 1997a,b), at least for two particular clones (ER and CR). Furthermore, if the complexity of the clonal populations had increased during the passages following cloning, with a concomitant effect on growth rate, virulence and transmission, the correlations may have been biased.

This study considered only one stage in the transmission cycle. There are many factors that determine whether a parasite is successful in its reproductive efforts. For example, the probability of transmission may be enhanced by higher numbers of oocysts and sporozoites in the mosquito, or by higher establishment rates in the liver and in the bloodstream upon rupture of the liver schizonts. The latter is one way in which early parasite replication rate may be genetically linked to transmission in the broadest sense.

One further observation in this study deserves mention here. Minimum red blood cell density (inversely related to anaemia) was negatively related to infectivity to mosquitoes at the genetic level (not significantly, r_G=-.40), consistent with the adaptive trade-off hypothesis and all other observed correlations between virulence and transmission. However, at the non-genetic level (within-clones), red blood cell density was positively related to infectivity (r_E=.60), and this was the only example of a clear conflict between genetic and non-genetic correlations. One hypothesis is that mosquitoes which do not get an adequate supply of nutrients from an anaemic host are not able to sustain an oocyst infection (reviewed by Hurd et al., 1995) thus potentially creating a negative non-genetic relationship between anaemia and infectivity to mosquitoes as observed here. Alternatively, the successful pairing of male and female gametes to produce oocysts may depend on having a high density of gametocytes in the blood meal. This latter hypothesis was examined by adjusting for gametocyte density by including it as a covariate in the model. It was found that even though the genetic correlation between anaemia and infectivity strengthened to r_G=-.75, but the phenotypic and residual correlations were unchanged (r_P=.17, r_E=.60). Thus these results do not support the hypothesis that infectivity is decreased by anaemia through an effect on gametocyte density, and are otherwise difficult to interpret. A further complicating factor in this relationship is that high virulence in the mammalian host could well be associated with high virulence in the mosquito host through excessive oocyst burdens. However, as the amount of mortality among mosquitoes was not recorded, it was not possible to evaluate this latter hypothesis.

The host, vector and environment were all foreign to the parasite in this study. Therefore it is perhaps expected that such large genetic variation was expressed in this artificial system. It is not known whether such variation exists in the natural environment of *P. chabaudi*, nor in other *Plasmodium* species, although theoretical models generally predict that it should. It is also possible that such variation was generated in the laboratory during the necessary passages prior to cloning. Such variation may have arisen through mutation and/or selection from the hosts. The possibility of non-genetic properties being acquired during repeated

passages, such as a new phenotypic antigenic type arising and becoming established within a cloned line, cannot be ruled out. However, it is difficult to see how an antigenic type in one clone would confer a growth advantage over another clone when grown in naive hosts. On the other hand, an increase in the degree of antigenic variation being expressed may have conferred an advantage to some clones. The high level of stability, or repeatability, of the traits over replicate experiments, and the lack of significance of the effect of the number of previous passages, suggest that non-genetic or genetic properties acquired during asexual passage were not important during the short series of asexual passages during these experiments. Nonetheless, even if such variation is not observed in the field, the demonstration in this study of intrinsic genetic ties between parasite growth and reproductive success supports the view that parasite virulence is driven towards a level which involves a compromise between the parasite and its host.

Chapter 3

Parasite genetics: a selection experiment for virulence in *Plasmodium chabaudi*

3.1 Summary

Within-clone selection for high or low virulence, as defined by the amount of weight lost by the mice which they infected, was performed for eleven generations in each of two clones (BC and CW) of *P. chabaudi* parasites. Parasites were transmitted by blood passage and did not undergo fertilisation and meiosis. It was found that, despite selection in both directions, all lines steadily increased in virulence over the generations. Associated with selection were increases in asexual parasitaemias and levels of anaemia: these potentially negative effects were rewarded by higher numbers of gametocytes. However, host mortality rates did not increase with selection. Strikingly, the clone which had very low virulence at the beginning of the experiment (clone CW) increased in virulence much more rapidly than the clone which was already virulent (clone BC). The results suggest that these parasites, when allowed to evolve in hosts and conditions which are different from those they encounter in their natural environment, are able to adapt rapidly to these new conditions. The opportunities for mutation and selection to occur within the vast asexual populations of parasites within individual hosts is suggested to be an important mechanism used by the malaria parasite to survive and persist in the face of strong acquired immunity by the host. The possible involvement of mechanisms relating to antigenic variation and cytoadherence in adapting to a new host are also discussed.

3.2 Introduction

In the previous chapter it was demonstrated that there was large genetic variation in virulence, replication rate and transmissibility among clones (genetically uniform lines) from a natural population of rodent malaria parasites when examined in a laboratory setting. This variation, which is probably due to many genes and gene combinations, was measured at the between-clone level. Between-clone variation is the substrate for the processes of selection and recombination to bring about genetic changes in these traits to suit the prevailing transmission and host environment. There is, however, another potential source of genetic variation on which within-clone, within-host selection can act - that of within-clone variation. Given the ample opportunity for mutations to arise from among the vast number of asexual parasites produced within the one infection, and the very expansive growth of parasite populations within a single host, some within-clone variation may also be expected. The amount will depend on the mutation rate and the selective advantage or disadvantage of the mutation, especially early on in the infection when the population is relatively small. Furthermore, within-clone 'genetic' variation can arise from the programmed switching of antigenic types on the surface of the red cell membrane (reviewed by Borst et al., 1995). Even though in the previous experiment the between-clone variation swamped the within-clone variation, selection within clones may still contribute much to the rate of parasite evolution.

There is much evidence that within-clone selection is responsible for genetic changes in parasites maintained in artificial laboratory environments. Increases in virulence with continual asexual or mixed asexual and sexual passages (i.e. including mosquito transmission) of various species of malaria parasites have often been reported (Bishop, 1954; Galli et al., 1967; Hartley, 1969, Sergent and Pancet, 1959, all cited by Alger et al., 1971; Contacos et al., 1962, Chin et al., 1968; Yoeli et al., 1975; Dearsly et al., 1990). Many of these changes have been rapid and have been associated with a distinct change in preference for the younger blood cell types (reticulocytes) instead of the normally preferred mature erythrocytes (Alger et al., 1971, Yoeli et al., 1975). Mutations with large effect, followed by

inadvertent selection for faster growing parasites, have been assumed to be the cause of such rapid changes. Walliker et al., (1976) demonstrated that this was likely to be the case for the virulent clone of Yoeli et al. (1975) because when crossed with a clone of low virulence, the progeny clearly segregated for high and low virulence. However, the segregation ratio was not 50:50 as expected from Mendelian inheritance: this may have been due to the difficulty of detecting avirulent clones. When this same virulent clone was further crossed with an another independently derived clone of intermediate virulence, 10% of the progeny were avirulent (Walliker, 1981) suggesting that the high and intermediate parent clones may have been carrying different mutations at separate loci. Walliker et al. (1976) also showed that the virulence phenotype was stable through mosquito transmission.

Decreases in virulence have also been shown to occur during maintenance of malaria parasites in laboratory animals (Carrescia and Arcolea, 1957, in Alger et al., 1971; Alger et al., 1971; Knowles and Walliker, 1980). However, while Knowles and Walliker (1980) found that mosquito passage restored virulence to these clones, Alger et al. (1971) repeatedly observed losses of virulence during mosquito passage. Similarly, maintenance of *P. falciparum* parasites in *in vitro* culture often results in loss of cytoadherence properties and the ability to form gametocytes: this has been found to be due to a deletion of a small region on chromosome 9 (Kemp et al., 1992; Day et al., 1993). As cytoadherence has been linked to virulence through its association with the most virulent form of the disease, cerebral malaria, in the field (MacPherson et al., 1985) this observation implies that virulence - at least that caused by cytoadherence – is reduced when the selection pressure for transmissibility is eliminated. *In vivo* studies have yet to confirm whether this observation holds up under natural conditions.

Thus, after ruling out the effects of diet, host genetics and contamination with other clones or other organisms (as most authors have done), the genetic behaviour of virulence and avirulence in laboratory strains of malaria parasites, and its relationship to transmissibility are unclear from these few investigations. Moreover, it appears that none of these studies made independent repeated tests of

the virulence of these clones, or performed contemporaneous comparisons, or analysed the data in a quantitative way. Thus a combination of anomalies and lack of appropriate testing render the available information difficult to interpret.

In the study presented here, artificial selection for high and low virulence was applied within *P. chabaudi* clones which were maintained by asexual passage in laboratory mice. The objectives were to determine whether there was enough within-clone genetic variation in virulence on which selection could successfully operate (without the constraints on transmission or fitness which would normally operate in a natural environment), to see whether the selection response was rapid or gradual, to determine the correlated responses to transmission, and to determine whether the genetic changes in virulence were asymmetric, i.e. biased towards higher or lower virulence when adapting to a novel host species.

3.3 Materials and Methods

3.3.1 Experimental design

Two clones were chosen from the panel of clones used in the experiment described in Chapter 2: one had high virulence (clone BC) and one had low virulence (clone CW). Within each clone, selection for high and low virulence was performed over eleven generations of asexual passage to give a total of four selection lines. These are denoted BC-A, BC-V, CW-A and CW-V where A (avirulent) and V (virulent) denote low and high virulence, respectively. The selection criterion for virulence was post-infection weight loss of the mouse. The selection procedure is described in the following.

Each generation, for eleven generations, the following steps were taken:

1. On the day of infection (Day 0), four mice per line (C57Bl/6, female, 6-8 weeks old, B & K Universal, U.K.) (eight per line in later generations, see Table 3.1) were injected with 5 x 10^4 parasites collected from each of two mice per line in the previous generation (see below) to give an initial inoculum of 10^5 parasites in total per mouse. In Generation 0, the initial inoculum was 10^6 parasites.

2. Liveweights were measured on Days 0, 6, 10 and 11 on all mice. For each mouse, the difference between the average of weights on Day 0 and 6 and the average of weights on Days 10 and 11 was calculated to obtain a measure of weight loss. Thin blood smears were made on Day 10 or 11 for parasitaemia counts, and red blood cell densities were also measured on these days in all generations except Generation 10.
3. On Day 12, blood was taken from two mice for inoculating the mice in the next generation (Step 1). In the high virulence lines, the two mice chosen as donors were those of their line group which had the highest weight loss. Similarly, in the low virulence lines, those with the least weight loss donated parasites to the next generation. Note that positive values of weight loss indicate high virulence.

Thus the selection regime focused on between-host differences, although the sampling of just 10^5 parasites from each host may also have allowed selection on within-host parasite variation. Selection intensity (proportion of hosts which donated parasites) was approximately 50% in Generations 0 to 7 and 25% in Generations 8 to 11 when the lines were expanded from four to eight mice. Details of numbers of mice which were infected, survived and donated in each generation are given in Table 3.1. In Generation 4, no selection was applied because of lack of availability of mice.

After eleven generations of selection, a separate experiment was conducted to compare the selected parasites from Generation 12 (all four lines) with their ancestors from Generation 0. In order to avoid possible genetic or other changes caused by deep freezing, parasites from Generation 12 were frozen down as they had been for Generation 0. After three weeks, parasites from these frozen stocks were injected into one mouse per line, and then a week later into another mouse per line. When these latter mice had reached 5-10% parasitaemia, they were used to donate 10^5 parasites to each of five mice per line to begin the experiment. One week later, a second replicate was established using parasites donated from one mouse per line in the first replicate. Liveweights were recorded throughout the infection. Red blood cell densities were recorded on Days 9 and 11. Asexual

parasitaemias were counted on Days 7, 13 and 15 (or 14 in Replicate 2). On Days 13 and 15 (or Day 14), gametocytaemia was also measured (as in Chapter 2).

3.3.2 Statistical analysis

A linear model was fitted to the data on weight loss, red blood cell density and parasitaemia from all the generations by analysis of variance using the SAS procedure GLM (SAS, 1990). Class effects in the model were day of measurement (Day 10 or 11), clone, selection line, and a two-way interaction for clone x selection line. Covariates included in the model were generation, two-way interactions for generation x clone and generation x line, and a three-way interaction for generation x clone x line. These covariates were intended to estimate the linear rate of change of the lines over the generations, and the class effects were to estimate the differences in intercepts.

Selection differentials (the deviation of selected mice from the group average) for weight loss for all mice contributing parasites to the next generation were calculated as the individual mouse's weight loss minus the mean weight loss for all mice in the selection line contemporary group. Cumulative selection differentials were calculated over the generations. Because selection intensity was doubled in Generation 8, the data were split into two parts (Generations 0 to 7 and Generations 8 to 11) and analysed separately. The same model as above was fitted to these data to estimate the average selection differential in each line. Non-significant terms ($P > .05$) were removed from the model. The average selection differential of those mice which died due to the infection was calculated and tested for a significant difference from the mean of all surviving mice.

For the analysis of the contemporaneous comparison of the lines at Generations 0 and 12, a model was fitted with effects for replicate, clone and line nested within generation, and all two-way interactions among these terms.

Table 3.1 Selection intensities in high (V) and low virulence (A) selection lines in two clones of *P. chabaudi* over eleven generations.

Generation	Selection intensity (No. mice selected/No. mice infected)			
	Clone BC		Clone CW	
	A	V	A	V
0[1]	2/8	2/8	2/8	2/8
1	2/4	2/4	2/4	2/4
2	2/4	1/3	2/4	2/4
3	2/4	2/4	2/4	2/4
4	2/2	2/2	2/2	2/2
5	2/4	2/4	1/3	2/4
6	2/4	2/4	1/3	2/4
7	2/4	2/4	2/4	2/4
8	2/7	2/7	2/7	2/8
9	2/8	2/8	2/8	2/8
10	2/8	2/8	2/8	2/7
11	2/8	2/7	2/8	2/7

1. In Generation 0, two high and two low virulence mice were selected from the same pool of eight mice per clone.

3.4 Results

3.4.1 Mortality rates

Eleven out of the 248 mice used in the selection experiment died during their infections, but two of these were due to cannibalism early in the infection and unrelated to virulence of the parasite. Of the remaining nine (3.6% of the total), eight had weights recorded on the day of death. The average of these eight was not significantly lower than the average of their contemporary group ($P > .05$) and so mortality had not greatly compromised the selection intensity, or caused bias to the estimated selection responses.

3.4.2 Selection responses

Changes in weight loss over the generations of selection for the four selection lines and the cumulative selection differentials are shown in Figure 3.1. The statistical analysis, summarised in Tables 3.2 for Generations 0 to 11 and in

Table 3.3 for the direct comparison of Generation 0 vs. Generation 12, showed that in all the lines there was an increase in weight loss over the generations, i.e. a significant generation effect. This increase was significantly higher ($P < .01$) in the clone which initially had low virulence (clone CW) than in the high virulence clone (BC), as indicated by the clone x generation interaction. Thus while the clones differed in virulence initially, they converged for virulence over time. All of these changes appeared to be gradual, i.e. there was no detectable rapid increase in virulence between consecutive generations.

Selection within clones on high or low weight loss was not effective, i.e. there were no significant line effects. However, the tendency was towards lines selected for low virulence to have a higher rate of increase in virulence over the generations which is the opposite of what was expected. The absence of significant line differences was in contrast to the highly significant selection differentials obtained by high versus low selection in both clones (Figure 3.1, Table 3.2). These averaged +0.4g and -0.4g per generation (or 2% of initial weight) in each of the clones for high and low weight loss, respectively, in Generations 0 to 7, and +1.4g (8%) and -0.7g (4%) in Generations 8 to 11. These correspond to between-line divergences of 0.8 and 2.4 standard deviation units for Generations to 0 to 7 and Generations 8 to 11, respectively, which compare quite well to expected values of 1.3 and 2.3 for selection intensities of two in four and two in eight mice, respectively (Falconer and Mackay, 1996), after allowing for lower selection intensities than these in some generations. Thus while significant selection differentials between high and low virulence lines were achieved, these did not produce corresponding selection responses. This indicates that there was no significant amount of heritable variation within clones dictating between-host differences in weight loss. Instead, some unintentional selection produced steady increases in virulence over time in all lines.

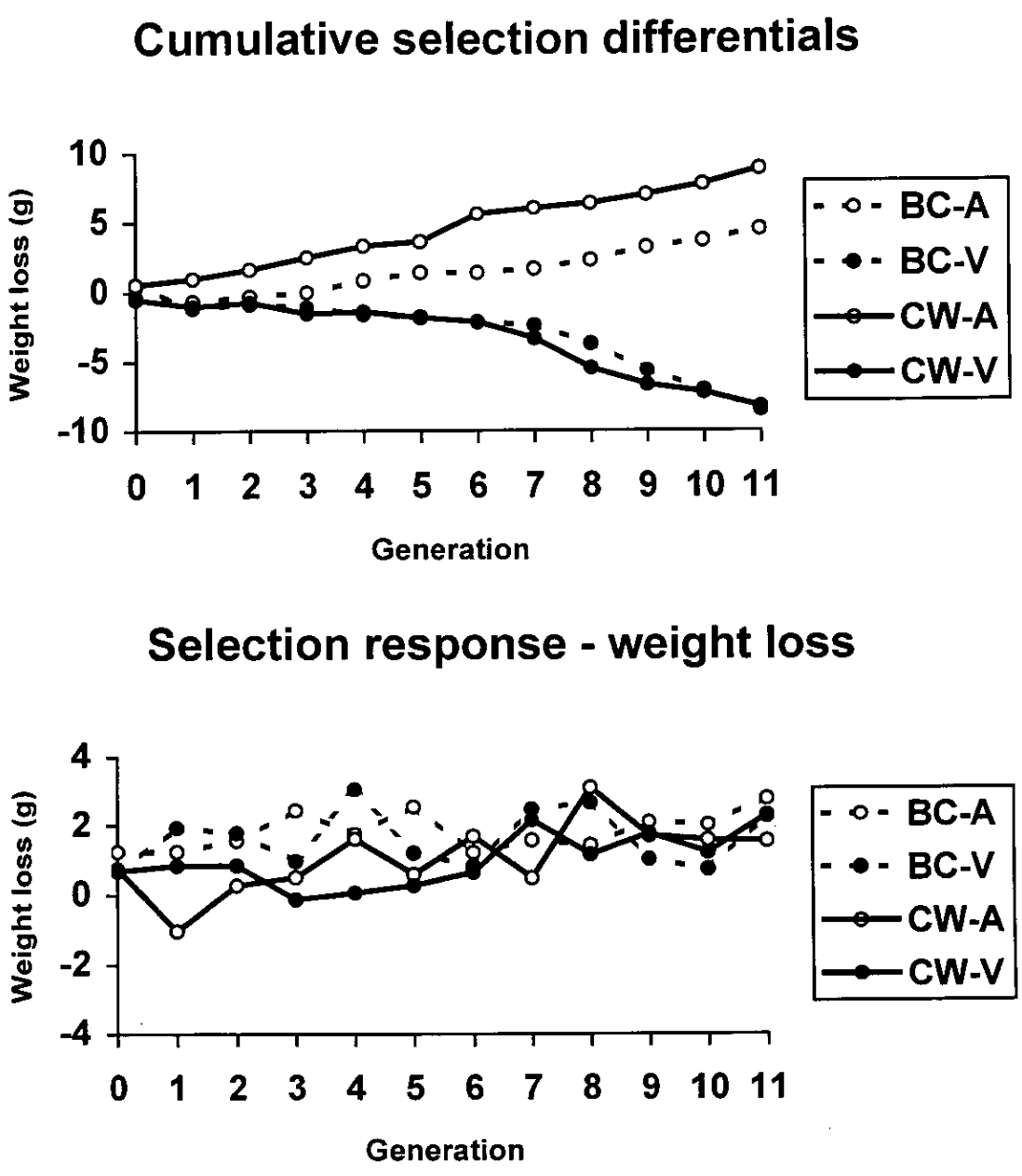


Figure 3.1. Cumulative selection differentials and response to selection for high (V) and low (A) virulence, as measured by weight lost by Days 10 and 11 post-infection, in *P. chabaudi* clones BC and CW.

Table 3.2. Estimates of responses to selection and selection differentials over eleven generations of selection for high (V) and low (A) virulence within clones BC and CW of *P. chabaudi*.

	Selection responses			Selection differential (g)	
	Weight loss (g)	Red blood Cell density ($x10^9$ rbc/ml)	Parasitaemia (%)	Gens. 0-7	Gens. 8-11
No. observations	247	204	205	56	32
Mean	0.83	1.99	11.06	0.03	-0.34
[1]R^2 (%)	15	16	20	15	61
[2]Intercepts					
[3]Significance					
Clone	***	**	n.s.	n.s.	n.s.
Line	n.s.	n.s.	n.s.	**	***
Clone x Line	n.s.	n.s.	n.s.		
[4]Estimates					
BC-A	1.45	1.70	15.9	-	-
BC-V	1.48	0.95	10.3	-	-
CW-A	0.09	2.70	10.2	-	-
CW-V	0.30	2.59	7.8	-	-
Standard error	0.47	0.52	3.8		
[2]Slopes					
[3]Significance					
Generation	***	n.s.	***		
Gen. x Clone	**	***	n.s.	n.s.	n.s.
Gen. x Line	n.s.	**	n.s.	**	***
Gen. x Clone x Line	n.s.	*	n.s.	n.s.	n.s.
[4]Estimates					
BC-A	0.07	-0.06	2.8	0.20	0.71
BC-V	0.02	0.19	1.6	-0.32	-1.53
CW-A	0.18	-0.13	1.3	0.68	0.70
CW-V	0.13	-0.11	1.5	-0.45	-1.24
Standard error	0.06	0.07	0.6	0.41	0.44

1. Proportion of variance explained by the model
2. Intercepts and slopes of the regression line fitting trait on generation
3. Significance levels based on F-tests in an analysis of variance
4. Least-squares estimates and average standard error of the selection line means

3.4.3 Correlated responses

The observed increases in weight loss were accompanied by correlated increases in anaemia and parasitaemia (Figure 3.2 and Table 3.1). Red blood cell density on Day 10 of the infection decreased over the generations over all lines (giving a generation effect), and this effect was most marked in the low virulence clone, CW, (giving a clone x generation interaction), consistent with the results for weight loss. However, in the high virulence line of the virulent clone (BC-V), in the last few generations, anaemia became much less severe. This was not due to outlier observations from individual mice because low anaemia was observed in several mice in each generation. This decrease in virulence, as measured by anaemia, occurred despite quite strong selection for high weight loss in this line in these generations, and therefore is difficult to explain. It is responsible for the significance of the generation x clone x line interaction. Parasitaemia also increased over the generations in all lines, but there were no line or clone differences in this rate of increase.

The experiment in which Generations 0 and 12 were compared contemporaneously confirmed that weight loss and anaemia had indeed increased over the 12 generations of asexual passage, and more so for CW than for BC (see the estimates of clone differences in Table 3.3). This is illustrated in Figure 3.3 where it is seen that CW caused no weight loss in Generation 0, but by Generation 12 was as virulent as BC. Parasitaemia early in the infection (Day 7) also increased during the experiment in both clones, and at a higher rate in the low virulence lines than the high virulence lines (see selection response estimates in Table 3.3). There was also an increase in the persistence of the infection over generations as evident by the higher parasitaemias and gametocytaemias after the peak of the infection (Table 3.3)

Table 3.3. Estimates of responses to selection for high (V) and low (A) virulence within clones BC and CW by direct comparison of Generation 12 with Generation 0.

	Weight loss (g)	[1]Red blood cell density ($x10^9$ rbc/ml)	Parasitaemia Day 7 (%)	[1,2]Parasitaemia Days 13-15 (%)	[1,2]Gametocyt-aemia Days 13-15 (No./1000rbc)
No. obs.	57	58	59	51	51
Mean	1.20	1.35	13.9	0.95	0.48
R^2 (%)	50	40	40	82	60
Estimates					
Clone means at Gen. 0					
BC	1.25	1.10	7.48	0.04	0.06
CW	-1.09	3.51	5.19	0.08	0.22
[3]Clone differences					
Gen. 0 BC-CW	2.34***	-2.41***	2.30	-0.04	-0.16
Gen. 12 BC-CW	-0.23	0.23	-0.64	3.80**	-0.89***
[4]Selection responses					
Gen. 12 BC-A vs. Gen. 0 BC	0.37	-0.23	14.76***	1.77***	0.25
Gen. 12 BC- V vs. Gen. 0 BC	0.51	0.61	5.47	2.96***	0.14
Gen. 12 CW-A vs. Gen. 0 CW	3.12***	-2.60***	15.39***	6.13***	1.09***
Gen. 12 CW-V vs. Gen. 0 CW	2.92***	-2.39***	10.72**	5.98***	0.77***
[5]Group standard error	0.54	1.38	3.94	1.69	0.13

1. Data were log-transformed prior to analysis. Estimates have been back-transformed to the original scale but significance levels apply to transformed data.
2. Averages are taken over Days 13 and 15 in Replicate 1, and Days 13 and 14 in Replicate 2.
3. Differences between the clones at the beginning and end of the experiment.
4. Selection line differences at Generation 12 cf. Generation 0.
5. Average standard error for selection line within generation.

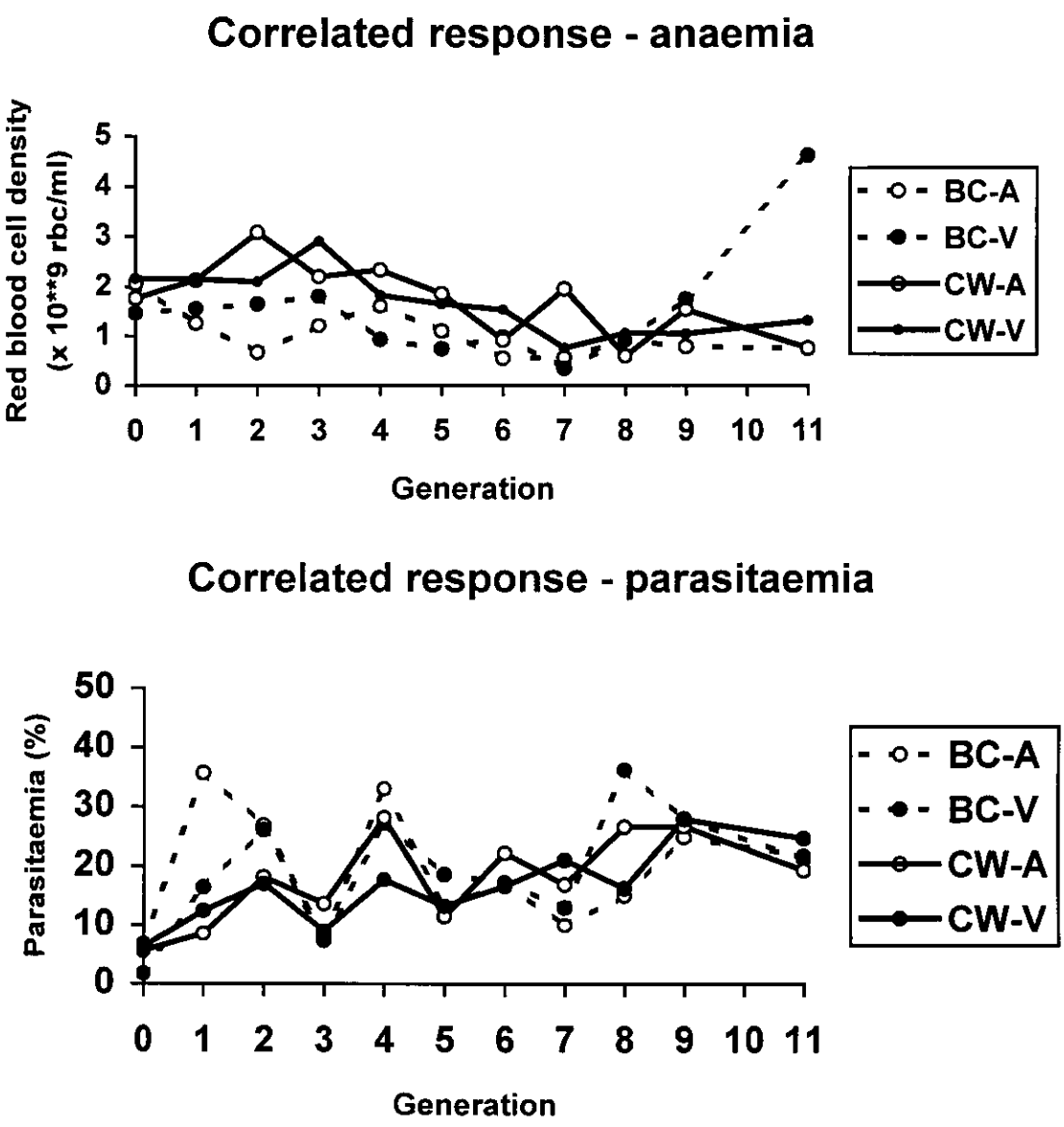


Figure 3.2. Correlated responses in anaemia and parasitaemia on Day 10 post infection to selection for high (V) and low (A) virulence in *P. chabaudi* clones BC and CW.

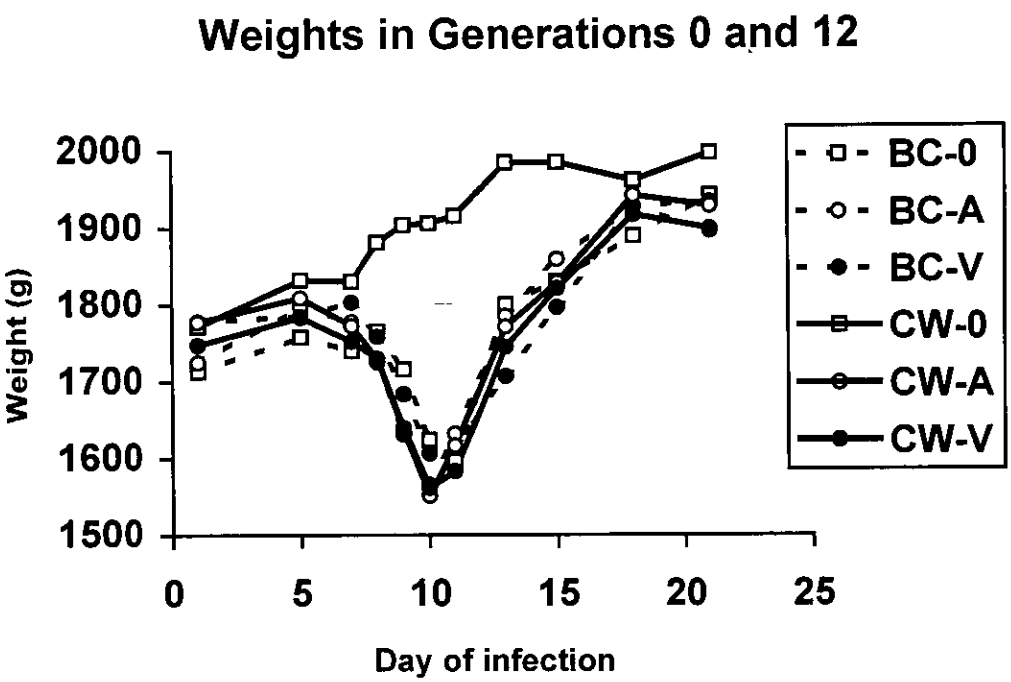


Figure 3.3 Liveweights over the course of infections with lines selected for high or low virulence in two clones (BC and CW) of *P. chabaudi*. Each line is the average of ten mice measured in two separate replicate experiments.

3.5 Discussion

Within-clone selection for virulence based on between-host variation was ineffective in this experiment. This was not due to a lack of selection pressure (reasonably high selection differentials were achieved), and therefore was either due to (a) lack of variation within a clone, (b) lack of stability upon transfer to a new host, or (c) to inadvertent and overriding selection for higher virulence in all lines resulting from the selection procedure. Lack of variation within a clone can be ruled out by the fact that the lines clearly changed genetically through time. The direct comparison of the selected lines with the original lines demonstrated that this was not due to a systematic change in the environment over time. This genetic change could only have arisen through selection (albeit in a different direction than intended) on variability either existing within the clone at the outset, or created during the course of the experiment. The lack of stability during serial passage explanation is in contrast to the wide variation and stability of virulence properties observed between clones in the previous experiment and therefore is unlikely to be true. The inadvertent selection explanation is likely to be true for the following reasons. The population of parasites which was transferred to new hosts each generation was only a sub-population of the total population in the host. If the parasite population evolved as the infection progressed as a result of negative selection by the immune system or positive selection for higher replication rates, the parasite population surviving on Day 12 would have been genetically different from the population causing the weight loss by Days 10 and 11. This would have caused selection for parasites able to survive best to Day 12 which is after the onslaught of the immune system when very high parasite mortality occurs. Certainly, an increased ability to persist through this crisis was evident by the higher parasitaemias and gametocytaemias in Generation 12 than in Generation 0. The intensity of such selection within hosts probably outweighed the artificial selection intensity between hosts, thus dominating the selection response. Thus selection within hosts seemed to be more potent than selection between hosts in this

experiment. In the natural situation, when only a few parasites are transmitted to the next sexual generation, this may not be the case. Nevertheless, the experiment demonstrated that rapid within-host evolution can occur in these parasites, even if it is only short-term.

This genetic change could only have arisen through selection on variability either existing within the clone at the outset, or created during the course of the experiment. Variation within the clone at the beginning of the experiment may have accumulated during the five passages between cloning and use in this experiment since the inocula at each passage were of the order of 10^5 to 10^7 parasites thus allowing maintenance of parasite variation. Two possible types of variants are envisaged. First, such variants could have been mutations that increased in frequency due to population expansion and selection within an infection. Possible mutations might be the deletion of a significant portion of the genome which would have allowed the parasite to replicate faster: this is unlikely because *P. chabaudi* has a synchronised 24-hour cycle of replication which would nullify any advantage in more rapid copying of the genome. Alternatively, mutations may have occurred which increased the number of merozoites per schizont, and hence population growth rate, or increased the ability of the parasite to adhere to host endothelial cells and thus avoid clearance by the spleen. As the latter involves a matching of host binding sites with parasite molecules, the observed adaptation to a new host genotype may well reflect an improvement of this mechanism. Theoretical studies on the potential rate of increase in frequency of favoured mutants within a host and across serial passages are required to establish whether mutation is an adequate source of variation to account for the observed rate of adaptation. Further experiments to explore whether host genotype at these receptor molecules correlates with parasite virulence should also be conducted.

The second source of new variation from within a clone is that of phenotypically variable antigens. Such antigens, expressed on the surface of infected red blood cells, continually switch expression of their epitopes during the infection thereby confounding the immune response: such a mechanism is clearly adaptive. While it is difficult to see how the new antigens *per se* could have a

selective advantage in parasite-naive hosts, it is possible that the efficacy of this survival mechanism was enhanced during the experiment through selection for persistence to Day 12 by immune evasion, e.g. through increased switching rates. An alternative possibility is that the binding affinity of the parasite to the host's receptors improved with repeated passage because it is the same molecule responsible for expressing phenotypically variable antigens that allows the parasite to attach to host endothelial cells (Baruch et al., 1995; Smith et al., 1995; Su et al., 1995). In other words, increased ability to cytoadhere and to express variable antigens are functionally linked through this molecule and so selection for persistence would be expected to induce an increase in cytoadherence ability. This ability in turn may have been the cause of higher virulence since cytoadherence helps the parasite avoid immune-mediated killing by the spleen and therefore probably allows higher parasitaemias to be reached. In addition, there may have been a direct effect of cytoadherence on virulence, as occurs in human malaria (MacPherson et al., 1985).

An intriguing alternative explanation for the increase in virulence over generations is that the within-clone variation itself was responsible for higher virulence. For example, in HIV and hepatitis B viral infections, immune antagonism among diverse parasites within a host appears to allow the pathogen to beat the immune system and hence survive and continue to replicate (Klenerman et al., 1994; Bertoletti et al., 1994). This has also recently been proposed to occur in malaria based on evidence of immune cell antagonism *in vitro* and from patterns of co-transmission of antagonistic parasite genotypes in field data (Gilbert et al. 1998). Moreover, this hypothesis is entirely consistent with the higher virulence observed in mixed-clone infections of *P. chabaudi* compared with single clone infections, a difference which was not simply accounted for by higher parasitaemias (Taylor et al. 1998). In this study, the data were not sufficient for testing this hypothesis by detecting increased variation in virulence or parasite growth rate over generations. One argument against this hypothesis as a general mechanism for increasing virulence is that the clones differed so markedly in their selection responses despite undergoing similar numbers of asexual passages prior to

and during the experiment. Nevertheless, empirical observations to date, accompanied by theoretical arguments for the success of this mechanism of within-host evolution (Davenport, 1995; Burroughs and Rand, 1998) suggest that this may be a potentially important area of further investigation. Furthermore, such within-host evolution is not necessarily in conflict with between-host evolution (Bonhoeffer and Nowak, 1994), and so it is believable that malaria parasites, which rely heavily on between-host fitness, employ such mechanisms.

This unintentional increase in virulence with repeated passage through novel hosts has been observed in other organisms, e.g. the influenza virus maintained in chickens (Schulmann, 1970), although sometimes viruses become less virulent ('attenuated') when maintained in novel hosts (Kilbourne et al., 1994). Theory surrounding the 'Red Queen' hypothesis - the favoured explanation of why hosts and parasites maintain genetic variation in resistance and virulence - predicts that parasites which are normally confronted with host parasite genetic variation, and then allowed to adapt in genetically uniform hosts, should evolve to higher levels of virulence. This is because the parasite is no longer trying to simultaneously overcome the variety of host adaptations, but instead can focus its selection on overcoming a single host genotype (reviewed by Ebert and Hamilton, 1996). The results from this experiment are in full accord with this prediction of the Red Queen hypothesis. On the other hand, the results do not support another prediction and empirically well supported part of the hypothesis which is that parasites cause more harm to hosts to which they are adapted than to novel hosts (Ebert and Hamilton, 1996). While not directly compared in these studies, the level of virulence observed in laboratory mice seems to be far higher than in the natural host, *Thamnomys rutilans* (Landau and Chabaud, 1994), although there have been few direct comparisons to support this view.

The fact that the mildly virulent clone (CW) markedly increased its virulence compared with the virulent clone (BC), which barely increased in virulence, is potentially very interesting. If it is assumed that the rapid change in CW was a result of selection on parasites varying in their ability to persist to Day 12, then it is expected that similar responses would be observed in clone BC, if such increases

did not result in major host death. However, death was not found to be a constraint in these experiments, and selection for a higher probability of death in two of the four lines was ineffective, suggesting that some other restraint was operating. One possible explanation is that anaemia constrains the parasite's population growth rate. Because parasites destroy up to 80% of the host's normal red blood cell population, they limit their own resources because there are not enough blood cells to invade. Theoretical and empirical studies have shown that the red blood cell population is a key limiting factor to the parasite's persistence (Hellriegel, 1992; Yap and Stevenson, 1994; Hetzel and Anderson, 1996; Gravenor et al., 1997). This hypothesis is strengthened by the result that the increase in parasitaemia over generations did not differ between the clones, even though virulence and anaemia did. The latter observation suggests that virulence is a product of the costs of host protection and not directly, but instead non-linearly related, to parasitaemia. The relationship between peak parasitaemia and the probability of host death (virulence) should be investigated further by infecting more susceptible mouse genotypes such as AJ (Stevenson et al., 1982) than the C57Bl/6 used here with the four selection lines to see whether differences between the lines in host mortality rate can be wholly accounted for by parasitaemia, or not.

Whatever the mechanism for increased virulence, the very rapid rate of adaptation by the parasite to a novel host observed in this experiment confirms that malaria parasites have the ability to exploit the resources for genetic change, whether these be mutation or antigenic variation. The fact that this change is not purely and open-endedly exploitative (i.e. the parasite seems to be self-restrained, and indeed, in the BC-V line appeared to be following an alternative strategy of causing low anaemia), but is instead adaptive, supports the idea that the ability to adapt is as critical as the adaptation itself. In this context, the very rapid rate of asexual growth within the host, which stimulates the immune system to kill the parasite, may, after all be the key for parasite success. If such rapidly expanding populations are able to furnish the parasite population with mutations or variants which are able to survive the immune system and persist to produce gametocytes, then the parasite will have a higher fitness. This within-clone variation would

therefore seem to be a beneficial mechanism for parasite evolution, although the effect of severe bottlenecking of the population during transmission must be considered. The maintenance of broad variation in virulence and its positive effect on transmission described in the previous chapter is consistent with such a hypothesis. Further experiments to determine whether such rapid parasite changes are truly adaptive could be done by challenging high and low virulence clones with a more susceptible host: in this case, will the too virulent clones evolve to become less virulent?

Chapter 4

Host genetic and environmental factors influencing virulence

4.1 Summary

Host factors contributing to the severity of malarial disease in a population living in an area of unstable malaria in Sri Lanka were determined from statistical and genetic analysis of sickness score data collected over a period of 38 months. The main findings of the study were that (1) older people were infected less often but experienced more severe symptoms than younger people, (2) people with recent infections were less sick than those free of infections for several months, but the number of infections over the patient's lifetime did not correlate with current levels of sickness, (3) people born in endemic areas were less sick than those which were not, (4) males were less sick than females, (5) *Plasmodium vivax* infections produced stronger symptoms than *P. falciparum,* (6) the use of bednets decreased the number of infections (especially of *P. falciparum*) but increased sickness scores once they became infected, (7) immunity to *P. vivax* may last longer or be stronger than to *P. falciparum*, (8) approximately 20% of the variation in sickness scores remaining after adjusting for these effects could be explained by repeatable differences between individuals, (9) heritabilities of sickness scores were low (generally less than 10%), but heritability of the number of clinical attacks acquired during the study period was moderate (36%) and for fever traits was 20%. Overall, these results highlight the strong influence of short-term immunity on the degree of sickness experienced by infected people, and the long-term (genetic and non-genetic) effects on the probability of developing a clinical infection.

4.2 Introduction

Why does disease severity vary so widely between malaria cases? Symptoms range from being life-threatening to being non-existent for patients of similar age, sex, and number of circulating parasites. Given the slow rate of acquisition of immunity to malaria, and its relatively rapid loss in the absence of reinfections, between-host variation could be largely explained by different levels of prior experience of the parasite. However, even in children with very little immune experience, variation in disease severity is great. If the reasons for this were better understood, prevention of the high mortality rates due to the most severe forms of the disease might be achieved. However, there are few clues as to what factors predispose some humans to life-threatening manifestations of malaria infection.

One of the longest known factors affecting malaria disease is host genetics. The existence of polymorphisms for many blood cell disorders (e.g. sickle cell anaemia, α–thalassaemia, β–thalassaemia, glucose-6-phosphate-dehydrogenase, ovalocytosis, Duffy antigens reviewed by Weatherall, 1987) in human populations of malarious areas of the world strongly suggests that these otherwise deleterious mutations are maintained by a selective advantage in the presence of malaria (Haldane, 1949; Allison, 1954; Flint et al., 1986; Ruwende et al., 1995). Certain alleles of the Class 1 and Class 2 human leucocyte antigens (HLA) of the major histocompatibility complex (MHC) (Hill et al., 1991), and some polymorphic alleles of the tumour necrosis factor gene (TNF, located within the MHC gene complex) (McGuire et al., 1994) have also been found to associate with cerebral and severe anaemia providing further evidence of host genetic variation in susceptibility to this disease. However, even though a causal basis can be argued for each of these associations based on the function of these genes in relation to malarial infection (Pasvol et al., 1977, 1978; Friedman, 1979; Hill et al., 1991; Luzzi et al., 1991; Yuthavong and Wilairat, 1993; Genton et al., 1995; Clarebout et al., 1996; Hood et al., 1996; Senok et al., 1997), the mechanisms of protection by these genes remain largely unknown. Moreover, most of these genes have been found to associate with cerebral or severe malaria which are relatively rare forms

of the disease (Brewster et al., 1990) and therefore these genes probably explain very little of the remarkable variation in malarial disease (Greenwood et al., 1991).

None of these associations, which are based on across-population disequilibrium between disease expression and certain alleles of highly polymorphic loci, have been confirmed using the more robust test of genetic linkage by within-family gene segregation studies. Because sub-divided populations vary in both their frequencies of alleles and their levels of disease across geographical space, 'linkage' disequilibrium can easily be generated in the absence of genetic linkage, thus yielding spurious 'genetic' associations (Flint et al., 1993). For the blood cell disorders, the fact that consistent associations occur throughout the world virtually rules out such spurious results. The less studied associations, however, such as for the HLA and TNF alleles, have not been confirmed by obtaining consistent results across populations: the evidence would be strengthened by family-based linkage studies. The family-based method of genetic analysis has been used to detect a segregating gene with a strong effect on parasitaemia levels in large families in Cameroon exposed to *P. falciparum* (Abel et al., 1992). Using segregation analysis, which does not rely on genetic markers to follow the segregation pattern of inheritance within families, but rather relies on bimodality in the phenotypic distribution of the trait to expose the difference in effect of alternative alleles within a family, a recessive gene with major effect was inferred. This gene has not yet been confirmed by marker-based linkage analysis. In mice, several genes conferring resistance (as measured by mortality rates) to *P. chabaudi* have been located to their chromosomes using linkage analysis in crosses of mouse inbred lines (Stevenson et al., 1988), but so far do not seem to have been identified. Thus there is considerable scope for closer genetic analysis of host resistance to malaria. This requires the collection of reliable phenotypic data and suitable family material.

A first approach to exploring genetic variation in host resistance to malaria is to quantify the proportion of phenotypic variance, which is genetic in nature. This can be done using a quantitative genetic approach to estimate the heritability of the trait based on some knowledge of genetic relationships between individuals in the population. It does not involve knowing the number or nature of genes

contributing to the variation, but quantifies their total contribution assuming many genes are involved. Such an approach does, however, benefit from a good understanding of the environmental influences on disease so that they can be separated out from the genetic effects. This, in itself, is a useful exercise in establishing the major factors contributing to the between-individual variation in disease. The benefit of taking the quantitative genetics approach is that the estimated heritability will establish an upper limit on the magnitude of putative major genes segregating in the population: if the heritability is found to be very low, it is unlikely that genes conferring a significant advantage on disease resistance will be detected by either population disequilibrium, segregation analysis or linkage analysis.

One of the reasons why family-based genetic studies on malaria have not been done has been the lack of suitable data. While pedigree data may be easy to collect in human populations, infectious diseases like malaria have a sporadic nature, which makes phenotypic data difficult to obtain. Moreover, the strong influence of non-genetic factors such as immune experience, age, sex, socio-demographic factors and chance are well recognised in malaria field studies and are likely to obscure any host genetic factors. Well-documented longitudinal studies in stable populations living in endemic areas are therefore required for this work. Such data are available in a population in Sri Lanka in which malaria is of low endemicity but disease incidence is high. The study presented here attempts to quantify the contribution of genetic factors to variation between individuals in malarial disease severity in this population making use of the large number of explanatory factors also recorded.

4.3 Materials and Methods

In this study, a rural population of individuals was monitored for their levels of sickness upon becoming infected with *P. vivax* or *P. falciparum*. Information on host factors such as age, sex, previous history of malaria infections, species of parasite, pedigree, the use of bednets, etc., was simultaneously collected and the effects of these factors on levels of sickness were analysed. (The statistical

analysis was my only contribution to this study: the design and data collection were performed by the team at the University of Colombo and the Kataragama Field Centre). Two separate studies were conducted. In the first (Study 1), the population comprised 1910 individuals living in eight contiguous villages in a total of 480 houses and monitoring took place for 19 months. Pedigrees for all individuals in this population were ascertained by interview and used to investigate whether there was a genetic component to the observed between-individual variation in sickness. In the second study (Study 2), which was also conducted over 19 months, but started two months after the end of Study 1, the population of 3964 lived in 944 houses in nine villages, four of which were also included in the first study. Pedigree information was not available for the majority of the individuals in the Study 2. However, in Study 2 bednets were used in half the villages with the objective of assessing whether this intervention had an impact on disease. Thus in Study 1, the emphasis of the analysis was on the contribution of genetics to disease severity, and in Study 2, on the influence of bednets. For the analysis of other host factors on disease severity (age, sex, etc.), the data from the two studies were combined.

4.3.1 Study area

The Kataragama area of south-east Sri Lanka ($6^0$25'N, $81^0$20'E) is situated in the dry lowland coastal plains and comprises an area of approximately 10km^2 of farmland. The incidence of *P. vivax* malaria is moderately seasonal, the bulk of transmission occurring in the monsoon season between October and January when most of the 1100-1300mm of annual rainfall occurs. Temperatures typically range from 25 to 35^{0}C and relative humidity ranges from 60 to 85%.

The area is considered to be one of 'unstable' but endemic malaria. The entomological inoculation rate, (EIR, the number of infectious bites per person per night) is of the order of 0.01 (Mendis et al., 1990) which compares with values of 0.4-1 in areas of stable malaria in Africa (Molineaux and Gramiccia, 1980; Trape et al., 1994). Most people have about one clinical attack per year and generally 1-2% of the population are infected with either *P. vivax* or *P. falciparum* at any

point in time (Mendis et al., 1990). Epidemics of *P. falciparum* malaria used to occur at 7-10 year intervals (Rajendram and Jayewickreme, 1951) but since 1986, *P. falciparum* has persisted (Gamage-Mendis, 1991). More than five anopheline species transmit malaria in this region, the predominant one being *A. culicifacies* (Mendis et al., 1990).

The use of bednets was minimal during Study 1. In Study 2, 1437 permethrin-impregnated bednets were supplied to 2071 individuals in 508 of the 944 households. The allocation of bednets was according to three trial units. Each trial comprised a control village (or group of villages) and a bednet treatment village. Pairs of control vs. treatment villages were geographically adjacent to each other and matched as closely as possible for various demographic and topographic variables. Bednets were reimpregnated halfway through the study with a compliance rate of 99.6%. Over the study period the usage rate was estimated by survey to be 83% (Mendis, K.N., pers. comm.). Two of the bednet trial units in Study 2 included most of study area in Study 1: the third trial was conducted at a separate site 22km away. Maps of the study site are given in Figure 4.1a and 4.1b and show the relative locations of the villages used in Study 1 and Study 2 together with significant topographical features such as forests, rice paddies and bodies of water.

4.3.2 Data collection

Incidence of malaria episodes was monitored by passive case detection. This meant that individuals showing symptoms would voluntarily present themselves at the research centre or occasionally at the local hospital in the study area (located bear the centre of the study area) at which they had been registered prior to commencement of the study. Upon being positively diagnosed as having malaria based on symptoms, supported by microscopic examination of thick blood films, a record of the variables described below was made. Patients were treated with curative doses of chloroquine and primaquine at this time.

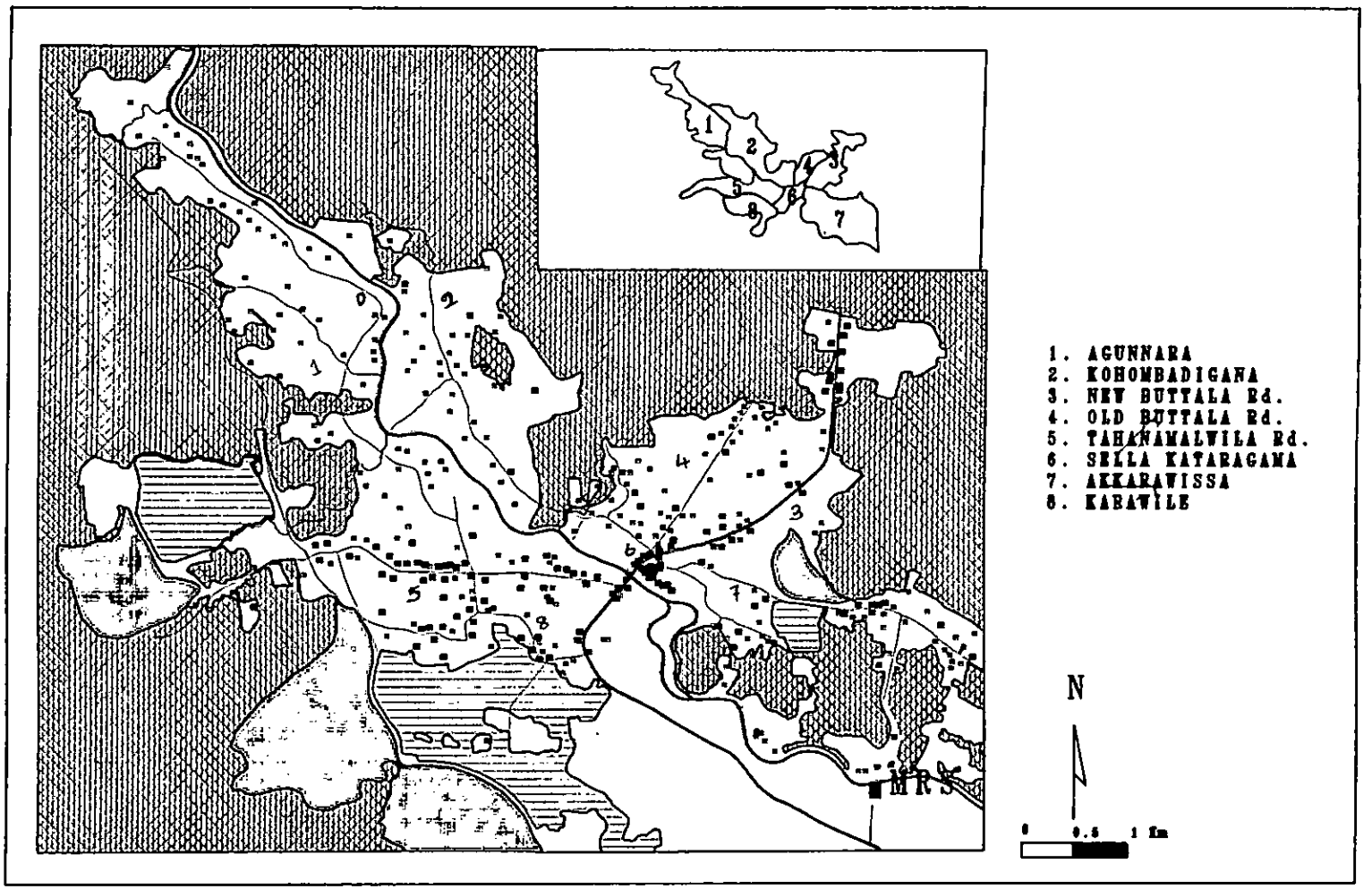


Figure 4.1 Map of study site in the Kataragama region of Sri Lanka showing villages in Study 1. Green hatched areas are scrub or forest. Green stripes are rice paddies. Blue areas are water. Red and black squares are houses. Red lines are roads. Blue lines are rivers. Continued overleaf.

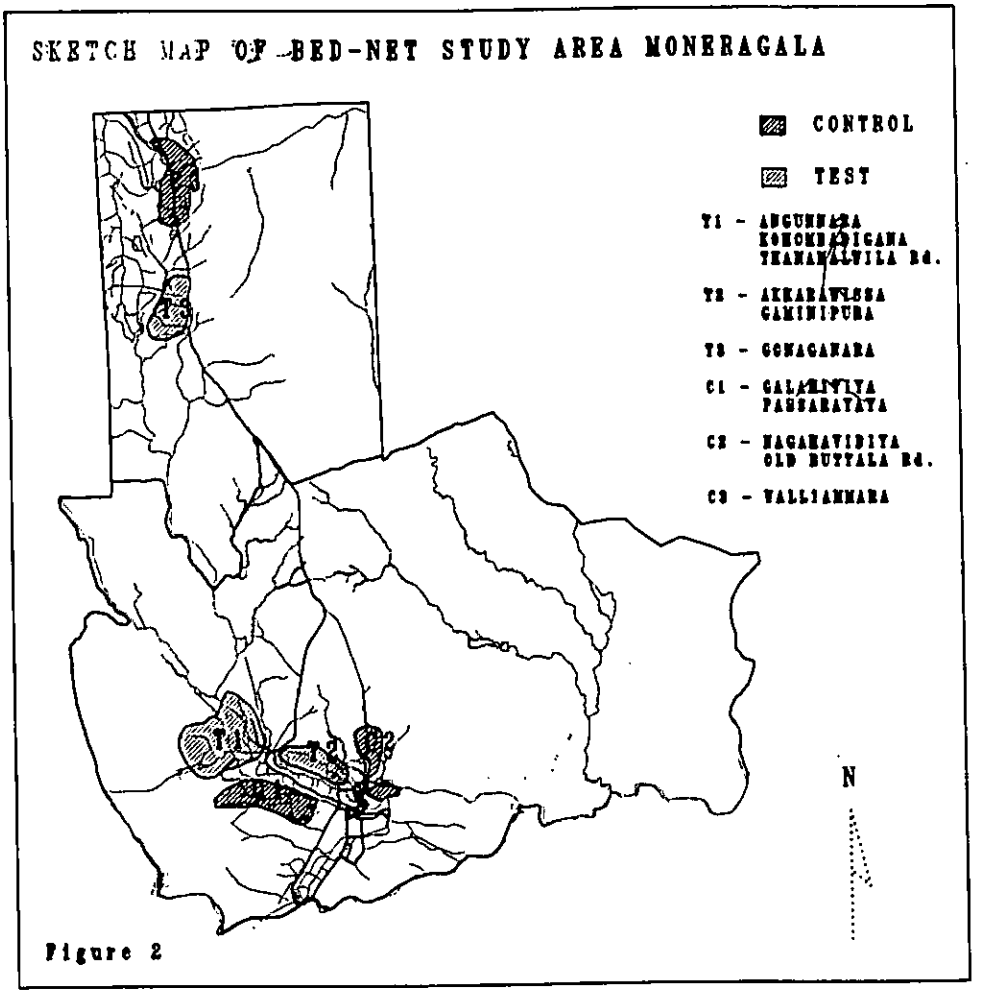


Figure 4.1 continued. Map of study site in the Kataragama region of Sri Lanka showing villages in Study 2. T denotes that bednets were used in these villages, and C denotes Control villages.

Factors relating to the patient

Sex

Age

Occupation

Village

House code

Factors relating to previous infections

Number of months since the last attack (obtained by interview).

Number of previous attacks during lifetime (obtained by interview).

Whether born in an endemic area or not (obtained by interview).

Species of previous infections (obtained from previous records).

Factors relating to the current infection

Species of parasite (obtained by examination of thin blood films).

Parasitaemia (obtained by counting approximately 500 fields of a thin blood film).

Gametocytaemia (as above).

Number of days since the onset of symptoms.

Number of hours since the last paroxysm.

Body temperature (^{0}C)

Degrees of the following symptoms (sickness scores), ranking from 0 (no symptoms) to 2 or 3 (maximum level of symptoms):

Backache

Arthralgia

Myalgia

Headache

Nausea

Vomiting

Anorexia

Cold

Shivering

Sweating

Hypochondrial pain

A total of these sickness scores was also calculated and weighted by the number of scores included in the sum. The identity of the interviewer at the clinic was also recorded to allow adjustment for any bias that interviewers might have in recording these scores.

4.3.3 Statistical analysis

4.3.3.1. Traits and transformations

The data were first edited for outlier values. If the datum was obviously a typographical error, it was excluded from the analysis. If the value was extreme, but probably correct, it was set at a 'reasonable' upper limit. For example, when the number of previous attacks was recorded as being >40, it was set to 40. The maximum values for other traits were 96 for the number of months since the last attack, 48 hours for the time since the last paroxysm, 14 for the number of days since onset of symptoms, 42^0 C for body temperature, 2% for parasitaemia, and 0.2% for gametocytaemia. These restrictions were imposed to avoid undue influence of extreme values on regression estimates.

The variables under analysis were:

the eleven sickness scores and their total

temperature and parasitaemia at time of presentation

number of infections within each of the 19 month study periods

species of parasite

the number of days since the onset of symptoms

whether the patient was gametocytaemic or not.

The analysis was divided into two parts - a fixed effects analysis to investigate systematic effects (such as age and sex) on these traits, and a random effects analysis to estimate variance components due to the additive genetic and the non-genetic contribution to the variation between people, i.e. the heritabilities and repeatabilities. The latter analysis was not done for the last two variables.

All fixed effects analyses were performed using the SAS statistical analysis package (SAS, 1990). Each sickness score was treated as an ordered categorical trait and was therefore analysed using logistic regression (using PROC LOGISTIC). This method models the trait as having an underlying normal distribution which, as the value on the underlying scale is increased such that it exceeds a certain threshold, moves the observed value into the next class upwards, e.g. from a sickness score of 1 to 2. The thresholds of each class, and the magnitude of the effects on them are able to be estimated for each class by using a logit transformation of the data which essentially models the data as probabilities of being in particular classes on the observed scale. The estimates of the effects are expressed as odds ratios because they represent the relative probability of falling into a particular class. The analysis assumes that fixed effects equally affect all threshold levels, e.g. an increase in the threshold of 20% for males compared to females is expected to apply to both the 1 to 2 threshold and to the 2 to 3 threshold. Thus the estimate reflected an average odds ratio over all categories. Significance tests for each level of a class against a common 'reference' class were based on a chi-squared statistic with one degree of freedom.

Total sickness score, temperature and number of days symptomatic were approximately normally distributed and were therefore analysed using standard regression techniques (PROC GLM). Parasitaemia was first transformed by taking the arcsine square root of the value multiplied by 10 and then analysed as a normally distributed variable. Species of parasite and whether gametocytaemic were analysed as binomial variables using PROC LOGISTIC.

The fact that repeated records on the same individual were in the data was ignored for the purpose of the fixed effects analysis of the above traits. The number of infections per person within the study period, for which there were no repeated records, was analysed as a Poisson variable, taking into account the Poisson error structure (PROC GENMOD). The assumption that the distribution is Poisson is approximate because the data were conditional on there being at least one infection, i.e. zero classes were missing from these data.

For the random effects analyses, categorical data were analysed under the assumption that the residuals were normally distributed, i.e. without prior transformation. In addition, the random effects analyses were also performed on the residuals on the transformed scale output from the fixed effects analyses. This was done because there are no computer packages available which fit mixed models (i.e. fixed and random effects) to ordered categorical data while simultaneously incorporating the additive genetic relationship matrix which allows separation of genetic from non-genetic variances (see below).

4.3.3.2. Fixed effects analysis

After preliminary exploration of the data, the class effects and covariates described below were chosen to be fitted in these models on the basis that each of them had a significant influence on one or more traits. All fixed effects (except those which were the dependent variable, e.g. number of days symptomatic) were retained in the model for all traits, even if not significant, so that results could be easily compared across traits.

Class effects

Sex	Male (M) or Female (F)	
Occupation	Inside (small businesses and government servants), or	
	Outside (farmers and paid labourers), or	
	Other (includes no occupation)	
Village/bednets	Study 1:	Angunara (SA)
		Old Buttala Rd. (SB)
		Kohobadigana (SC)
		Karawile (SK)
		New Buttala Rd. (SN)
		Sella Kataragama (SS)
		Thanamalwila Rd. (ST)
		Akkarawissa (SW)
	Study 2:	Trial 1 Control - Passarayaya (PP)

	Trial 1 Bednets - SA,SC,ST
	Trial 2 Control - Nagahaveediya (NN)
	Trial 2 Bednets - SW
	Trial 3 Control - Walliammara (WW)
	Trial 3 Bednets - Gonaganara (GG).
Interviewer:	Study 1: coded as GUN, JAG, KAN, SUD, OTHER.
	Study 2: not recorded and so included in 'OTHER'.
Current parasite species	*P. vivax* or *P. falciparum*
Previous parasite species	None, i.e. no previous attacks within this study period
	All *P. vivax*
	All *P. falciparum*
	Mixture of *P. vivax* and *P. falciparum.*
Season of year	October-January (Season 1)
	February-May (Season 2)
	June-September (Season 3)
Parasitaemia (%)	<0.1, 0.1-0.2, >0.2
Temperature (^{0}C)	36-37.5, 37.5-39.0, >39.0
Gametocyte positive	Yes or No

Continuous covariates

Age and age^2

Number of previous attacks (NA) and NA^2

Months since last attack (LA) and LA^2

Hours since last paroxysm

Number of days since onset of symptoms

4.3.3.3. Random effects analysis

For estimation of heritability and repeatability, the above fixed effects models were expanded to include two random effects - one for house and one for person. The person effects were partitioned into additive genetic versus non-genetic components by simultaneously fitting pedigree information (see below) in

the mixed model equations. The model may be represented by the following equation:

$$Y = Xb + Wh + Za + Zc + e \qquad (4.1)$$

where Y is the vector of observations on individual people,

X is known incidence matrix for the fixed effects,

Z and W are the known incidence matrices for the random effects of person and house, respectively,

b is a vector of unknown fixed effects,

h is a vector of unknown random house effects,

a is a vector of unknown random additive genetic effects,

c is a vector of unknown random permanent environmental effects (non-genetic 'person' effects),

e is a vector of unknown random residuals.

The variances of the random effects are assumed as follows:

var(Y)= σ^2_P, the total phenotypic variation after accounting for fixed effects

var(h)= σ^2_H, the between-house variance

var(a)= $A\sigma^2_A$, the additive genetic variance among *related* individuals (see below)

var(c)= σ^2_C, the variance due to non-genetic 'permanent environmental' person effects

var(e)= σ^2_E , the residual variance, i.e. (σ^2_P-σ^2_H-σ^2_A-σ^2_C). Covariances between h, c and a are assumed to be zero.

Since individuals in this study are genetically related, the observed additive genetic variance (var(a)) among these people is less than would be observed in a population of unrelated people. The amount of shrinkage can be specified by the numerator relationship matrix, A, which is the matrix of coefficients of relationship (Wright, 1922) among all the individuals in Z, and is easily computed. For example, a coefficient for a parent-offspring pair is 0.5 and for two half-sibs is 0.25. By incorporating A into Eq.4.1 (see below), the observations in Y are, in effect, also regressed on degree of relationship, thus allowing separation of additive genetic from other non-genetic effects on the individual person (Henderson, 1973).

To estimate the variance components, the following mixed model equation, which is a matrix version of Eq. 4.1, was used to compute the likelihood of the data under the model (Smith and Graser, 1986). This likelihood was then maximised with respect to the variance components to obtain the best estimates using the DFREML programme of Meyer (1991):

$$\begin{bmatrix} X'X & X'W & X'Z & X'Z \\ W'X & W'W & W'Z & W'Z \\ Z'X & Z'W & Z'Z+(\sigma_P^2/\sigma_E^2)^{-1} & Z'Z \\ Z'X & Z'W & Z'Z & Z'Z+(A\sigma_A^2/\sigma_E^2)^{-1} \end{bmatrix} \begin{bmatrix} b \\ h \\ c \\ a \end{bmatrix} = \begin{bmatrix} X'Y \\ W'Y \\ Z'Y \\ Z'Y \end{bmatrix} \quad (4.2)$$

From these, heritability was calculated as:

$$h^2=\sigma^2_A/(\sigma^2_P-\sigma^2_H)$$

and the proportion due to permanent environment as

$$c^2=\sigma^2_C/(\sigma^2_P-\sigma^2_H)$$

giving a repeatability of

$t=c^2+h^2$ which is the proportion of variance attributable to individuals, both genetic and non-genetic.

Standard errors of the estimates and the sampling correlations between them were approximated from the information matrix which essentially uses information about the shape of the likelihood surface around the maximum to determine the confidence limits around the parameter estimates. As there are no computer packages available which fit both logistic regression models to ordered categorical data and a genetic relationship matrix, the data were analysed under the assumption that the residuals were normally distributed.

Since members of the same family usually lived in the same house, there was potential for confounding between genetic and house effects. Information for disentangling these two comes from the data structure: relatives living in different houses enable separation of these effects. An example of how house, genetic and non-genetic effects are disentangled is given in Figure 4.2.

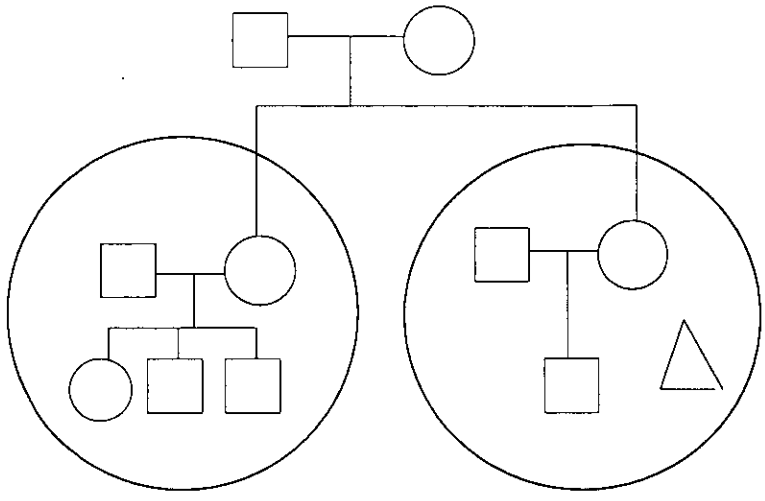


Figure 4.2. Illustration of how house effects are able to be separated from genetic effects. Large circles are houses. Within each house there is a nuclear family (males are squares, females are circles. Horizontal lines represent matings and vertical lines represent parent-offspring pairs). There may also be an unrelated individual (triangle). If people are related across houses, e.g. through the grandparents, then the house effect can be separated from the genetic effect by regressing out this relatedness. Also, if two people living in the same house have a different relationship with a third person (e.g. one is an offspring of this person, and the other is not), then there is some information to separate genetic effects from house effects. However, if only one of the family members has a record, and this person has no relatives with records, then house is completely confounded with person, and the two effects cannot be separated.

Two alternative models to the full model described above (Model 1) were fitted to determine whether heritability estimates were affected by the choice of model. Model 2 was Model 1 minus the random effect for house: this was done to see whether the estimates of additive genetic effects were traded off against house effects as it was known that pedigree and house were heavily confounded. Model 3 was Model 1 minus the covariates for the number of previous attacks and months since last attack: this was done to determine whether these factors had removed some of the genetic variation between individuals. The goodness of fits of the alternative models were tested for statistical significance using log likelihood ratio tests, assumed to be distributed as half a chi-squared variable.

Principal components analyses were also performed on groups of sickness scores using PROC PRINCOMP of SAS (SAS, 1990). This effectively 'collapses' correlated traits into one trait which can then be analysed further. The grouping of traits was as follows: shivering, sweating and cold (called 'fever traits'), vomiting, nausea and anorexia ('gastrointestinal traits'), and arthralgia, myalgia, backache and hypochondrial pain ('pains'). This analysis involved estimating the first principal component (the linear combination of traits which accounts for most of the variation in all of them) of each group after first adjusting for the fixed effects. These principal components were then analysed to estimate heritabilities and repeatabilities as described above.

4.4 Results and preliminary discussion

Because of the interrelationships between the large number of traits analysed in this study and the factors which affect them, this section includes some explanation and interpretation as it proceeds. The Discussion section is then reserved for a general interpretation of the main findings of the study and their relevance to malaria pathology.

4.4.1 Data distributions

In Study 1, 39% of the population (742 people) became infected at least once during the 19 month period. In Study 2, 40% (764 people) of those not

sleeping under bednets, and 12% (239 people) of those sleeping under bednets became infected at least once during this second 19 month period. Across both studies, of those which had an attack, 54% only had one attack, 23% had two attacks and the remaining 23% had three or more attacks. Figure 4.3 shows distributions of the number of attacks per person during the study period (average of 1.9), the number of previous attacks before the study (average of 7.8) and the number of months since the previous attack at the commencement of the study (average of 9.4 months). The time for which the patient had been symptomatic by the time they presented at the clinic averaged 2.6 days, with 48% of the population reporting within two days. The high literacy rate in this population and ready availability of medical attention was probably responsible for this early reporting (Gamage-Mendis, 1991).

The distribution of number of clinical attacks per month is shown in Figure 4.4, together with monthly rainfall. Incidence of clinical attacks was moderately seasonal, the majority of them occurring late in the monsoonal season between October and January. Out of all infections, *P. falciparum* accounted for 44% of them which is far greater than the typical value in this area of $<1\%$ before 1986 (Gamage-Mendis, 1991) when *P. falciparum* prevalence increased markedly and has since persisted (Mendis et al., 1990). Studies on the infectious reservoir of these species have not revealed a clear explanation as to why this change has come about although part of the cause may be the development of low levels of chloroquine resistance in Sri Lanka (Ratnapala et al., 1984; Mendis et al., 1990) and an increase in vectorial capacity (Gamage-Mendis et al., 1991).

Distributions of sickness scores and number of infections per person are shown in Figure 4.5, and of parasitaemia and temperatures in Figure 4.6. The average of the total of 11 symptoms scores was 10.3, and thus clinical symptoms were quite strong, despite low parasitaemias. In general, then, malaria in this area is of low endemicity, and this maintains high levels of disease symptoms because low transmission intensity and low inoculation rates (Mendis et al.,1990) are presumably not high enough to maintain clinical immunity.

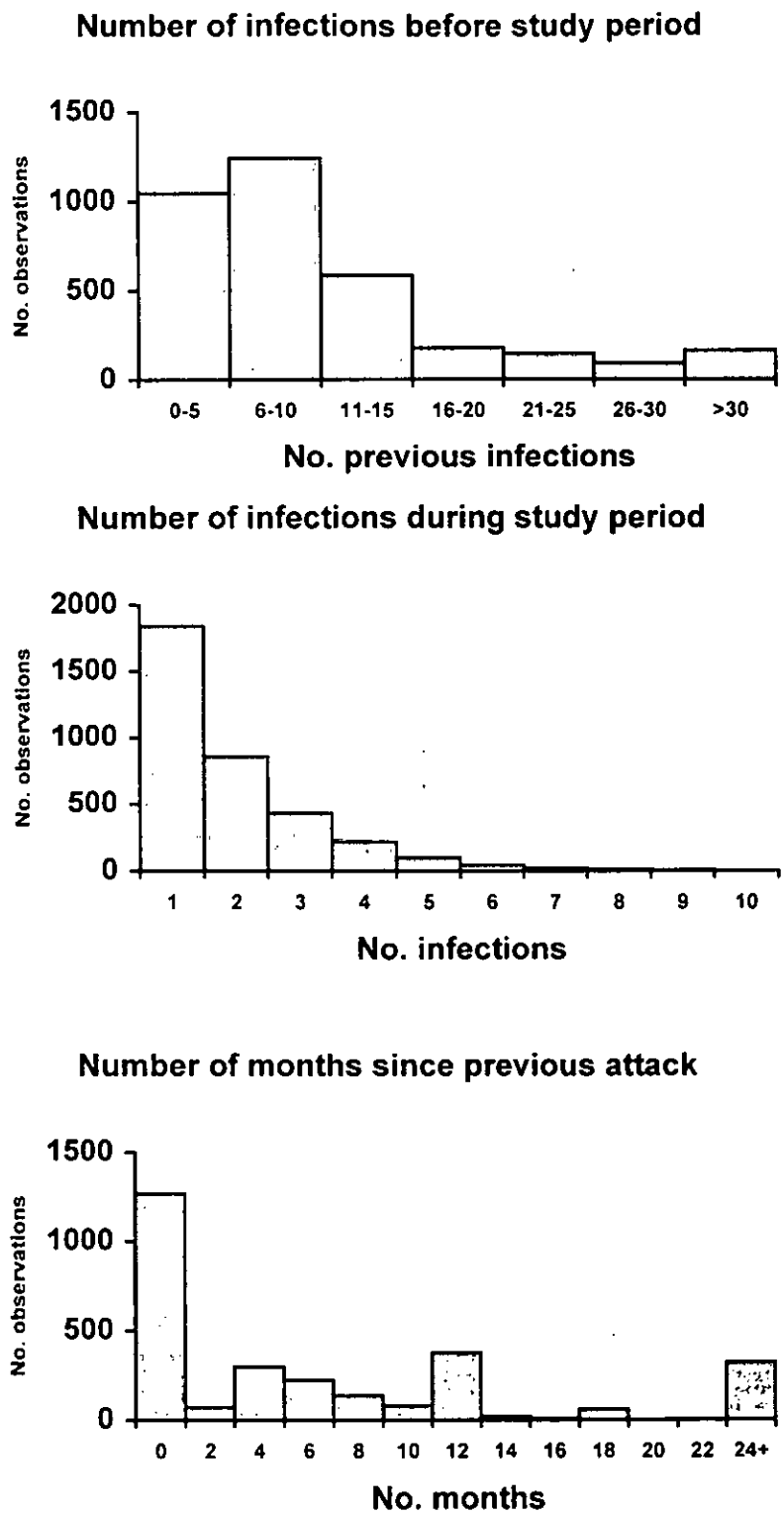


Figure 4.3 Number of infections per person recorded during the study period, before the study period and duration (months) between the last and current attack.

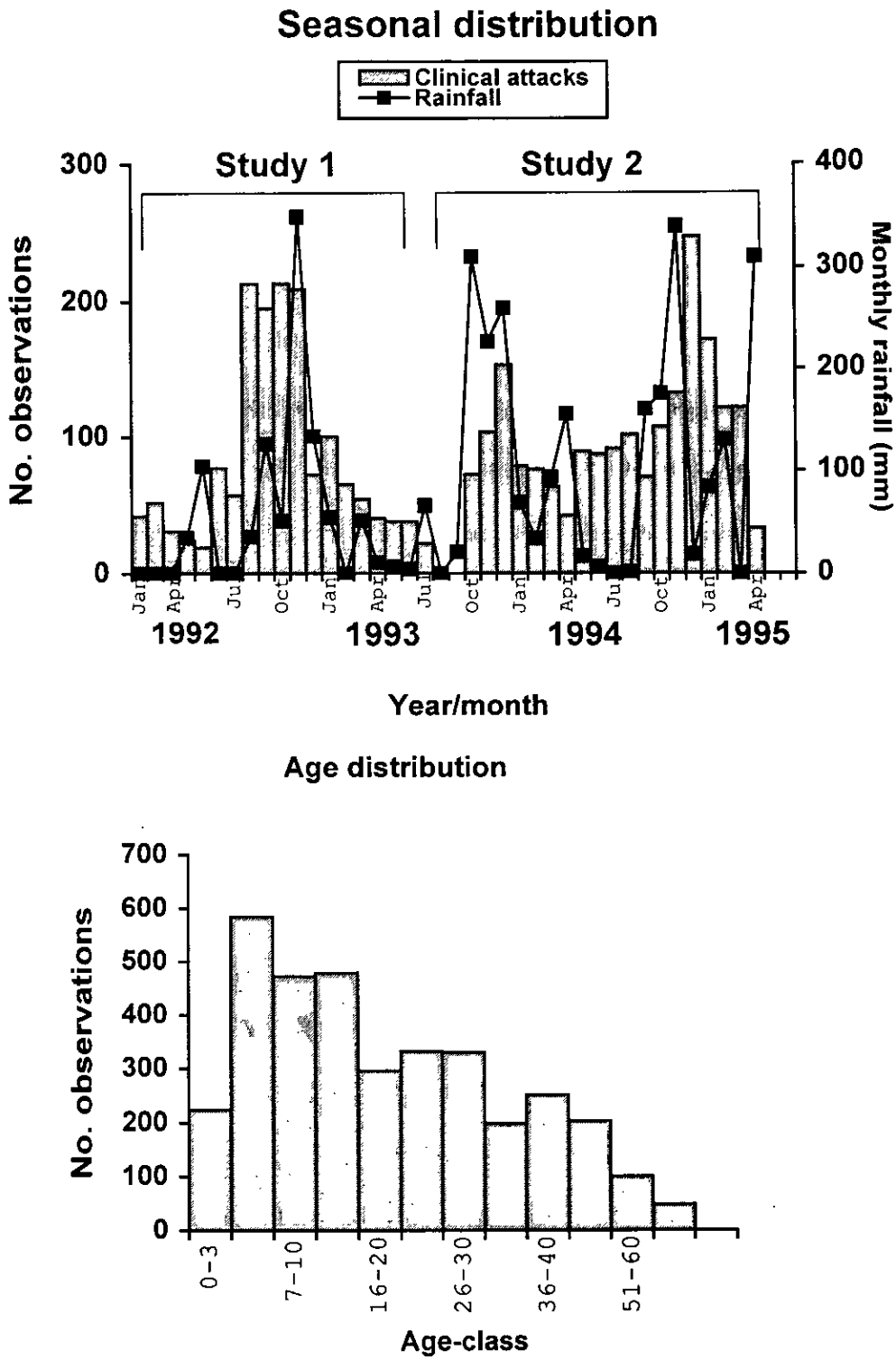


Figure 4.4. Distributions of recorded clinical attacks by season (upper) and age-class (lower). Monthly rainfall is also shown in the upper figure.

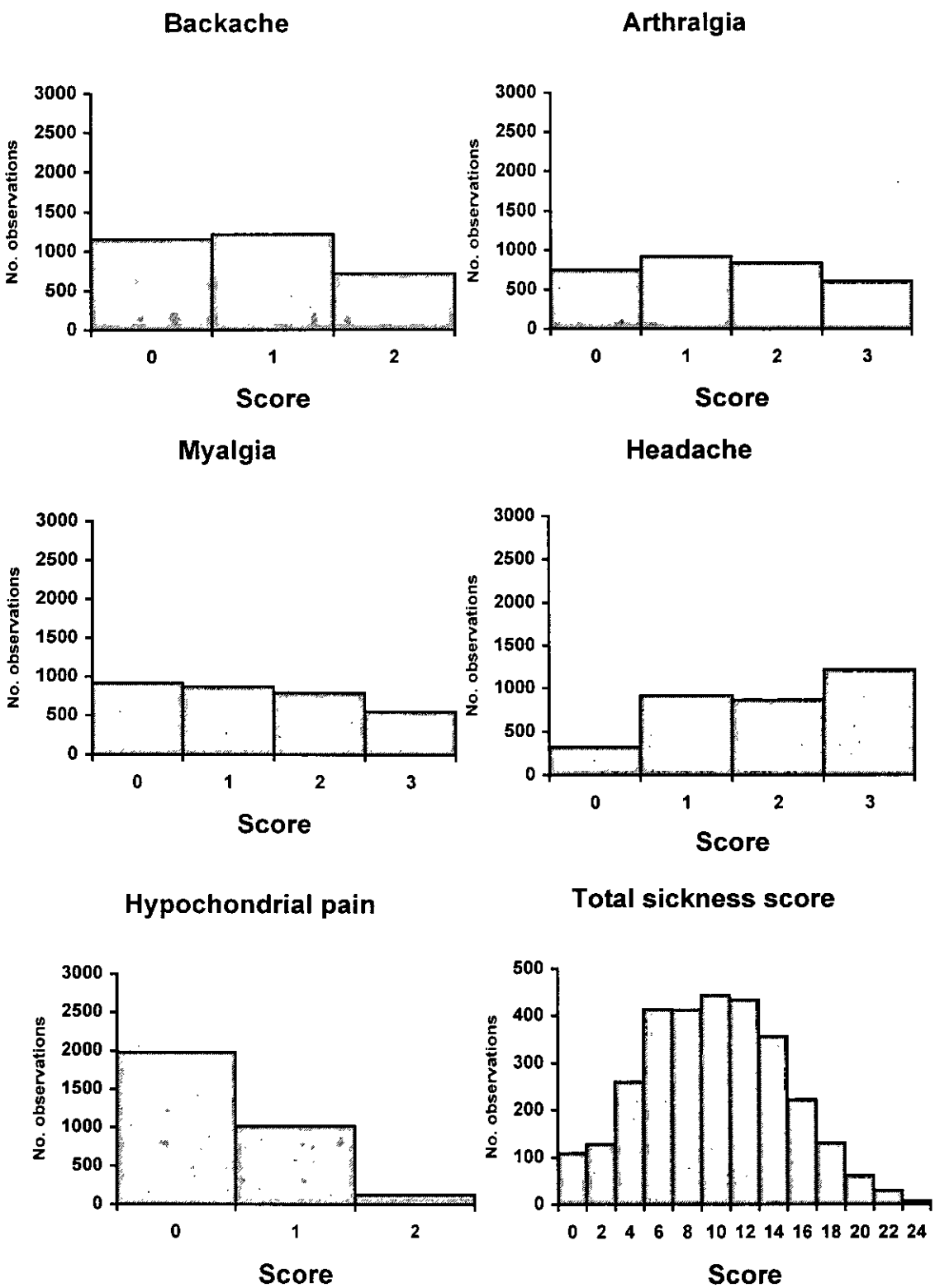


Figure 4.5 Frequency distributions (no. of reported cases) for sickness scores. Numbers on the horizontal axis represent upper boundaries for the class. Continued on next page.

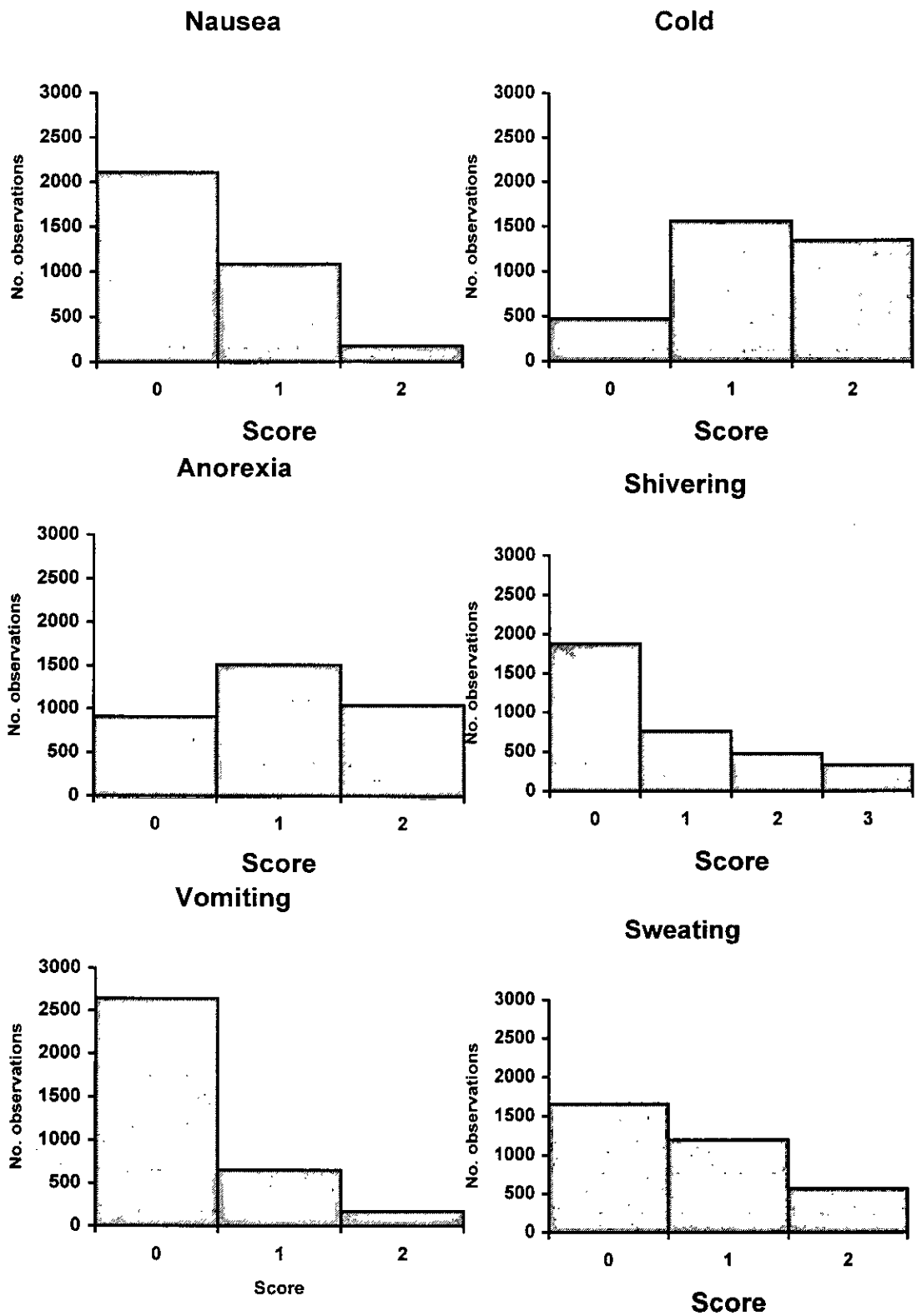


Figure 4.5 continued. Distributions of sickness scores.

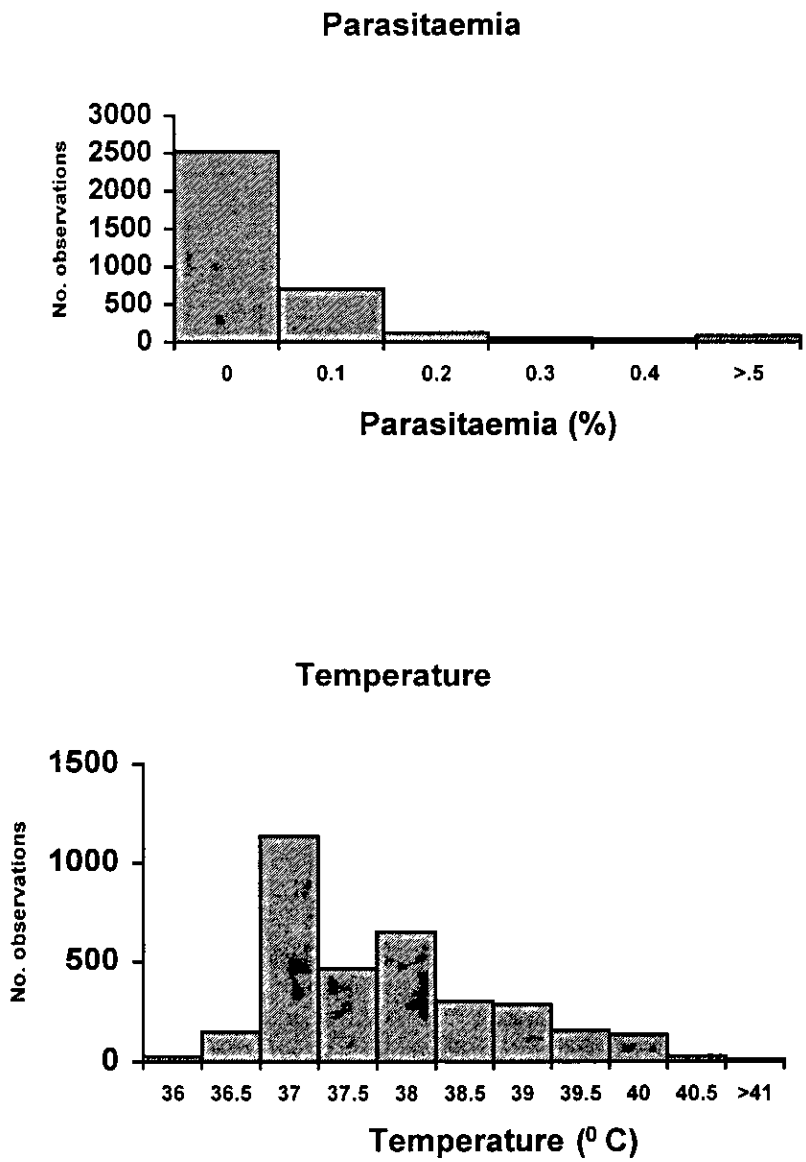


Figure 4.6. Distributions of parasitaemia and temperatures at the time of presentation at the clinic. Numbers on horizontal axis indicate lower boundaries for the class.

4.4.2 Fixed effects

Before describing the systematic effects on disease severity, it may be helpful for interpretation of the results to delineate between several sets of contributing factors. The first set of factors relate to the current infection state: they include the immediate influences on sickness such as the stage of infection, the degree of parasitaemia, and the infecting parasite species. The second set of factors are the predisposing factors which influence how sick the person is likely to become given that the first set of factors are held constant. These include previous immune history (number of previous attacks, time since last attack, species of previous infections) and physiological state (age, sex). Finally, there is a third set of factors which, unlike the first and second factors, are extraneous to the host. However, they may (or may not) influence the level of the first and second sets of factors and thereby indirectly influence the degree of sickness. Such factors include season, occupation, village, bednet treatment, and interviewer.

It is also useful to distinguish between clinical immunity (anti-disease or anti-toxic) and anti-infection immunity. People with high levels of anti-infection immunity would not be observed in this study because data were only collected on symptomatic people. However, the number of clinical attacks per person within the period of study may reflect a mixture of anti-infection and anti-disease immunity because repeated exposure can raise the level of anti-disease immunity to the point where people carry the parasite but do not express symptoms (Cox et al., 1994). In relation to symptomology, it is also useful to bear in mind that some symptoms are thought to be indicators of the host controlling the infection, while others are thought to indicate that the host is unable to control the infection. For example, fever is thought to be a mechanism for controlling parasitaemia (reviewed by Kwiatkowski, 1995), and higher levels of hypochondrial pain may also reflect a greater rate of clearance of parasites by the spleen. On the other hand, symptoms such as nausea, vomiting and aches seem to play no obvious role in controlling the parasite infection. For this reason, the results for groups of related symptoms (i.e. aches and pains, gastrointestinal traits, fever traits) are sometimes discussed

separately because they may indicate different levels of clinical immunity in the host.

Finally, the symptoms recorded in this study do not all appear simultaneously during the course of an infection and are transient in nature. Therefore time of reporting to the clinic must be allowed for in the interpretation of results. Pains and aches tend to occur early in the infection around the time when the infection becomes patent in the blood – the so-called prodromal period (Kitchen, 1949). Nausea and vomiting can also begin to occur at this time. These are then followed by a primary attack consisting of intense cold and shivering for up to an hour, then high fever and sweating for up to two hours, and then tiredness but normal temperatures for the remainder of the 48 hour period between attacks. Vomiting and intense headache often accompanies these paroxysms (Kitchen, 1949; Harinasuta and Bunnag, 1988). After the primary attack there may be secondary attacks caused by recrudescences of uncleared parasites for a period of up to two years (James et al., 1936). In *P. vivax* malaria, recurrences (distinct from recrudescences by the fact that they are caused by a new release of 'dormant' parasites from liver schizonts rather than increase in numbers of parasites causing the primary attack) typically occur about 6-8 weeks after the primary attack (James et al., 1936). Recrudescences are probably rare in this study because every reporting patient was treated with drugs and drug resistance is not very frequent in this area (Mendis et al., 1990), although the contribution of recurrences is not known.

The results from the fixed effects analysis are summarised in Table 4.1 (for sickness and total scores) and Table 4.2 (for parasitaemia, temperature, species and number of attacks during the study period). These are described and discussed in more detail below.

(i) *Immediate effects - parasitaemia, temperature, stage of infection and parasite species*

Sickness scores were higher for people with higher levels of parasitaemia and elevated temperatures (Table 4.1). Sickness scores also increased as the number of days since becoming symptomatic increased (Table 4.1), which itself was positively

correlated to parasitaemia (P>.05) and temperature (P<.05) (Table 4.2). Similarly, higher levels of parasitaemia were associated with higher temperatures (Table 4.2). These results are most probably a reflection of the increase in symptoms as the infection progresses from the incubation period through the prodromal period (when parasitaemia becomes patent) to the fully symptomatic stage when parasitaemias are maximal.

Parasite species affected the degree of sickness where *P. vivax* infections caused more aches and fever symptoms than *P. falciparum*, consistent with the more pronounced paroxysms associated with this species (Kitchen, 1949; Covell and Nicol, 1951). However, *P. vivax* tended to cause milder gastrointestinal symptoms, although this was significant for anorexia only (Table 4.1). These species differences in sickness scores were not able to be accounted for by a difference in the time between becoming symptomatic and reporting the illness (Table 4.2), nor by different levels of parasitaemia or temperature.

Patients were more likely to be gametocyte positive when they were showing greater gastrointestinal and fever symptoms (but not aches) (Table 4.1) and if parasitaemias were high (Table 4.2). This probably reflects the fact that gametocytes are only produced later in the infection. *P. vivax* infections were eight times more likely to be gametocytaemic than *P. falciparum* infections. This is probably due to the shorter maturation period (two days) of gametocytes of *P. vivax* (Boyd and Kitchen, 1937; Coatney et al., 1971) compared with 8-10 days for *P. falciparum* (Smalley, 1976). There may also be a bias towards *P. vivax* gametocytes because of some confusion between the morphology of asexual parasites and gametocytes in this species (Gamage-Mendis, 1991). The number of gametocyte positive people declined as the hours since the last paroxysm increased, probably also reflecting production of gametocytes later in the infection when paroxysms have declined.

(ii) Predisposing factors - sex and occupation

Males consistently reported lower clinical illness than females for all traits except fever traits for which there was no sex difference (Table 4.1). This was despite having higher parasitaemias at presentation (Table 4.2) suggesting that

Table 4.1. Estimates[1,2,3] of fixed effects on sickness scores. See footnotes for details.

Effect	[4]No./class	[2]Total Score	Backache	Arthralgia	Myalgia	Headache	Anorexia	Nausea	Vomiting	Cold	Shivering	Sweating	Hypo. Pain
[4]No./trait	3519	3519	3085	3115	3119	3304	3457	3371	3469	3368	3452	3419	3127
[5]Mean			0.86	1.42	1.31	1.89	1.04	0.43	0.29	1.26	0.79	0.68	0.41
[5]Standard deviation			0.77	1.06	1.07	1.01	0.75	0.59	0.55	0.69	1.01	0.74	0.57
[6]χ^2_{40}			***847	***725	***976	***523	***338	***186	***239	***478	***292	***223	***132
[7]% concordant			74	70	73	67	65	63	67	68	63	63	62
[8]R^2 (%)		20	25	21	27	14	9	6	7	13	8	6	4
Fixed effects													
Species													
P. falciparum	1539	0	1	1	1	1	1	1	1	1	1	1	1
P. vivax	1980	*0.44	1.08	1.15	**1.23	**1.24	*0.84	1.12	0.95	1.05	***1.35	1.09	1.02
[9]Previous species													
None	1799	0	1	1	1	1	1	1	1	1	1	1	1
F,FF->F	372	-0.27	1.01	0.93	0.82	1.04	0.93	1.02	0.95	0.79	0.81	0.90	**1.51
V,VV->F	271	0.12	1.23	0.90	0.97	1.19	0.92	1.11	1.17	0.99	0.83	*0.65	1.22
V,VV->V	496	0.12	1.17	1.15	1.11	1.03	0.91	0.83	0.84	1.12	0.99	0.95	0.99
F,FF->V	157	-0.02	1.10	1.08	1.05		0.84	0.90	1.04	0.91	1.04	0.95	1.28
Other	399	-0.09	1.20	1.23	0.99	1.03		1.16	1.06	1.00	*0.75	1.01	1.25
V vs. F	Vs	0.09	1.08	1.07	1.10	1.01	1.00	0.80	0.84	1.09	1.09	1.00	0.97
Het. vs. Hom	vs.	0.10	1.09	1.04	1.00	1.12	1.03	1.08	1.20	0.99	0.99	0.82	1.11
[10]Parasitaemia													
<0.1%	3061	0	1	1	1	1	1	1	1	1	1	1	1
≥0.1%, <0.2%	241	*0.86	1.05	1.31	1.24	0.99	1.22	1.00	0.98	1.26	***1.88	*1.33	1.26
≥0.2%	217	***2.07	1.26	**2.11	***1.95	**1.46	***1.59	**1.46	**1.60	*1.39	2.33	**1.57	1.21
Gametocytes													
Negative	3083	0	1	1	1	1	1	1	1	1	1	1	1
Positive	436	0.47	0.91	0.94	0.96	1.07	*1.27	**1.39	**1.58	***1.42	1.18	*1.27	0.93
Temperature													
<37.5°C	1779	0	1	1	1	1	1	1	1	1	1	1	1
37.5-39.0°C	1320	***1.62	***1.55	***1.70	***1.58	***2.04	***1.66	**1.29	1.16	***1.65	***1.42	0.94	***1.41
>39.0°C	420	***2.53	***2.02	***2.02	***1.84	***2.78	***2.21	***1.80	***1.57	***2.49	***2.11	0.85	***1.79

Table 4.1 continued

Sex													
Female	1515	0	1	1	1	1	1	1	1	1	1	1	1
Male	2004	***-0.93	***0.65	0.87	*0.84	***0.76	***0.67	***0.57	***0.59	0.96	1.08	0.87	0.89
Occupation													
Unknown	3017	0	1	1	1	1	1	1	1	1	1	1	1
Inside	79	0.74	0.98	1.41	1.40	0.79	0.97	1.12	1.37	1.47	1.22	1.09	1.33
Outside	423	*0.63	*1.36	1.14	1.17	0.78	1.01	1.01	0.90	1.26	1.18	1.17	1.12
Born endemic area													
No	116	0	1	1	1	1	1	1	1	1	1	1	1
Yes	3403	**-1.28	0.79	0.86	*0.62	0.70	**0.55	0.87	1.02	1.02	***0.52	0.81	0.76
Season													
Oct.-Jan.	1700	0	1	1	1	1	1	1	1	1	1	1	1
Feb.-May	868	0.17	0.86	0.92	1.05	1.02	1.08	1.02	1.04	1.05	0.91	***1.40	*1.26
June-Sept.	951	0.10	0.91	**0.76	**0.79	1.07	*1.21	1.05	1.06	1.10	1.02	***1.47	1.09
[11]Village/bednets													
Study 1													
SA	330	*1.08	0.99	1.15	**1.82	1.30	*1.71	1.02	0.66	***3.33	1.25	1.49	0.75
SB	106	0.33	0.74	0.85	1.28	1.51	1.08	0.84	0.59	**2.49	1.33	1.29	0.69
SC	321	0.96	0.97	1.20	*1.62	1.21	**1.89	0.86	0.67	***2.98	1.15	1.52	0.66
SK	118	0.70	0.66	0.65	0.96	1.06	*1.94	1.14	0.53	***2.97	1.60	1.51	0.60
SN	42	0.86	0.67	0.63	0.98	1.81	*2.33	1.06	1.08	**2.75	1.91	1.34	*0.40
SS	139	0.77	0.88	1.04	1.34	0.89	**2.22	1.00	0.89	**2.29	1.30	1.40	***0.33
ST	290	0.35	0.80	1.00	1.33	1.06	1.36	1.06	0.74	**2.13	0.86	1.53	*0.55
SW	215	0.17	0.87	1.20	*1.81	0.84	1.44	0.73	*0.43	**2.33	0.94	1.42	*0.50
Study 2													
Trial 1 Control	454	0	1	1	1	1	1	1	1	1	1	1	1
Bednets	207	*0.53	0.89	1.14	1.05	1.17	1.01	*1.31	1.26	1.07	***1.44	0.89	0.95
Trial 2 Control	352	0	1	1	1	1	1	1	1	1	1	1	1
Bednets	117	0.25	1.17	0.91	0.95	0.92	*0.75	**1.48	***1.85	**0.74	**1.38	**0.72	1.02
Trial 3 Control	684	0	1	1	1	1	1	1	1	1	1	1	1
Bednets	144	***2.05	***2.45	***2.44	***2.15	**1.69	1.29	1.36	*1.58	0.96	0.99	1.01	1.36

Cont.....

Table 4.1 continued

Effect	No./class	Total Score	Backache	Arthralgia	Myalgia	Headache	Anorexia	Nausea	Vomiting	Cold	Shivering	Sweating	Hypo. Pain
[12]Covariates													
Age (years)	3519	***12.38	***4.75	***5.11	***4.72	***2.90	**-0.86	0.10	***-1.35	0.28	0.82	0.05	-0.05
Age^2 (years^2)	3519	***-.10	***-0.05	***-0.05	***-0.04	***-0.04	***0.02	0.00	***0.02	0.00	-0.01	0.00	0.00
Last attack (mo)	3519	***4.79	0.16	0.63	0.46	**0.95	*0.56	0.14	0.07	***0.89	0.44	*0.52	0.01
Last attack ^{2} (mo^2)	3519	-.35	0.00	0.00	0.00	*-0.01	0.00	0.00	0.00	**-0.01	0.00	-0.01	0.00
No. attacks	3519	2.90	0.33	0.36	*1.25	-0.90	-0.58	0.25	-0.23	-0.32	0.17	**-1.38	-0.21
No. attacks^2	3519	0.07	0.00	0.00	-0.02	0.03	0.02	-0.01	-0.01	0.00	0.00	*0.03	0.001
Last paroxysm (hrs)	3519	*1.91	0.22	**-0.36	**-0.53	-0.05	-0.11	0.15	0.19	-0.11	-0.02	***0.68	0.07
Days symptomatic	3519	***35.38	*1.54	*2.88	***3.56	1.13	***3.16	***1.54	**1.46	0.93	**3.08	***3.33	***2.64

1 Estimates for sickness scores are from logistic regression analyses of categorical ordered data. They are expressed as odds ratios, i.e. the probability that the observation in the particular class takes the highest possible value relative to the probability that the observation of a 'standard' class (shown with value '1' in the table) takes this maximum value. For example, an odds ratio of 2 for males versus females means that males have twice the probability of having the maximum sickness score as females. This ratio is assumed to apply to all classes except the minimum class.

2 Total score was analysed by normal regression. Estimates are expressed as absolute deviations from the mean of the class shown with an estimate of zero.

3 Significance levels are for contrasts relative to the category with estimate '1' for sickness scores, '0' for total score. $P<.001$ (***), $P<.01$ (**), $P<.05$ (*) .

4 Refers to maximum numbers used in the analysis because 560 records did not have number of previous attacks or months since last attack recorded.

5 Raw means and standard deviations before adjustment for fixed effects.

6 χ^2_{40} is twice the log ratio of the likelihood of the data under the fitted model to the likelihood with no effects fitted, assumed to be distributed as a chi-squared variable with 40 degrees of freedom. It describes the goodness of fit of the model to the data.

7 % concordant is the proportion of observations which are the same as those predicted by the model based on the estimated probabilities of falling in each class.

8 R^2 is the proportion of the variance explained by the model when analysed using normal regression. It measures the goodness of fit of the model to the data.

9 F,FF->F means the current infection is *P. falciparum* and the previous one, two or more infections were also *P. falciparum*. Similar notation applies to the other classes. 'None' includes patients with no recorded previous infection during the study, and 'Other' includes patients with a history of both species in previous infections during the study. Pooled estimates and significance tests are shown for the effects of previous *P. vivax* infections vs. previous *P. falciparum* infections (V vs. F), and for previous infections of heterologous vs. homologous species to the current infecting species.

10 <0.1% includes 4 zero observations.

11 Estimates for Study 1 are expressed relative to the mean of Study 2. Estimates for the effects of using bednets in Study 2 are expressed relative to their appropriate controls.

12 Estimates for covariates are expressed in absolute values instead of as odds ratios, as obtained from analyses using normal regression. All values have been multiplied by 100.

Table 4.2. Estimates of fixed effects for other traits. See footnotes for this table and Table 4.1 for explanation.

Effect	[1,2]Parasitaemia	[1]Temperature	[3]No. of infections	No. of days symptom.	[4]Species	Gametocyte positive
No./trait	3006	3336	1805	3517	3518	2959
Mean	0.481	37.84	1.91	2.56	0.563	0.124
Standard deviation	0.317	0.95	1.28		0.496	0.330
χ^2			***1383		***525	***620
% concordant					70	83
R^2 (%)	15	23	12	17		
Fixed effects						
Species						
P. falciparum	0	0	-	0	-	1
P. vivax	0.010	-0.025	-	-0.11	-	***7.41
Previous species						
None	0	0	-	0		1
F,FF->F	0.007	***-0.19	-	***-0.39	-	1.89
V,VV->F	0.010	0.00	-	***0.53	-	0.73
V,VV->V	***.074	0.01	-	**0.08	-	1.12
F,FF->V	0.049	0.00	-	*-0.05	-	1.14
Other	0.036	-0.06	-	***-0.50	-	0.93
V vs. F	0.028	*0.119	-	0.00	-	0.61
Het. vs. Hom.	-0.019	0.073	-	-0.13	-	0.62
Parasitaemia						
<0.1%	-	0	-	0	1	1
≥0.1%, <0.2%	-	***0.204	-	-0.03	***2.08	***4.83
≥0.2%	-	*0.124	-	0.15	***1.79	***3.61
Gametocytes						
Negative	0	0	-	0	1	-
Positive	***0.366	0.030	-	***0.45	***7.52	-
Temperature						
<37.5°C	0	-	-	0	1	1
37.5-39.0°C	**0.038	-	-	***0.27	0.97	1.00
>39.0°C	***0.092	-	-	0.11	1.06	1.32
Sex						
Female	0	0	0	0	1	1
Male	*0.027	-0.029	1.02	0.02	*0.836	1.10
Occupation						
Unknown	0	0	0	0	1	1
Inside	**-0.109	-0.101	0.93	0.15	*1.83	0.98
Outside	*-0.059	***-0.231	1.06	-0.21	0.75	0.99
Born endemic area						
No	0	0	0	0	1	1
Yes	-0.005	***-0.308	0.90	-0.24	*0.64	0.80

Cont...

Table 4.2. continued

Effect	[1,2]Parasitaemia	[1]Temperature	[3]No. of infections	[1]No. days symptomatic	[4]Species	[5]Gametocyte positive
Season						
Oct.-Jan.	0	0	0	0	0	0
Feb.-May	-0.017	0.025	-	-0.03	***1.61	1.32
June-Sept.	**-0.042	***0.208	-	0.00	*1.24	1.22
Village/bednets						
Study 1						
SA	0.063	0.541	1.00	***0.82	0.93	*2.06
SB	0.052	0.692	1.10	*0.68	*2.09	2.10
SC	0.040	0.603	1.18	***0.77	1.40	*2.36
SK	0.061	0.608	0.68	*0.64	**2.62	**3.48
SN	-0.02	0.588	0.71	*0.82	1.56	*3.89
SS	0.101	0.721	0.90	***0.91	*2.22	2.04
ST	0.041	0.543	0.88	**0.57	1.50	2.03
SW	0.003	0.621	0.93	***0.60	***3.19	2.04
Study 2						
Trial 1 Control	0	0	0	0	1	1
Bednets	0.023	***0.309	*0.89	0.12	1.20	**0.63
Trial 2 Control	0	0	0	0	1	1
Bednets	-0.025	*0.258	*0.88	*0.47	1.09	0.64
Trial 3 Control	0	0	0	0	1	1
Bednets	***-0.161	*0.208	***0.72	*0.38	***2.04	1.67
[6]Covariates						
Age (years)	-0.116	-0.700	***-3.92	**2.05	***-0.87	-0.18
Age^2 (years^2)	0.001	0.003	***0.04	0.00	***0.01	0.00
Last attack (mo)	0.067	0.141	-	-0.12	***0.91	*-0.25
Last attack^2 (mo^2)	0.000	0.001	-	0.00	***-0.01	0.00
No. attacks	-0.295	0.801	***8.01	**2.66	***1.32	-0.21
No. attacks^2	0.004	-0.020	***-0.16	*-0.05	*-.03	.00
Last paroxysm (hrs)	-0.055	***-3.340	-	***2.32	**-0.23	**-0.18
Days symptomatic	-0.079	1.300	-	-	-0.40	***0.94

1. Analysed using normal regression.
2. Estimates are on the transformed scale.
3. Analysed assuming a Poisson distribution using a log link function. Estimates expressed as odds ratios.
4. Analysed by logistic regression after recoding *P. falciparum* to zero and *P. vivax* to one. Estimates expressed as odds ratios. Higher values are interpreted as a tendency towards *P. vivax*.
5. As for 4 but with gametocyte positive being coded as one and gametocyte negative coded as zero.
6. Analysed using normal regression. Estimates have been multiplied by 100.

males had a higher level of clinical immunity than females. This may arise through being exposed more often due to their jobs predominantly as farm or forest workers. However, males did not have significantly more clinical attacks than females within the period of this study (Table 4.2). There was also no significant effect of occupation on sickness (Table 4.1) or number of infections (Table 4.2) after adjusting for sex, although outside workers did have higher levels of parasitaemia and lower temperatures than inside workers, consistent with the lower sickness and higher parasitaemias in males. Even though parasitaemia and occupation were included in the model for sickness scores, and therefore should have accounted for any such effects on males, it is possible that these factors did not account for all of the hypothesised higher immunity in males. Therefore, another analysis was performed on sickness scores in which occupation, parasitaemia and number of previous attacks was dropped from the model (data not shown). This did not alter the sex effect and so the lower sickness in males cannot be wholly explained by greater levels of immune experience given the available data. This does not rule out the possibility that males were exposed more often: they may have been so, but because of higher levels of immunity, did not experience symptoms, in which case they were not included in the data set. However, the higher numbers of observations in males compared with females does not support this. Thus in general, the data do not indicate that the sexes differ in their levels of sickness because of different frequencies of exposure.

Another interesting difference between the sexes was that males had a higher proportion of *P. falciparum* infections than females which does suggest that the sexes had different patterns (but not necessarily frequency) of exposure, or immunity. This does not explain a higher level of immunity in males because, as discussed later, immunity to *P. vivax* appeared to be stronger or longer than for *P. falciparum*. There was no evidence for a sex by species interaction affecting any of the traits (data not shown).

The question of whether the sex difference was due to a difference between males and females in the time of reporting to the clinic was also addressed by analysing the number of days symptomatic fitting all the usual fixed effects. Sex

and occupation effects were not significant, but people infected with *P. falciparum* infections took longer to report than people with *P. vivax* infections. However, this still does not explain why males were less sick than females because species and sex were both included in the model simultaneously. When species was dropped from the model, the sex effects on the number of days symptomatic remained non-significant.

Finally, an alternative explanation for the sex difference might be that females perceived or genuinely experienced more severe symptoms for reasons which were not related to prior exposure and immunity. Given the physiological. endocrinological and behavioural differences between the sexes, this finding is not surprising: for such reasons sexes differ in host resistance to many parasite species (Roberts et al., 1996). For example, several studies in mice using *P. chabaudi* have shown that testosterone suppresses the ability to acquire immunity and resolve primary infections. However, once immunity has been acquired, there is no further effect of this hormone (Wunderlich et al., 1992; Benten et al., 1997; Mossmann et al., 1997).

(iii) Predisposing factors – long-term immunity, short-term immunity and age

People born in endemic areas were less sick for most traits than people who were not (Table 4.1) although they had the same number of attacks during the period of study. This effect was present even after accounting for the number of previous attacks and age, and therefore may reflect long-term immune memory. This is consistent with the observation from Madagascar, where malaria was eradicated but reappeared after 30 years, that people who had been exposed as children, but not since, developed less severe symptoms than people who had not (Deloron and Chougnet, 1992). It is also consistent with the hypothesis that the acquisition of strain-transcending immunity through repeated exposure is long-term (Brown, 1991) and acquired in a non-linear way, i.e. once it reaches some threshold level, it is almost complete (Gupta and Day, 1994a). Interestingly, people from endemic areas had a higher proportion of infections which were *P. falciparum* infections than people not from endemic areas (Table 4.2). This will also be discussed later. There are many reasons to believe that such long-term

immunity is not responsible for these effects of endemic history, however. For example, people born in endemic areas may differ from immigrants in many aspects such has nutritional history, socio-economic status and genetics. These alternative explanations cannot be ruled out and so the existence of very long-term immunity is still unsure.

The present study also illustrates that there is shorter-term immunity acquired through repeated exposure, as expressed by a combination of age effects, the number of previous attacks during the individual's lifetime, and the time since last attack, as discussed below. Total sickness score increased with age ($P < .001$, Table 4.1), though there were differences between the individual sickness scores in the direction of these effects. Aches and pains increased with age, but gastrointestinal traits decreased with age, and fever was unaffected by age (Figure 4.7). However, older people had fewer infections within the period of this study and reported later to the clinic than younger people (Table 4.2). Thus it seems that there was an increase in the threshold for clinical attacks (anti-disease immunity) with age. While higher numbers of previous attacks during the lifetime generally did not lead to less sickness (except for myalgia and shivering), longer durations between the current and previous infection did (Table 4.1). This was further examined by dropping age from the model: this did not reveal an effect of the number of previous attacks. (The correlation between age and number of previous attacks was 0.27). There are two possible explanations for these observations. Either the number of previous attacks was a very inaccurate measure of previous history of exposure, (as is likely given that this information was obtained by interview only), thus leaving anti-disease immunity with a long-term component to explain these age differences. Or, anti-disease acquired immunity is short-lived and not responsible for age-related differences in disease severity: in this case, age-related decreases in frequency of clinical attacks must then be explained by anti-infection immunity with long-term protection acquired with age. The former hypothesis has been favoured by Cox et al. (1994) who argue that anti-disease immunity must be strain-specific and long-lasting to explain observed age patterns in incidence of febrile illness in children between 2 and 15 years old.

Similarly, Gupta et al. (1994a) argue that differences between curves of prevalence and disease as a function of age indicate an acquisition of strain-specific immunity which gives long-term protection against disease, rather than infection. The lack of prevalence data in this study preclude drawing such a conclusion here. In favour of the latter hypothesis – that anti-disease immunity is short-term – is the observation that the severity of illness increased with the amount of time since the previous clinical attack.

An additional question is whether age effects on anti-disease or anti-infection immunity reflect the acquired effects of repeated exposure, or of some other age-dependent mechanism, such as a reduced ability to mount an immune response in children (Baird et al., 1991). These effects could not be separated out in the present study because infection by patterns (as distinct from clinical attacks by age) were not directly observed, and concomitant data on immune response parameters (e.g. antibody titres) were not obtained. Further discussion of this issue has been published (Baird (1994), Gupta and Day (1994b), Roberts et al. (1994)).

A further point in relation to age effects on anti-disease immunity is that older people experienced different symptoms than younger people: older people suffered more back, muscle and bone aches than children, whereas children tended to throw up! It is suggested that the age trends in symptom scores, as distinct from the frequency of symptomatic episodes, is a reflection of natural susceptibility of certain age-groups to particular symptoms (e.g. older people experience feel more aches, regardless of malaria), rather than of differential pathology. Wherever age effects were significant, the age-squared effect was almost always significant too, showing that the relationship between age and sickness was not linear. This was partly due to the propensity of old people (60-80 year-olds) to suffer more for all symptoms. This is illustrated in Figure 4.7 where age effects on the summed score for groups of related traits (aches, fever and gastrointestinal traits) are shown. However, when data from old people were dropped from the data set, the age-squared effects did not change in their significance level, and the linear age effects simply became stronger (data not shown). Thus the nature of the age effects on disease severity appeared to be truly curvilinear over the entire age range. This is

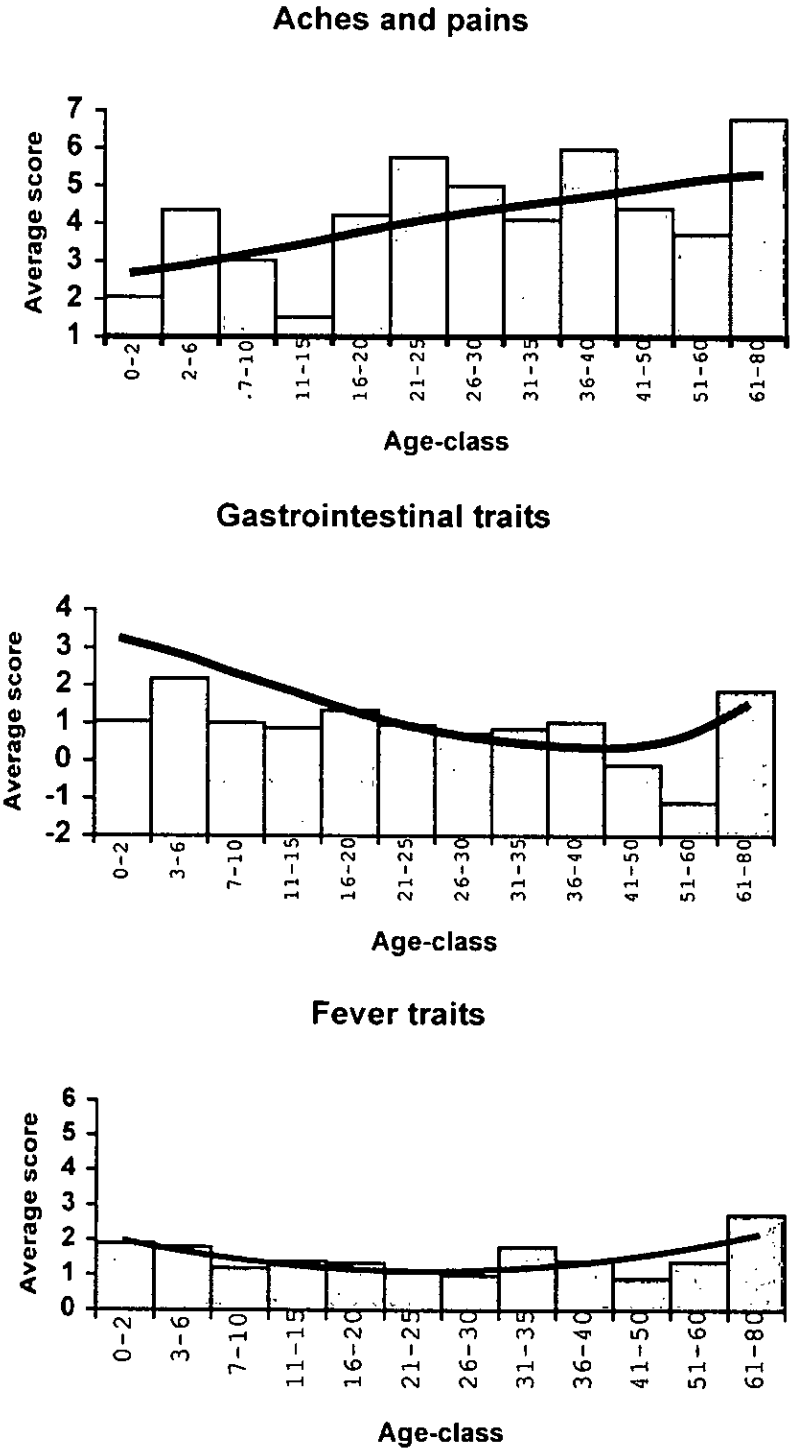


Figure 4.7. Age effects on aches (backache+arthralgia+myalgia+headache), gastrointestinal traits (anorexia+vomiting+nausea) and fever traits (cold+shivering+sweating). Heights of bars are least-squares means for age class and curved lines are linear and quadratic regression lines. The same scale is used for each graph, though the means are different.

consistent with the notion that some physiological transition taking place as the person develops from a child to puberty to full maturity is responsible for age differences in sickness, i.e. that there is an age effect on sickness operating which is independent of previous exposure.

The other issue regarding age effects is species of parasite. It was found that older people acquired relatively more *P. falciparum* infections than younger people (Table 4.2). This may indicate that immunity acquired through repeated infections lasts longer for *P. vivax* infections than for *P. falciparum* infections, in agreement with many other data (reviewed by Taliaferro (1949)). To determine whether this might be the cause, an age by species interaction was added to the model for analysis of the number of infections during the period of study (anti-infection immunity) and of sickness scores (anti-disease immunity). It was found that people tended to acquire immunity to *P. vivax* at an earlier age than they did for *P. falciparum,* as shown in Figure 4.8, but this age by species interaction was not significant. Nor was there as significant age by species interaction on total sickness score, although individual symptoms showed some differential species effects. The increase in aches as age increased was consistently lower for *P. falciparum* than for *P. vivax* ($P < .10$ for backache, $P < .05$ for arthralgia and myalgia). In contrast, though not statistically significant, the decline with age of all three gastrointestinal symptoms was slower for *P. falciparum* than *P. vivax*. Fever traits showed smaller and inconsistent age by species interactions. If aches and pains, which tend to occur in the prodromal period, were positively associated with anti-infection immunity, and gastrointestinal traits were negatively associated with anti-infection immunity, as appeared to be the case with age effects, then this would help explain the lower number of *P. vivax* infections in older people. However, as it is not understood why people develop aches and nausea in relation to immunity to malaria, this is somewhat speculative. In general, however, it can be said that the data do suggest that anti-infection immunity to *P. vivax* lasts longer and, in this region develops earlier, than to *P. falciparum*.

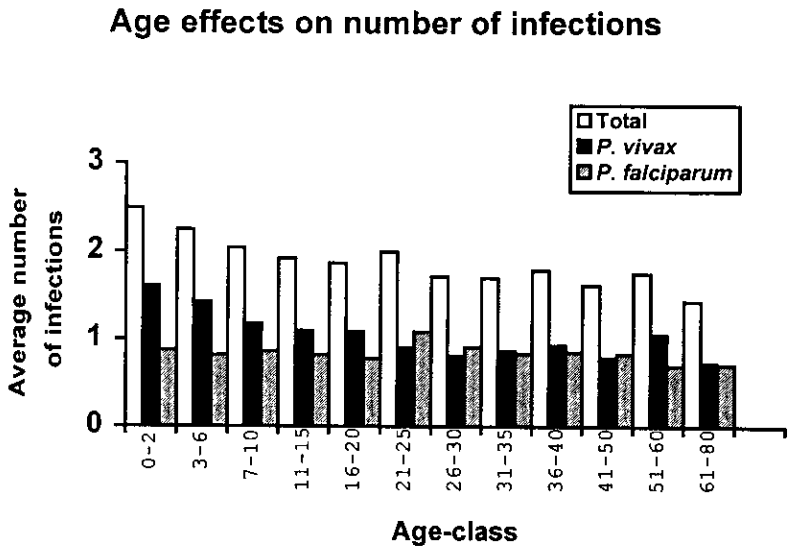


Figure 4.8. Mean number of clinical attacks per person occurring within a 19-month period as a function of age. This is shown separately for *P. vivax* and *P. falciparum* infections.

An alternative explanation for the age difference in species acquired is that because *P. falciparum* was relatively rare before 1986 (Gamage-Mendis, 1991), older people may have been less exposed to this species in their lifetime than younger people. This may also explain why people from endemic areas had proportionally more *P. falciparum* infections than people from non-endemic areas ($P < .05$, Table 4.2). This explanation requires that anti-infection immunity is to some degree species-specific, as implied above and for which there is good evidence (Taliaferro, 1949).

The relationship between previous exposure and species was further examined by fitting the effect of species previous to the current infection. It was found that having at least one previous infection (of any species) within the study period tended to reduce the level of total sickness, the temperature at presentation, and the time to reporting. However, parasitaemia levels increased which is probably because the higher levels of clinical immunity in these patients allowed them to reach higher parasitaemia levels before reporting ill. However, these effects were generally not significant. There was also no strong evidence of species-specific (homologous) immunity, or of non-reciprocal species-specific immunity as has been reported for many other pairs of species (reviewed by Maitland et al., 1997). This result is also in marked contrast with the result from a previous analysis of the data from Study 1 by Gunewardena et al. (1994) who found that successive infections of *P. vivax* conferred some clinical tolerance to *P. falciparum* infections but that *P. falciparum* infections did not confer tolerance to *P. vivax*. Possible reasons for this discrepancy are that Gunewardena analysed median sickness scores, and did not adjust for other effects (e.g. interviewer or village) which caused imbalances in the data. However, a simple analysis along the lines of Gunewardena's still did not show his result, and careful checking of the data did not reveal any reasons for this discrepancy between analyses.

In summary, the analysis of species effects and interactions with age leads to the conclusion that species-specific immunity is not important in the short-term for protection against disease, but that in the long-term (or perhaps the short-term also) it affects the acquisition of anti-infection immunity. This is in accord with the

strong and lasting acquired species-specific immunity observed during the era of malaria therapy (reviewed by Taliaferro, 1949). It is proposed that though species-specific, this is a reflection of the 'strain-transcending' type of immunity which takes some time to acquire, but is long-lived, and provides anti-infection immunity. This is contrasted with the short-lived immunity which protects against severe disease and may or not be strain-specific.

(iv) Other effects – interviewer, season, village and bednets.

In general, season did not affect the level of sickness despite the fact that most infections occurred in the monsoonal period (Figure 4.4). Exceptions to this were a lower degree of arthralgia and myalgia in the dry season of June to September, and a lower degree of sweating in the monsoonal months. *P. falciparum* infections were more prevalent during the monsoonal season than in other seasons (Table 4.2), also supporting the conclusion below that this species is less robust to unfavourable transmission conditions.

Interviewer had a very strong effect on sickness scores (data not shown) probably reflecting differences in interpretation of the largely subjective assessment of disease severity. On the whole, interviewer 'GUN' was consistently higher in recording all traits. In Study 2, interviewer was not recorded and so this large source of variation could not be accounted for in half the data.

In the absence of bednet use (Study 1), villages varied in the mean levels of sickness (Table 4.1), but not in the number of infections per person acquired during the study period (Table 4.2). This may be partly due to the variation between villages in the type of species to which they were exposed (Table 4.2), although the inclusion of species in the model for analysis of sickness scores should have accounted for such an effect.

The effect of bednets (Study 2) was to markedly decrease the number of infections per person, increase the proportion of infections which were *P. vivax*, and decrease the probability of the person being gametocyte positive. This was associated with an increase in total sickness score, and most individual scores, especially in Trial 3. Temperatures at presentation were also higher in the bednet groups, but effects on parasitaemia differed between the trials. Time to reporting

to the clinic also was higher in bednet villages, perhaps suggesting a longer incubation period resulting from smaller inoculum size (Kitchen, 1949). Thus in general the results are consistent with the hypothesis that people sleeping under bednets are infected less often, and as a result lose some anti-disease immunity so that the next time they are infected they show more severe symptoms. This is consistent with the previous results which show a strong short-term component to acquired anti-disease immunity.

An interesting feature of the bednet study was that *P. vivax* infections became relatively more common when bednets were used. This is also consistent with the previous results relating species to anti-infection immunity. It may also reflect a shift in the epidemiological balance between length of infection (which is usually longer in *P. vivax* than *P. falciparum*) and other factors related to the inherent transmissibility of the parasite which could have caused a change in prevalence of the two species in the villages in which bednets were used. Spatial clustering of vector and parasite species within the villages could have occurred because bednet and control villages were physically separated. Even though these villages were closely situated and matched for as many factors as possible, the prevalence and species-specific immunity effects cannot be truly separated in this study. Indeed, the two may interact. For example, when a vector control programme was carried out in New Guinea where four species of malaria parasite were present, the species balance was altered (Metselaar, 1960). The higher persistence of *P. vivax* compared with other parasite species has been noted previously (Taliaferro, 1949).

4.4.3 Heritabilities and repeatabilities

The population from Study 1 lived in 480 houses out of which 293 had at least one person with at least one clinical attack. Only 54% of those people that had an attack also had their parents identified and therefore could be assigned to nuclear families, i.e. to mother-father couples with one or more children. In these families (which almost always lived in the same household) the average number of children per couple was 2.9, with an average of 1.6 of these having at least one attack. In

59% of such cases, only one child per couple had an attack. In 60 of the couples (24%), either the mother or father (or both) also had a clinical attack at some point during the study period thereby giving offspring-parent pairs of records. Thus information on a genetic component came from either pairs of full sibs from 41% of couples, or from offspring-parent pairs in 24% of couples, or a mixture of the two. Of the 240 couples, 36 (15%) had grandchildren with records, and 6 of these grandparents also had records. Children and grandchildren of these grandparents almost always lived in different houses. Thus in summary, the pedigree information consisted mainly of two-generation nuclear families living in the same house with about 30% of affected people also having affected full-sibs or parents living in the same house, and 15% having cousins, uncles, aunts or grandparents living in separate houses. For the remainder of people, house, genetic and non-genetic effects could not be disentangled because there was only one affected person per house/family. Thus there was a large degree of confounding between genetic relationship and house. However, this was by no means complete and therefore, in theory at least, the genetic components should be able to be separated from house and non-genetic person effects.

Table 4.3 shows the phenotypic variances (after adjusting for fixed effects), and the proportions of variance due to house, due to additive genetic effects (heritability) and due to individual non-genetic effects (permanent environmental) for sickness scores and other measures of the infection. Estimates are presented for the full model (Model 1), the model without house fitted (Model 2), and the model without covariates for number and time since previous infections (Model 3). Significance tests for fit of the alternative models based on log likelihood ratios are also shown.

House explained between 0 and 7% of the phenotypic variance in all traits, and in most cases, this was less than 3% and not significantly different from zero. Heritabilities for sickness scores ranged between 0 and 21% with an average of 7%. The highest values were for backache and shivering which were significantly different from zero. Combinations of traits using principal components showed higher heritabilities than individual traits, especially for combined fever traits

Table 4.3. Variance components for sickness scores and other parameters relating to disease severity

Trait	[1]σ^2_P	[2]Variance components (s.e.)								[3]LRT	
		[4]House		[5]Genetic (h^2)			[6]Permanent environment (c^2)			Model	
[7]Model		1	3	1	2	3	1	2	3	1 vs 2	1 vs 3
Total	17.47	0.02 (0.03)	0.02 (0.03)	0.09 (0.06)	0.11 (0.05)	0.09 (0.06)	0.07 (0.06)	0.10 (0.06)		0.2	24.0
Backache	0.485	0.00	0.00	0.16	0.17 (0.07)	0.16	0.02	0.02 (0.07)	0.03	0.0	30.3
Arthralgia	0.952	0.02 (0.03)	0.01	0.04 (0.07)	0.06 (0.05)	0.05	0.15 (0.06)	0.14 (0.06)	0.14	0.1	27.8
Myalgia	0.860	0.01 (0.03)	0.00	0.04 (0.06)	0.05 (0.05)	0.05	0.14 (0.06)	0.14 (0.06)	0.14	0.0	30.6
Headache	0.786	0.000	0.00	0.03	0.00	0.01	0.22	0.21	0.23	0.9	29.7
Anorexia	0.484	0.02 (0.03)	0.01	0.05 (0.06)	0.08 (0.05)	0.05 (0.06)	0.11 (0.06)	0.10 (0.06)	0.10 (0.06)	0.2	34.5
Nausea	0.360	0.04 (0.02)	0.06 (0.03)	0.10 (0.07)	0.16 (0.05)	0.04 (0.07)	0.06 (0.06)	0.03 (0.05)	0.10 (0.06)	0.9	31.4
Vomiting	0.281	0.07 (0.06)	0.07 (0.03)	0.000 (0.07)	0.11 (0.05)	0.00 (0.04)	0.07 (0.05)	0.03 (0.05)	0.00 (0.04)	1.9	34.2
Cold	0.411	0.02 (0.02)	0.01 (0.03)	0.07 (0.07)	0.08 (0.05)	0.07 (0.07)	0.04 (0.06)	0.04 (0.06)	0.04 (0.06)	0.0	27.1
Shivering	1.059	0.00	0.00	0.19	0.18 (0.07)	0.19	0.05	0.05 (0.07)	0.04	0.0	31.9
Sweating	0.503	0.01 (0.03)	0.01	0.01 (0.07)	0.02 (0.04)	0.01	0.04 (0.05)	0.03 (0.05)	0.04	0.0	34.1
Hypo. Pain	0.287	0.02 (0.03)	0.02 (0.03)	0.07 (0.08)	0.10 (0.07)	0.06 (0.08)	0.05 (0.08)	0.04 (0.07)	0.06 (0.07)	0.3	35.2
PC-Aches	1.907	0.02 (0.03)	0.02 (0.03)	0.06 (0.07)	0.08 (0.06)	0.05 (0.07)	0.14 (0.07)	0.14 (0.07)	0.14 (0.06)	0.2	32.2
PC-Gastro	0.634	0.05 (0.04)	0.05 (0.04)	0.11 (0.09)	0.18 (0.07)	0.11 (0.08)	0.06 (0.07)	0.03 (0.07)	0.06 (0.07)	1.2	34.2
PC-Fever	1.107	0.01 (0.03)	0.01 (0.03)	0.21 (0.10)	0.23 (0.11)	0.21 (0.09)	0.01 (0.08)	0.01 (0.11)	0.01 (0.09)	0.1	33.3
P/mia	0.086	0.000 (0.03)	0.00 (0.04)	0.01 (0.09)	0.000 (0.07)	0.01 (0.08)	0.07 (0.09)	0.08 (0.07)	0.08 (0.08)	0.1	35.7
Temp.	0.871	0.01	0.01 (0.03)	0.00	0.01	0.09 (0.04)	0.04	0.04	0.02 (0.04)	0.3	27.9
No. of infections	2.126	0.04 (0.05)	0.04 (0.05)	0.34 (0.12)	0.41 (0.09)	0.29 (0.13)	-	-	-	0.4	7.7
Species	0.200	0.03 (0.03)	0.03 (0.03)	0.000 (0.07)	0.02 (0.05)	0.00 (0.07)	0.15 (0.07)	0.17 (0.06)	0.14 (0.07)	0.8	30.6

1. Total phenotypic variance after accounting for fixed effects, but not house.
2. Some standard errors were not estimable in which case they are left blank.
3. LRT is the likelihood ratio test of Model 2 vs Model 1, or Model 3 vs Model 1. It is assumed to be distributed as $\chi^2/2$. Significance tests based on this distribution with 1 and 4 degrees of freedom, respectively, were $P>0.05$ for all Model 1 vs. 2 comparisons, and $P<0.001$ for all Model 1 vs. 3 comparisons, except for no. of infections where $P<0.05$.
4. Proportion of σ^2_P explained by house.
5. Heritability, i.e. proportion of σ^2_P minus house variance explained by additive genetic effects
6. Proportion of σ^2_P minus house variance explained by non-genetic person effects.
7. Model 1 included all fixed effects, house, additive genetic and permanent environmental effects. Model 2 was as for Model 1 without house fitted. Model 3 was as for Model 1 but without covariates for number of previous attacks and time since last attack.

(h^2=0.21). A further 0 to 22% (average of 7%) of variation in sickness scores was explained by non-genetic variation between individuals, this being greatest (and significant) for aches and pains. Estimates of genetic and permanent environmental effects tended to trade against each other with the estimated correlation between these estimates ranging from -0.51 to -0.81. Thus the repeatability of sickness scores, (i.e. the sum of heritability and permanent environmental effects) ranged from 4 to 25% with an average of 14%, and was typically 15-20%. These results show that there was considerable semi-permanent between-individual variation in disease symptoms, some of which was genetic.

Heritabilities and repeatabilities of parasitaemia and temperature upon presentation were very low (<10%) probably reflecting the transient nature of these traits. In contrast to the clinical symptoms, the heritability of the number of attacks during the study period was moderate (36%) suggesting that there is considerable genetic variation in susceptibility to developing a clinical infection. This is an important result and should be analysed further for major genes segregating within families. Repeatability (but not heritability) of the species infecting an individual was higher than for other traits, perhaps reflecting a long-term species-specific effect, as concluded from the fixed effects analysis of age by species interactions.

When house was dropped from the model, there was usually an increase in the heritability of 1-2%, except for vomiting and nausea where the increase was 10% and 6% respectively. This increase in heritability was largely due to a transfer of house variance into genetic variance because these factors are largely confounded. The estimated correlation between estimates of house and additive genetic variances ranged between -0.75 and -0.86 confirming the high degree of interdependence between house and family relationships. The fit of the model was not significantly reduced by dropping house from the model because the phenotypic variance did not change.

When previous immune history was not accounted for in the model, the phenotypic variance significantly increased because of the strong influences these covariates had on sickness, especially those relating to recent history. This

generally did not alter the heritability estimates or permanent environment estimates (except for vomiting) and so the effects of recent immune history cannot be considered to be responsible for the remaining repeatable differences between individuals in their degree of sickness. Other effects, such as house construction type, nutrition, sleeping and clothing habits, and behavioural differences in reporting may contribute to this variation (El Samani et al., 1987; Gamage-Mendis et al., 1991).

In summary, the analysis showed that individual people carried permanent differences in the degree of disease which they experienced which were not already explained by known immune history and other fixed characteristics such as sex and age. The proportion of variation explained by individuals was approximately the same amount explained by fixed effects (20-25%). A small proportion of the between-individual variation (<5%) may be attributable to between-house variation, a small proportion (<5%) seems to be genetic in nature and the remainder (15%) seems to be due to a mixture of long-term immunity acquired through repeated infection. In general then, a large amount of the total variation in sickness was not able to be explained. This is probably due to many factors, namely, the short-term nature of anti-disease immunity, transient nature of the disease symptoms, stochastic nature of infection rate, inaccuracy of the data obtained by interview, and perhaps genetic variability in virulence among the parasite population. In contrast, the ability to prevent clinical infections and to prevent reinfection with a species experienced many times previously (in this case *P. vivax*) seemed to have a more permanent basis in the individual host which could be genetic.

4.5 General discussion

This study identified some important factors influencing the degree of disease severity experienced by clinically ill people living in an area of low endemicity in Sri Lanka. Drawing together a number of different results from the analysis, the following three-tiered model is suggested to explain some (as little as 25%) of the wide range in clinical and anti-infection immunity observed in this

population. First, there appears to be innate variation among people in the ability to acquire protective immunity (i.e. protection from clinical attacks), and also in the expression of parasite-controlling mechanisms through fever, but not in the severity of other clinical symptoms. This is consistent with other observations of host genetic components to protection against progression towards severe disease (Hill et al., 1991; Abel et al., 1992). Mechanisms for such protection are not understood but in general have been postulated to be a function of anti-parasite activity either by specific immune killing mechanisms (Hill et al., 1992; Luzzi et al., 1991) or by growth-inhibiting mechanisms independent of the immune system (Pasvol et al., 1977, 1978; Friedman, 1979; Abel et al., 1992), as opposed to reduced immune pathology (but see Carter et al., 1992; Dieye et al., 1997). The moderately high frequencies of haemoglobinopathies and other red blood cell disorders in the Indian sub-continent (Livingstone, 1967) may explain some of this variation in disease susceptibility. More detailed within-family studies, concentrating especially on sub-clinical infections, would confirm whether the moderate heritability for the frequency of clinical attacks is true. Linkage analysis with candidate genes may reveal which loci are involved.

Second, there appears to be a long-term effect on the degree of clinical immunity (and perhaps protective immunity) which is acquired through repeated (but not necessarily chronic) exposure. This was evident by the reduction in sickness in people born in an endemic area and the slower rates of acquisition of infections in older people. This could be the outcome of two different mechanisms operating simultaneously – strain-transcending immunity which is acquired slowly and non-linearly and protects against infection, and strain-specific immunity which, because of the delay in experiencing all parasite genotypes, appears to be slow to acquire, linearly related to the number of previous infections and an important determinant of anti-disease immunity (Gupta and Day, 1994a). It has been proposed by Gupta and Day (1994a) that these types can be separated out by comparing age profiles of infection (clinical or sub-clinical) with age profiles of disease (clinical episodes). However, in the present study, this was not possible because parasite prevalence rates were not obtained by continuous active case

detection. Such long-term immunity is suggested to be cell-mediated, with its chief function being to limit the degree of pathogenesis caused by the parasite. This may be through fever (Kwiatkowski, 1989), through cytokine-assisted clearance by the spleen (Taverne et al., 1986), or by non-specific damage caused by cytokines (Clark et al., 1981; Bate et al., 1988; Clark et al., 1989; Grau et al., 1989). A role for TNF in mediating these effects has been shown for all of these pathologies. This long-term acquired immunity may have a genetic basis in the host since heritabilities of combined fever traits were 21%, and TNF-α alleles have been associated with disease severity in an endemic area (McGuire et al., 1994). Expression of this trait would depend on having previously experienced a clinical attack.

Third, short-term immunity seems to be the chief cause of variation in disease severity, as evident by the strong effects of recent infections on reducing sickness, and the relatively low contribution of genetic effects and low repeatability of sickness scores within the 38 month period. It is suggested that this is mediated through specific mechanisms such as antibody lysis of infected cells (Marsh et al., 1989) which are short-lived, and once waned, take time to regenerate in the event of a subsequent infection. The overriding importance of short-term immunity in reducing disease symptoms was clearly demonstrated by the adverse effects of bednets on levels of clinical immunity.

The three proposed general components of immunity to malaria described above, as deduced from this analysis of field data, are in accord with the general picture of immunity to malaria built up over the years from both controlled laboratory experiments and observations from the field. The overall consensus (obtained from reviews of the subject by Taliaferro, 1949; Garnham, 1966; Cohen, 1979; Molineaux and Gramiccia, 1980; Newbold, 1985; Mendis and Carter, 1995) is that (1) general immunity is slowly accumulated through repeated infections (2) specific immunity is short-lived, and (3) immunity has as much to do with tolerance to the infection than with parasite killing. The latter is an interesting and important concept for malaria. As discussed by Mitchell (1991) on the basis of ideas by Sprent (1962), host immunity can be seen to be a balance between two strategies:

either the host produces an exaggerated response which clears the parasite but damages itself (because of the cost of proliferation of immune cells and the triggering of cytokine release with toxic consequences); or, the host produces no response and is overwhelmed by high parasite numbers. Tolerance or resilience to the consequences of the infection lies between these two extremes, and in malaria seems to be the optimal strategy for the host. Given these alternatives, it is perhaps not surprising that such wide variation in disease severity is expressed in malaria. Moreover, the high degree of antigenic diversity in malaria parasites (Kemp et al., 1990) and its ability to generate diversity within infections would select for an immune response which not only is able to target conserved epitopes of the parasite, but is also able to control new infections with different parasite genotypes instead of eliminating all new infections.

An interesting feature of this study was the mixture of *P. vivax* and *P. falciparum* populations in the area. The interaction between these species and the consequences for epidemiology have been reviewed and discussed by Maitland et al. (1997). In this study, from various different standpoints, *P. vivax* appeared to be the dominant parasite. For example, it was the preferred species when levels of immunity were low, as in younger age-groups and in people sleeping under bednets. As a corollary, it appeared to generate longer or stronger clinical immunity than *P. falciparum* and this had long-term consequences for maintenance of clinical immunity. The importance of interactions between *P. vivax* and *P. falciparum* for levels of mortality and morbidity of host populations was argued by Williams et al. (1996) for a population in Vanuatu. In their study, they found that carrying the mildly deleterious α-thalassaemia mutant predisposed children towards acquiring a malarial infection, usually *P. vivax*. This predisposition was proposed to confer a lifetime fitness advantage by providing some protection from the much more lethal parasite *P. falciparum*. This argument for maintenance of an otherwise deleterious gene through *increased* susceptibility to malaria would at first seem to be paradoxical. However, the results from the present study, in particular the maintenance of host genetic variation for the ability to prevent clinical episodes, may reflect a similar phenomenon in this Sri Lankan population. The importance

of maintaining clinical immunity to avoid high levels of morbidity was clearly illustrated when bednets were introduced into this population.

Chapter 5

A theoretical model for predicting the rate of spread of drug resistance

5.1 Summary

Forces determining the rate of spread of drug resistance which has already become established in a population of malaria parasites were explored using a genetics-transmission model which takes account of the strong population structure of these parasites. It was shown that the rate of change of frequency of drug resistant mutants in the parasite population is primarily a function of the proportion of hosts treated with drugs and parasite transmission rates. With high transmission, selection by drugs is more effective than with low transmission because the resistant mutant passes on more copies of itself to the next generation. Recombination between resistance loci only mildly counteracts this force by breaking up resistance combinations. Thus reducing transmission rates, either at the overall population level, or from drug-treated individuals should be effective in curbing the spread of resistance. An exception to this is when the following conditions occur: two unlinked genes act jointly (not independently) to confer resistance, the prevailing transmission rate is already low, and drug use is minimal. Reductions in fitness of the mutant in the absence of drugs, (i.e. a fitness cost to resistance) and the degree of joint action of the mutants do not alter these conclusions.

5.2 Introduction

Drug resistance, especially multiple drug resistance, is disastrous for treatment and control of malaria. Resistance to almost all available drugs, most notably chloroquine, mefloquine and pyrimethamine, continues to spread throughout the tropical world, and the need for new drugs and a strategy to prevent further spread is urgent (Peters, 1987; Warhurst, 1989; Wernsdorfer, 1991; Schapira et al., 1993). In an influential study, Curtis and Otoo (1986) showed that the prevalence of drug use was the most important factor in determining the rate of spread of resistance, and that resistance to two drugs would spread more slowly than resistance to one drug. Thus

they were able to make strong recommendations on how to prolong the useful life of drugs.

The model used to reach this conclusion was simple: it assumed that parasites randomly mate with each other, as they might, for instance, if the entire parasite population were to be found in a single large vat of blood just prior to mating. However, this is unrealistic because malaria parasite populations are strongly sub-structured into hosts. This has the effect of severely restricting the pool of sexual partners during mating and therefore often forcing the parasite to self-mate (inbreed). Indeed, one of the major advances in the field of malaria in the last decade has been to describe just how much inbreeding does occur in natural parasite populations. It is now clear that each host typically carries between one and four genetically distinct parasite types at any one time, the number per host tending to be higher in high transmission areas (Carter and McGregor, 1973; Joshi et al., 1989; Conway and McBride, 1991; Babiker et al., 1994; Hill and Babiker, 1995; Hill et al., 1995; Paul et al., 1995; Babiker et al., 1997; Joshi et al., 1997). So how does this new information on population structure influence our understanding of how drug resistance spreads?

Several authors (Curtis and Otoo, 1986; Paul et al., 1995; Dye, 1991; Dye, 1994; Dye and Williams, 1997; Hastings, 1997) have pointed out that in more outbred populations, such as occur in high transmission areas, there is greater effective recombination breakdown between the two or more loci encoding resistance. It has therefore been suggested that multiple drug resistance develops more slowly in such areas (Paul et al., 1995). Analytical examination of this hypothesis has indeed shown that outbreeding can slow the spread of multiple drug resistance, though only under a limited set of conditions, namely, when none of the resistance genes confer significant protection on their own, when the genes are rare and when selection pressure is low (Dye, 1994; Dye and Williams, 1994; Hastings, 1997). However, this issue of recombination has somewhat diverted attention from the main force driving frequency increases in resistance genes - that of drug selection itself - and so in this study it is also considered how transmission rate influences the effectiveness this selection. Using a deterministic simulation model to pitch the force of effective

selection against the force of effective recombination as determined by population structure, the rates of spread of drug resistance for a range of transmission rates, drug pressures and mechanisms of drug resistance are predicted.

5.3 The basic model and assumptions

The following paragraphs describe the model. First the basic transmission-genetics cycle is described, followed by a description of the assumptions made about the transmission process, epidemiology, population structuring, and drug and natural selection. Finally, the equations describing the changes in frequencies of resistance genes each generation are given. Table 5.1 summarises the notation used.

5.3.1 Transmission cycle and population structuring

As shown in Figure 5.1, in each generation the parasite population flows through hosts (boxes) and mosquitoes (circles) undergoing a series of events which alter the genetic composition of the population. At Stage 1, the parasite is transmitted to new hosts as sporozoites. Each transmission event is represented by a small box within each host: in this diagram there are three independent inoculations to each host. Each inoculum may contain a mixture of genetically different parasites which have probably derived from a bite on just one infectious host. The couplets of letters each represent a parasite's two-locus haploid genotype (haplotype) which is distinct with respect to resistance or susceptibility to two drugs denoted α and β. Haplotype AB is resistant to both drugs, Ab is resistant to only drug α, aB is resistant to only drug β and ab is susceptible to both drugs. The resistance loci are assumed to be genetically linked with a recombination fraction of r. At Stage 2, frequencies of the haplotypes within the host are adjusted according to the fitness of the mutant parasites relative to their susceptible counterparts. At Stage 3, drugs are administered to some of the infected hosts. (In this diagram, two drugs are administered together, but the model allows for single drugs to be used). If the host is carrying a resistant parasite all the susceptible parasites are eliminated leaving only resistant parasites. At Stage 4, pairs of parasite gametes form diploid zygotes in the gut of the mosquito (left side of the circles) and then undergo meiosis to produce four haploid meiotic products per zygote

(right side). If the mosquito has bitten a treated carrier host, all the meiotic products are doubly resistant. If the donor host has not been drug treated, there is recombination breakdown of the doubly resistant haplotypes which reduces their frequency. At Stage 5, some of these meiotic products are transmitted (Stage 6) in the form of sporozoites to new hosts which become superinfected as in Stage 1.

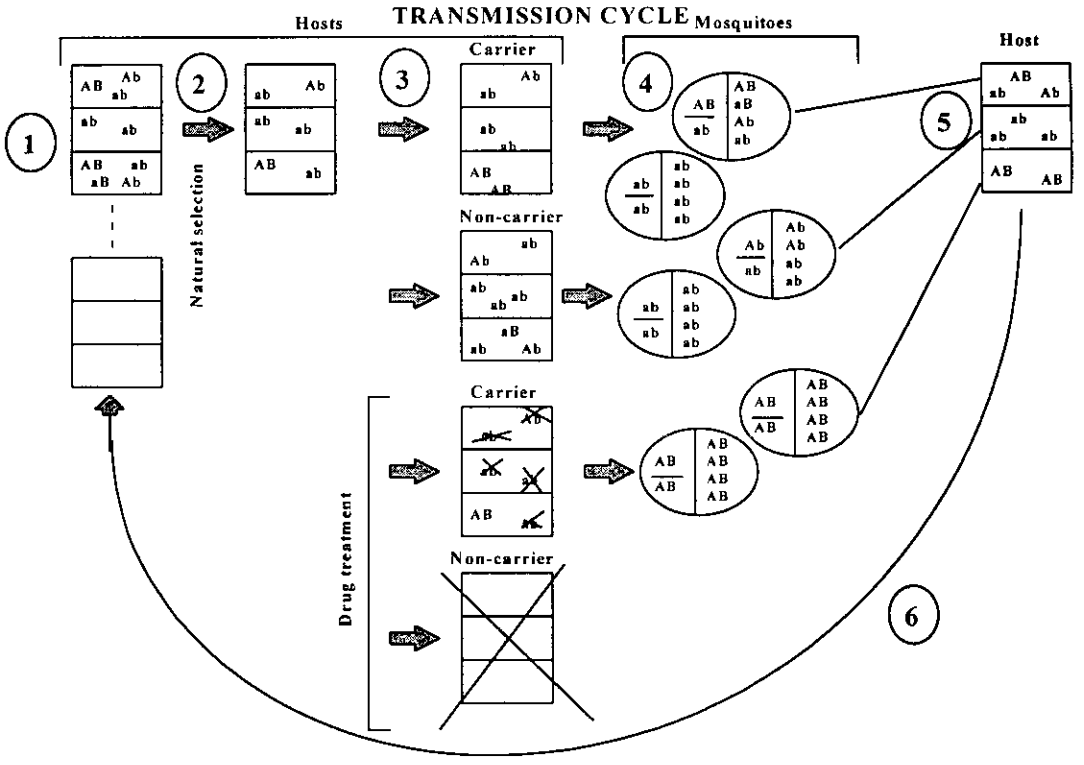


Figure 5.1. Transmission cycle of malaria parasites showing the processes of selection and recombination contributing to gene frequency changes. See text for details. Circled numbers denote the stages represented by the mathematical model described in Figure 5.2.

Table 5.1. Notation and typical values used in the model.

Symbol	Meaning	Values used
Transmission		
c	Mean number of independent infections received by each host	1.1, 3
M	Mean number of transmitted meiotic products per oocyst	1, 4
z	Mean number of oocysts formed per mosquito	1
m	Mean number of genetically distinct haplotypes among gametocytes	1.1, 3
S	Proportion of parasites which self-fertilize	$1/m$
I	Proportion of parasites which inbreed	$1/c$
Genes and frequencies		
α, β	Two drugs which kill malaria parasites	
A, B	Mutant alleles encoding resistance to drugs α and β respectively	
a, b	Wild-type alleles to A and B which are susceptible to drugs α and β, respectively	
r	Recombination fraction between the two loci	
$P=[p_{AB}\ p_{Ab}\ p_{aB}\ p_{ab}]$	Vector of population frequencies of two-locus haplotypes	
$P=[p_A\ p_a]$	Vector of population frequencies in the one-locus case	
P^T, P^U	Vectors of haplotype frequencies within treated and untreated hosts	
Σ	Sum of the elements in a vector of frequencies used for re-scaling	
Σp_i	Sum of frequencies of carrier (resistant) haplotypes where i denotes a carrier type	
C	Proportion of hosts which carry drug-resistant parasites ('carriers')	
D	Population disequilibrium between the two loci	
d	Disequilibrium as a proportion of the maximum disequilibrium	
Selection		
T	Proportion of the host population which are treated with drugs	0.05,0.1,0.2
t	No. of transmissions from a treated host relative to an untreated host	1, 0.2,0.33
W_T	Vector of fitnesses of the haplotypes in the presence of drugs	
	For the two-locus, two-drug (*2-2*) case $W_T=[1\ 0\ 0\ 0]$	
	For the two-locus, one-drug (*2-1*)case $W_T=[1\ w_T\ w_T\ 0]$	$w_T=0.5$
	For the one-locus, one-drug (*1-1*) case $W_T=[1\ 0]$	
W_U	Vector of relative fitnesses of the haplotypes in the absence of drugs	
	For the two-locus cases $W_U=[w_U^2\ \ w_U\ \ w_U\ \ 1]$	$w_U=1, 0.9$
	For the one-locus, one-drug case $W^U=[w_U\ \ 1]$	

5.3.2 Assumptions and notation

The mathematical model is based on the following assumptions:

1. Malaria is stable, i.e. the population of infected hosts and parasites remains large through time such that changes in total parasite population size do not affect the changes in frequency of the mutant genes.
2. Each host receives c independent infections each of which may comprise up to Mz ($M=1..4$) meiotic products or distinct haplotypes among the sporozoites inoculated into the host from a mosquito which has formed z zygotes (oocysts). These c infections give rise to m haplotypes which simultaneously have sexual forms of the parasite (gametocytes) in the blood and $m \leq Mzc$.
3. If frequencies of the haplotypes in the blood are equal, the frequency of selfing (mating between identical parasites) is $S=1/m$, the frequency of inbreeding (mating between parasites derived from the same mosquito, including selfing) is $I=1/c$, and the frequency of outbreeding is 1-I.
4. In the presence of drugs, the vector of fitnesses of the haplotypes *[AB Ab aB ab]* is $W_T=[1 \quad w_T \quad w_T \quad 0]$. This notation can represent two possible cases: either two different drugs are used which both kill parasites carrying the wild-type alleles and only parasites carrying both mutant alleles survive (the two-locus, two-drug case with $w_T=0$, denoted the *2-2* case or 'the epistatic model'); or a single drug is used (i.e. α is the same as β) and each of the mutant alleles confer partial resistance to this drug such that a proportion, w_T, of the parasites carrying a single mutant allele survive after drug treatment (the two-locus case, one-drug case denoted the *2-1* case or 'the additive model'). A third case, in which only one locus is involved and only one drug is used (denoted the *1-1* case), is also considered here. The one-locus vector of fitnesses for haplotypes A and a in the presence of the drug α is represented by the vector $W^T=[1 \quad 0]$.
5. In the absence of drugs, the mutant allele confers a lower fitness on the parasite such that its fitness is reduced to a fraction, w_U, ($w_U<1$) of the fitness of parasites carrying the wild-type allele. Fitness is assumed to act multiplicatively so that the vector of fitnesses without drugs is represented by $W^U=[w_U^2 \quad w_U \quad w_U \quad 1]$ in the two-drug case. For the one-locus case $W^U=[w_U \quad 1]$.

6. A proportion of the host population, T, is treated with drugs and the drugs are fully effective in killing non-mutant parasites.
7. The number of transmissions from a treated host relative to an untreated host is t, i.e. if $t<1$ then drug treatment not only reduces asexual parasites but also reduces the infectiousness of the host to mosquitoes. If $t>1$ then the drugs promote transmission.

5.3.3 Rate of change in frequency of drug resistant mutants

The equations in Figure 5.2 describe the change in frequency of the haplotypes from one generation to the next using the process described above and in Figure 5.1. It has been written in the most general form which is the *2-1* case, but the *1-1* and *2-2* cases can easily be recovered by substituting in $r=0$ and $w_T=0$, respectively. The method requires keeping track of the haplotype frequencies in the parasite population which are stored in a vector $P=[p_{AB}\ p_{ab}\ p_{Ab}\ p_{aB}]$ which at some stages is split into two separate vectors for frequencies among treated and untreated hosts, P^T and P^U. These haplotype frequencies can also be represented by the frequencies of the individual alleles, p_A and p_B, and disequilibrium, D, which represents the deviation of haplotype frequencies from their expected frequencies if the population is randomly mating and not subjected to selection. For example, $p_{AB}=p_Ap_B+D$ where $p_A=p_{AB}+p_{Ab}$ and $p_B=p_{AB}+p_{aB}$. Disequilibrium after meiosis is:

$$D^* = \frac{D}{2}\left[1-r+\frac{S}{2}+\sqrt{(1-r+\frac{S}{2})^2-2S(1-2r)}\right]$$ (Weir et al., 1972).

In this study it is assumed that the two resistance loci are unlinked i.e. $r=0.5$ in which case disequilibrium changes can be calculated as $D^*=½D(1+S)$ if parasites self and $D^*=½D(1+I)$ if parasites inbreed. As the equation for the case of mixed selfing, inbreeding and random mating has not yet been derived, the equation for purely selfing is used in the algorithm because it yields the maximum rate of recombination breakdown and therefore is the least conservative with respect to the recombination effect. In treated hosts, the within-host haplotype frequencies become uneven due to the drug and so selfing rate is not $1/m$ as for treated hosts, but is increased by a factor of $(1+2w_T^2)/(1+2w_T)^2$.

Stage 1. Set initial values

$$P = [p_{AB} \quad p_{Ab} \quad p_{aB} \quad p_{ab}]$$
$$C = \Sigma p_i$$

Stage 2. Natural selection within blood of hosts

$$P^* = [p_{AB} w_U^2 \quad p_{Ab} w_U \quad p_{aB} w_U \quad p_{ab}] / \Sigma$$

Stage 3. Drug selection

Untreated hosts	Treated hosts
$P^{*U} = P^*$	$P^{*T} = [p^*_{AB} \quad p^*_{Ab} w_T \quad p^*_{aB} w_T \quad 0] / \Sigma$

Stage 4. Meiosis in the mosquito

4a) Degree of selfing

$$S^U = \frac{1}{m} \qquad S^T = \frac{1 + 2 w_T^2}{(1 + 2 w_T)^2}$$

4b) Recombination breakdown

$$D^U = p^{*U}_{AB} p^{*U}_{ab} - p^{*U}_{Ab} p^{*U}_{aB} \qquad D^T = p^{*T}_{AB} p^{*T}_{ab} - p^{*T}_{Ab} p^{*T}_{aB}$$

$$D^{*U} = \frac{1}{2} D^U (1 + S^U) \qquad D^{*T} = \frac{1}{2} D^T (1 + S^T)$$

$$p^{*U}_A = p^{*U}_{AB} + p^{*U}_{Ab} \qquad p^{*T}_A = p^{*T}_{AB} + p^{*T}_{Ab}$$

$$p^{*U}_B = p^{*U}_{AB} + p^{*U}_{aB} \qquad p^{*T}_B = p^{*T}_{AB} + p^{*T}_{aB}$$

$$P^{**U} = [p^{*U}_A p^{*U}_B \quad p^{*U}_A p^{*U}_b \quad p^{*U}_a p^{*U}_B \quad p^{*U}_a p^{*U}_b] \qquad P^{**T} = [p^{*T}_A p^{*T}_B \quad p^{*T}_A p^{*T}_b \quad p^{*T}_a p^{*T}_B \quad p^{*T}_a p^{*T}_b]$$
$$+ \quad [D^{*U} \quad -D^{*U} \quad -D^{*U} \quad D^{*U}] \qquad + \quad [D^{*T} \quad -D^{*T} \quad -D^{*T} \quad D^{*T}]$$

Stage 5. Transmission to new hosts

5a) Frequencies in total surviving parasite population

$$P^{***} = \frac{(1 - T) P^{**U} + TCt P^{**T}}{1 - T + TCt}$$

5b) Frequency of carriers in new hosts

$$C^* = 1 - \left[\frac{(1 - T)(1 - \Sigma p_i^{**U})}{1 - T + TCt} \right]^m$$

Stage 6. Re-start cycle

$$P = P^{***}$$
$$C = C^*$$

Figure 5.2. Cyclic algorithm for predicting the frequencies of drug resistant mutants under drug selection over generations. Asterisks indicate where values have been updated from the previous equation. Notation is defined in Table 5.1.

5.4 Predictions from the model

Predictions of the change in frequency of resistance over generations are shown in Figure 5.3 for when drug resistance is controlled by one or two loci and when one or two drugs are in use. The results for a set of conditions (T=.1, t=1, w_U=1, c=3, M=1 and r=.5) are shown in Figure 5.3a and the effects of changing any one of these parameters are shown in Figures 5.3b-f. The following sections discuss the results.

5.4.1 General

Frequency changes are very slow at low frequencies (not shown) thus giving a long lead-in time (hundreds of generations) before drug selection brings a new mutant to a detectable frequency (Anderson and May, 1991; Dye and Williams, 1997). However, once the frequency reaches about 10%, the rate of increase is rapid and if drug pressure is uninterrupted can reach a frequency of 90% within 20 to 60 generations (approximately 2 to 5 years in areas of high transmission, depending on the average generation interval which is expected to decrease as transmission increases). Thus strategies to prevent a drug resistance problem increasing further should be implemented as soon as possible after the problem is detected.

5.4.2 Genetic mechanism of resistance

Two-locus mutants always increase at a slower rate than one-locus mutants because they are continuously broken down by recombination. Therefore it is better to use multiple drugs simultaneously rather than sequentially, as is well established (Curtis and Otoo, 1986; Dye, 1991; Dye, 1994). A further result is that when only one drug is used but is under the control of two loci, if single mutants confer partial resistance (i.e. when two-locus resistance is partly additive), resistance increases faster than if both mutant alleles are required for any degree of resistance (i.e. when two-locus resistance is epistatic). Dye and Williams (1997) have examined this in more detail, and note that the effect of recombination in retarding multi-locus resistance (see below) is eliminated when the genes act in an additive way. This is

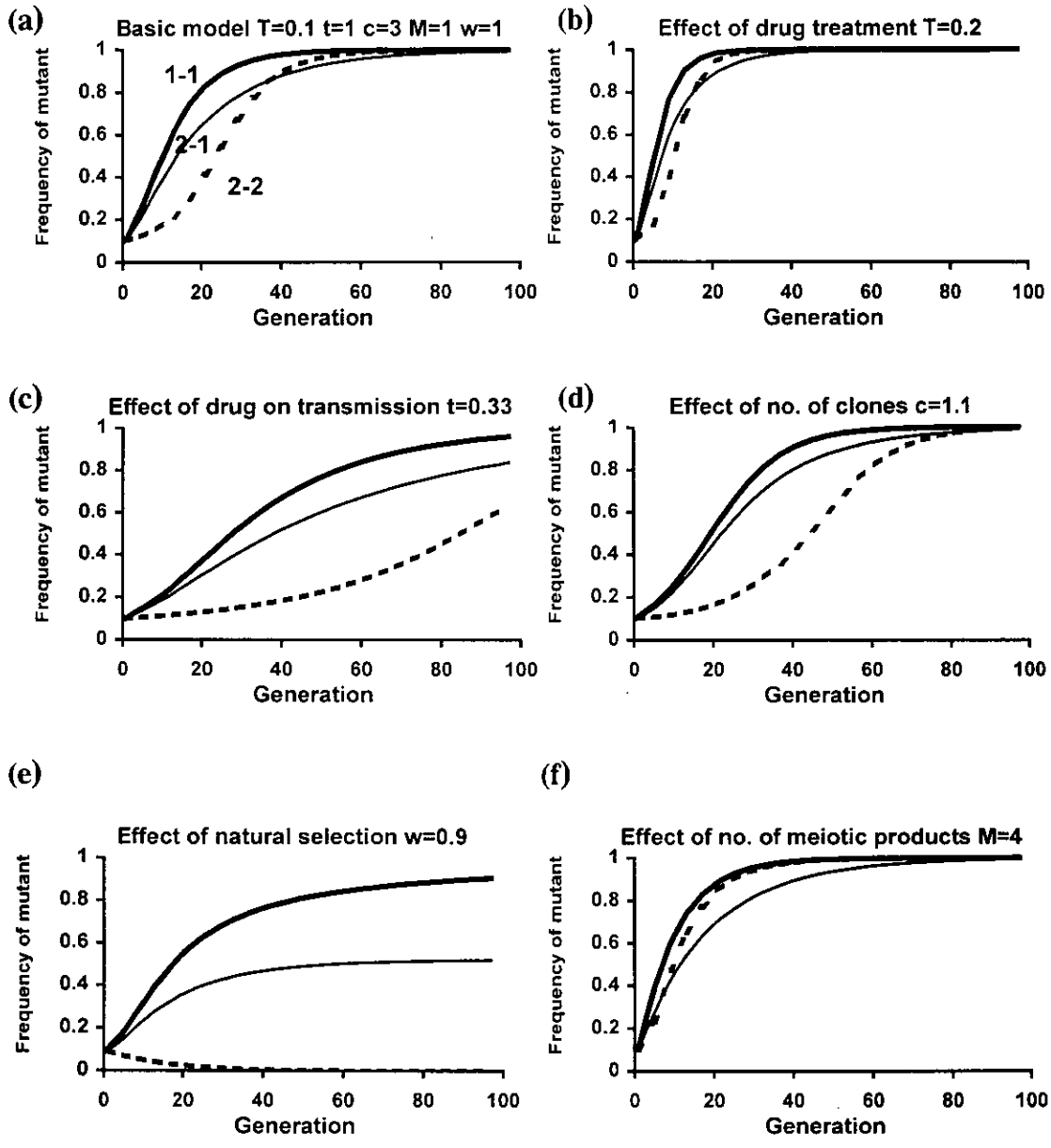


Figure 5.3. Change in frequency of mutant alleles (*A* or *B*) over generations for the one-locus, one-drug (*1-1*, thick filled lines), two-locus, two-drug (*2-2*, dotted lines) and two-locus, one-drug (*2-1*, thin filled lines) cases. (a) Parameter values are $T=0.1$, $t=1$, $c=3$, $M=1$, $w_U=1$, $r=0.5$. (b)-(f) Same conditions as in (a) except for a change in value of one of the parameters, as shown in the headings on each graph.

relevant to chloroquine resistance where probably several genes are required to confer resistance to a single drug (Foote et al., 1990; Wellems et al., 1991; Su et al., 1997) but it is not clear whether these genes act in concert (epistatically), or independently and additively. Multiple mechanisms of resistance are expected to evolve more easily than joint (interdependent) mechanisms which strengthens the case for using two drugs simultaneously instead of one.

5.4.3 Drug pressure (*T*)

The most important influence on the rate of spread of resistance is the proportion of hosts treated. If 20% of the host population is treated with drugs ($T=.2$), it will take about half as long, or 5, 7 and 13 generations less in the *1-1*, *2-1* and *2-2* cases respectively for a mutant allele at a frequency of 10% to increase to 50% than if $T=.1$ (Figure 5.3b vs Figure 5.3a). These differences are somewhat dependent on the starting frequency: the corresponding differences are 10, 11 and 64 generations if the starting frequency is 0.1%. Nevertheless, with any significant level of drug pressure (e.g. $T>.1$) the rate of spread is very rapid in all cases. This result is well known but is emphasised here because it dominates all further considerations. In many circumstances, however, drug treatment is essential to prevent mortality in which case reducing drug pressure is not an option.

5.4.4 Effect of drug on transmission (*t*)

The second major influence on the rate of spread is the degree of transmission from treated hosts carrying resistant parasites (Figure 5.3c vs. Figure 5.3a). If the drug does not reduce transmission, the surviving resistant parasites, in effect, replace susceptible parasites by transmitting to the next generation and thus gain a selective advantage. This advantage is even greater in high transmission areas because the number of copies transmitted to the next generation is directly related to the average number of infections per host. When the drug does succeed in reducing transmission, however, the spread of resistance is dramatically curbed. This leads to the conclusion that the useful life of a drug would be significantly prolonged if drug-treated carrier hosts were prevented from transmitting. This might be achieved through using drugs which are effective against transmission stages, or through simultaneous use of other

control measures such as bednets. The adage that 'sub-curative use of drugs generates resistance' is particularly pertinent here because there is some evidence to suggest that chloroquine, the most widely used drug, when applied to resistant parasites or at sub-curative levels does not impair, and may sometimes promote transmission (Ramkaran and Peters, 1969; Wilkinson et al., 1976; Ichimori et al., 1990; Handunetti et al., 1996; Robert et al., 1996; Buckling et al., 1997). If true, overcoming this problem would be of considerable practical benefit in reducing the rate of spread of resistance.

5.4.5 Number of clones per host (m)

Figure 5.3d shows that if there are few haplotypes per host ($m=c=1.1$ vs. $m=c=3$ in Figure 5.3a), as occurs at low transmission intensities, the rate of increase in frequency of drug resistance mutants is slower than if there are more haplotypes per host. There are two reasons for this which both contribute to greater effective selection in high transmission areas. The first is that there is greater *opportunity* for selection: when hosts carry more haplotypes, the chances of the host carrying a drug resistant mutant (i.e. being a carrier) is higher than if each host carries only one haplotype which means that the mutant is exposed to the drug more often. This effect is only important when frequencies are not low, e.g. above 10%. The second effect is that there is a greater *outcome* of selection. As described above, if the drug does not impair transmission from a treated carrier host, the resistant mutant transmits more copies to the next generation when prevailing transmission rates are high compared with when they are low. This has the effect of amplifying the selective advantage of the resistance genes and thus accelerating the spread of drug resistance in high transmission areas (Hastings, 1997). The third and opposite effect of high transmission rates is that there is more effective recombination when there are more clones per host which retards the rate of spread of resistance when multiple genes are involved (Curtis and Otoo, 1986; Dye 1991; Dye, 1994; Dye and Williams, 1997; Hastings, 1997). This recombination effect is only appreciable when the number of clones per host is very low ($c<1.5$) and at low levels of drug pressure ($T<.2$). In such cases, it would be detrimental to decrease the number of clones per host in the

general population because high levels of inbreeding helps maintain resistance gene combinations. Reducing transmission from *individual* drug-treated hosts is still beneficial, however: it is only the *overall* population transmission rate which is relevant to the recombination effect. The issue of recombination versus selection is discussed in more detail in a later section.

5.4.6 Cost of resistance (w_U)

Figure 5.3e shows that if, in the absence of drugs, the parasite pays a cost in fitness for harbouring resistance genes, this will retard the spread of resistance. In the case of two loci where the cost of each allele is 10% so that the relative fitness of the double mutant is 81%, this is sufficient to counteract the force of drug selection in the two-drug cases and to limit the rate of spread in the one-drug case. If 10% of the host population are treated with drugs, reductions in fitness of greater than 10%, 6% and 25% for the *1-1*, *2-1* and the *2-2* cases, respectively, are sufficient to prevent the further spread of drug resistance by causing a slow decline in the frequency of the mutants. However, for 20% drug treatment, the mutant would have to be 25%, 18% and 60% less fit to counteract drug selection. In other words, drug selection is far more potent than natural selection. There is no good evidence from the field that there is strong natural selection against any of the drug resistant mutants, and in the laboratory the stability of resistance and persistence of mutants is well established (Rosario, 1976; Rosario et al., 1978). Further information is required on the fitness costs of drug resistance in order to establish whether complete withdrawal of a drug from a region will result in a decline in the frequency of resistance and ultimately to renewed effectiveness, as seems to have occurred with some anticoccidial drugs used in poultry (Chapman, 1993).

5.4.7 Number of meiotic products per inoculum (M)

Figure 5.3f shows the effect of the number of distinct 2-locus meiotic products ($M=4$ cf $M=1$ in Figure 5.3a) among the sporozoites which successfully infect a new host per transmission event. If many sporozoites successfully infect a new host such that the probability of getting all four meiotic products (from an outbred mating) is high, then the frequency of carriers, and hence the effective

selection pressure is increased. Thus decreasing the number of sporozoites per mosquito or their establishment in the liver through use of transmission-blocking or liver stage vaccines would help retard the spread of resistance. Even though this is a relatively unimportant effect, it is, nevertheless, a key parameter for more general population genetics models of malaria parasite gene flow. The lack of data on this question calls for further study.

5.5 When does recombination beat selection?

It has been postulated that in high transmission areas, the associated greater amounts of outcrossing will slow the spread of drug resistance compared with low transmission areas (Paul et al., 1995). This depends on whether the increase in effective recombination more than counteracts the concomitant increase in effective selection. In this section, the conditions under which recombination outweighs selection are described, i.e. when drug resistance spread is promoted by inbreeding. This is done by solving the equation which predicts the change in frequency from one generation to the next, viz:

$$\begin{aligned}\Delta p_{AB} &= p_{AB}^{***} - p_{AB}^{U} \\ &= \frac{p_{AB}^{**U}(1-T)+TCt}{1-T+TCt} - p_{AB}^{U} \\ &= \frac{[\frac{1}{2}D(1+S)+p_A p_B](1-T)+TCt}{1-T+TCt} - D - p_A p_B\end{aligned} \qquad (5.1)$$

When this is positive, drug resistance will spread, i.e. when

$$\frac{TCt}{1-T}(1-D-p_A p_B) \geq \frac{1}{2}D(1-S) \qquad (5.2)$$

The left side of Equation (5.2) represents the force of effective selection: this is strongest when drug pressure (T), transmission following drug treatment (t) and the frequency of carriers (C) is high, and when the frequency of the resistance alleles (p_A and p_B) and disequilibrium (D) are low. The right hand side represents effective recombination: it is weakest (i.e. most easily overcome by drug selection) when selfing rate (S, inbreeding) is high which occurs in low transmission areas. The important point is that recombination is only relevant when there is some positive disequilibrium. Disequilibrium levels are maintained by a balance between the

positive force of selection for the double mutant by drugs and the negative force of recombination breakdown through outcrossing (Dye and Williams, 1997). Thus the trade-off between effective selection and effective recombination involves disequilibrium. Therefore, in order to predict whether drug resistance will spread, it is necessary to quantify the amount of disequilibrium generated through the selection process. In Figure 5.4a the disequilibrium levels generated as the mutant alleles increase in frequency from .01 to 1 under drug pressures of $T=.05$ and $T=.1$ for a low transmission ($c=1.1$) and high transmission ($c=3$) environment are shown for the two-drug, two-locus case: these were calculated using the algorithm in Figure 5.2. The results are expressed in terms of normalised disequilibrium, d, which is disequilibrium as a proportion of its maximum value and is calculated as $D=dp_A(1-p_B)$. It can be seen that higher amounts of disequilibrium are caused by higher drug pressures and fewer clones per host, but these are well below the maximum possible disequilibrium ($d=1$). This leads to the conclusion that the recombination effect is only important when there are few clones per host: this is illustrated in Figure 5.4b where the change in frequency of the double mutant (p_{AB}), in the generation immediately after p_{AB} has reached a value of 0.1, is shown for drug pressures of $T=.05$ and $T=.10$. There is a sharp decline in the rate of change between $c=1.05$ and $c=1.5$ after which the rate of change increases with transmission rate. Figure 5.4b also shows that this recombination effect is more easily overcome when drug pressure becomes stronger, and virtually disappears when the drug reduces transmission (i.e. t decreases).

In summary, this section has shown that the conditions under which decreasing the average number of clones per host promotes double drug resistance are approximately when $c<1.5$, when drug treatment rates are below 20%, and when the frequency of both mutants, each of which confer no significant protection on their own, are low. These conclusions concur with those of Dye and Williams (1997) even though the model used here was quite different. They are different from the conclusion of Hastings (1997) in which it was assumed that disequilibrium would always be maximal, a condition which over-emphasises the effect of recombination in retarding the spread of multi-locus drug resistance.

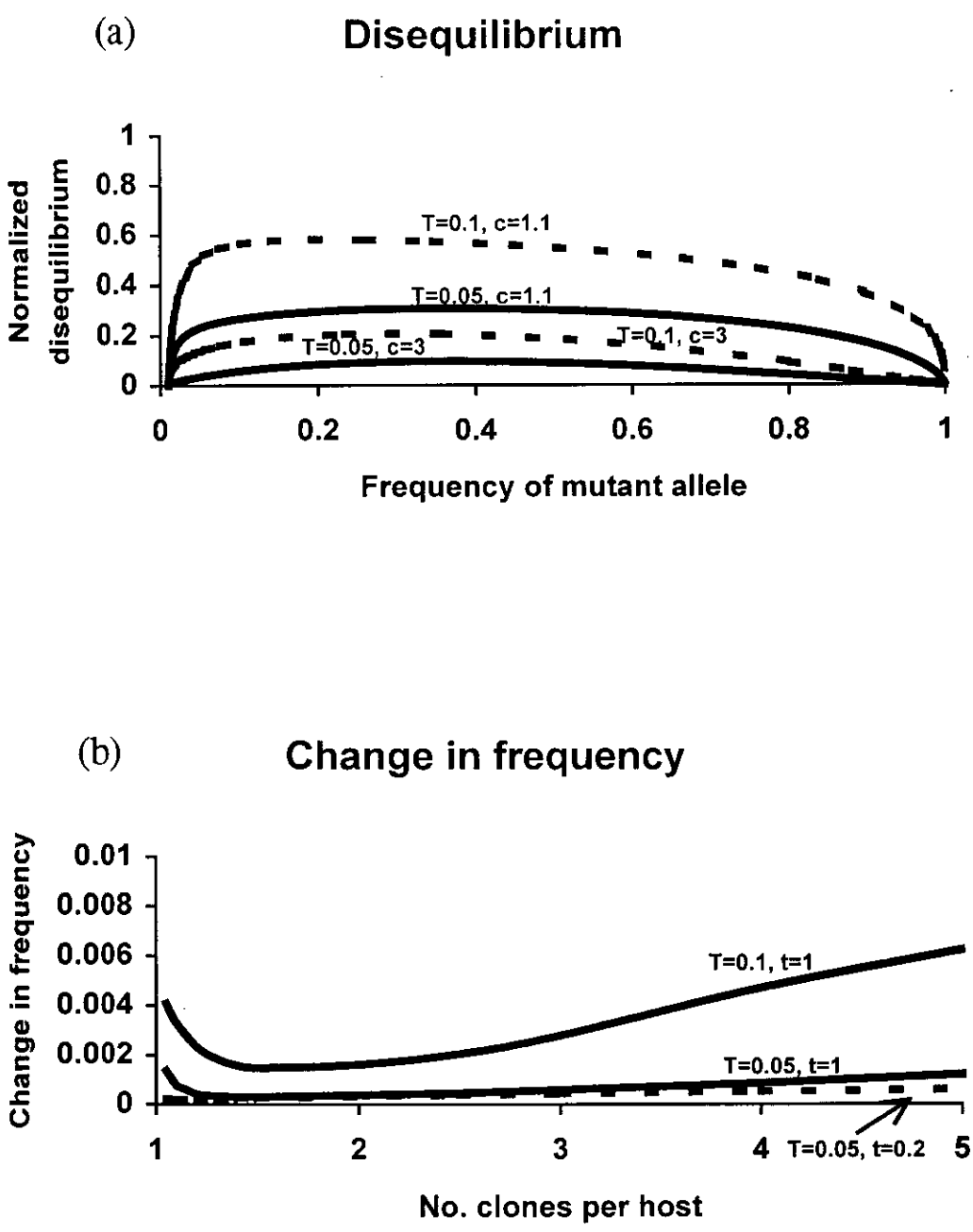


Figure 5.4. (a) Normalised disequilibrium as a function of mutant allele frequency in the two-locus case for two levels of drug pressure (T=0.05 and T=0.1) and two transmission levels (number of clones per host, c=1.1 or 3). (b) Change in frequency of the double mutant haplotype (*AB*) in the generation when the frequency of both resistance alleles has reached 0.1 for two levels of drug pressure (T=0.05 and T=0.1) and when the drug reduces transmission rate from treated carrier hosts to 20% that of untreated hosts (t=0.2).

5.6 Discussion

Building population structure into models to predict the rate of increase in drug resistance does not alter the two well-established principles regarding the spread of resistance, namely: (1) drug resistance will relentlessly increase to the point of drug treatment failure in the majority of the host population for as long as there is any significant drug use in areas of endemic malaria, and (2) the use of two drugs simultaneously will slow the rate of spread, especially when the frequency of the mutant alleles are both rare. A more novel conclusion from this study, however, and one which has been the issue of some debate (Dye and Williams, 1997; Hastings, 1997; Mackinnon, 1997, Chapter 6), is that in the *majority* of cases, high transmission rates promote the spread of drug resistance rather than hinder it, as proposed by Paul et al. (1995). This is because selection is more effective in high transmission areas where resistance genes under drug selection are transmitted to the next generation in greater copy number than in low transmission areas. The higher effective selection in high transmission areas outweighs the corresponding higher rate of effective recombination which opposes the spread of resistance. This finding generates two new recommendations. The first is that transmission should be minimised from individuals who are treated with drugs. This would be especially effective in areas of high transmission intensity, though is helpful in all environments. In the short term, this could be achieved through use of bednets by treated patients, proper administration of drugs, (i.e. avoiding sub-clinical doses), or in the longer term, developing drugs which are effective against transmission stages. The second recommendation is that if strategies for managing drug resistance are implemented, these should be accompanied by simultaneous efforts to reduce overall transmission rate in the population because the two strategies act synergistically. The exception to this is managing multi-locus resistance in areas where the average number of infections per host is very low (<1.5), drug pressure is also low, and the mutant genes are rare (Dye and Williams, 1997; Chapter 6, Hastings and Mackinnon, submitted). In this case, the force of recombination in breaking up resistance gene combinations is sufficient to oppose the force of drug selection thus causing a decrease in the rate of spread as

transmission is further reduced. Both of these recommendations are tall orders: malaria parasites seem to be particularly adept at increasing their transmission output when faced with difficult conditions in the host such as toxic drugs or immunity (Carter and Graves, 1988; Alano and Carter, 1990; Sinden et al., 1996; Buckling et al., 1997). This is why a better understanding of how the parasite adapts genetically and facultatively in response to host-imposed selection pressures is critical for devising better control strategies. As shown here, population structure can have a profound effect on whether the parasite succeeds in adapting to these control measures and so is an integral part of this understanding.

Chapter 6

Survival probability of drug resistant mutants in malaria parasites

6.1. Summary

This study predicts the ultimate probability of survival of a newly arisen drug resistant mutant in a population of malaria parasites with a view to understanding what conditions favour the evolution of drug resistance. Using branching process theory and a population genetics transmission model, the probabilities of survival of one and two-locus new mutants are calculated as functions of the degree of drug pressure, the effect of the drug on transmission, the mean and variation in transmission rate, and the degree of natural selection against the mutant. It was found that probability of survival increases approximately linearly with drug pressure, the relative transmission output from drug-treated hosts, and mean transmission rate; it decreases with variability in transmission rate and with natural selection. A complex interplay between transmission rate, drug pressure and the effect of the drug on transmission leads to one exception to these general rules: when resistance is coded for by two loci, when drug pressure is low, when transmission rate is low and when the population has stabilised to a new equilibrium following the introduction of drugs, a decrease in transmission rate can favour the survival of a new mutant. This is because under these conditions recombination between the resistance genes has a sufficiently strong negative effect on the frequency of double resistance to counteract the positive effects of drug selection and higher relative transmission rate of mutants compared with non-mutants. In all other circumstances, however, the results show that reducing transmission rate in the general population, especially in drug treated hosts, when combined with minimising drug usage, will have a significant impact on preventing new drug resistance mutations becoming established in parasite populations.

6.2. Introduction

Malaria parasites have a remarkable ability to develop resistance to drugs. This has created an urgent problem because resistance to all of the available drugs has arisen at least once (Bjorkman and Phillips-Howard, 1990), and the development of new drugs has virtually stalled. Evidence from field and theoretical studies indicates that resistance continues to spread as long as there is any drug pressure, and that beyond a certain frequency, the rate of spread is very rapid (Curtis and Otoo, 1986; Dye 1991; Wernsdorfer 1991; Dye, 1994; Dye and Williams, 1997; Hastings, 1997; Mackinnon and Hastings, in press, Chapter 5). It therefore seems wise to try and prevent or delay the development of resistance in the first place.

The spread of a new drug resistant mutant, or set of mutants, in a malaria parasite population depends on the relative forces of selection by drugs, natural selection (presumed to be unfavourable), recombination between resistance loci in the case of multi-locus resistance, and the probability that the mutant is transmitted. Theoretical models have been formulated around these factors to predict how rapidly the frequency of drug resistant mutants increases (Curtis and Otoo, 1986; Cross and Singer, 1990; Dye and Williams, 1997; Hastings, 1997; Mackinnon and Hastings, 1998, Chapter 5). These models, deterministic in nature, have shown that the key factors leading to a more rapid rate of spread of drug resistance are high drug pressure and high transmission rate. An exception to this is when high levels of inbreeding prevail. In such cases, at low drug pressure, when the mutant is rare, and when resistance is coded by two loci which jointly confer resistance, with neither of the loci conferring significant resistance on its own, increasing transmission rate can slow the spread of drug resistance, and even decrease its frequency. The reason why this occurs is because resistance gene combinations are broken down more often when outbreeding is common, and this eroding force on frequencies is sometimes sufficient to counteract the opposite of force of drug selection. Under most conditions, however, the mutant is predicted to increase in frequency, and increase faster when any of these restrictions is relaxed.

These previous studies address only one of the two main issues regarding the evolution of drug resistance, namely, for a mutant which is already established in the

population, how rapidly it increases (or decreases) in frequency. The other important issue is how easily resistance can develop in the first place. This depends primarily on the stochastic events surrounding the mutant's arrival in the population. In this study, a stochastic population genetics model is used to predict the probability that a newly arisen mutant survives the first few rounds of transmission until selection by drugs can bring it to a 'safe' frequency, i.e. beyond the risk of being lost to the population due to chance events during transmission. Thus it addresses the issue of how readily a drug resistance problem can arise from a new mutation event, or from the arrival of a new migrant parasite into a resistance-free population, and therefore has implications for limiting the evolution of drug resistance.

6.3. The basic model and assumptions

In the following section the assumptions made in the model about the epidemiology, transmission cycle, drug selection and relative fitness of mutants are given. These are the components required for the branching process theory subsequently used to predict survival probability of a new mutant.

6.3.1. Distribution of number of transmissions per host

Transmission of malaria is characterised by its variability due to a multitude of host, vector and parasite-related factors. Though transmission rate is usually described in terms of a single parameter, R_o, which is often thought of as a constant because it represents the average number of transmissions from one infected host to other hosts, it is recognized that there is variability around R_o, i.e. in the number of transmissions from individual hosts (Koella, 1991). It is this variability which makes new mutants vulnerable to loss during the first few rounds of transmission. If the average R_o is one, then on average the total parasite population replaces itself each generation. However, individual parasites with distinct multi-locus haplotypes may not always replace themselves once because of sampling variation in the transmission process. Thus when considering the population genetics of parasites, it is necessary to account for the uneven redistribution of genes from one generation to the next.

In the model presented here the transmission rate is modelled by a variable, R, which is distributed as a negative binomial with a mean of $\overline{R}$ and variance of σ_R^2. The parameters of the negative binomial distribution, p, and k, are related to the mean and variance in the following way:

$$\overline{R} = \frac{k(1 - p)}{p} \qquad (6.1)$$

$$\sigma_R^2 = \frac{k(1 - p)}{p^2}$$

The parameter k can be thought of as the aggregation or shape parameter: as k increases, the less 'clumped' are the data and when $k = \infty$, the distribution is Poisson. The parameter p can be thought of as the zero probability parameter: the frequency of the zero class is given by p^k so that the mean increases as p decreases. When transmission rate has a mean of $\overline{R}$, the mean number of successful (non-zero) transmissions is $\overline{R}/(1-p^k)$.

The above applies to the number of transmissions in the absence of drugs. An additional parameter, t, is introduced to the model to allow for the fact that drug treated hosts may transmit parasites at different rates from treated hosts. To reflect this, the negative binomial distribution for the number of transmissions is now assumed to have a mean value of $t\overline{R}$ where t is the relative transmission from treated hosts compared to untreated hosts which carry the mutant, and may vary from zero upwards, but not conceivably beyond a value of $t=10$. For example, if drugs increase the transmission rate per host by a factor of five, then $t=5$. When $t=1$ the drugs are assumed to have no effect on transmission. The variance is assumed to be reduced by a factor of t^2.

6.3.2. Number of clones per host

It is assumed that a host carries an average of c independent infections, each infection comprising one clone. It is also assumed that c is the number of haplotypes which simultaneously have sexual forms of the parasite (gametocytes) in the blood and therefore are potential mates during fertilization after the mosquito has taken a blood

meal. Here c is assumed to be constant even though these c infections come from a variable number of transmissions. In this study c is defined as being equal to $\overline{R}$ for simplicity. However, $\overline{R}$ could be considered to be an underestimate for c because it represents the number of non-zero transmissions. On the other hand, it may be an overestimate because it may be that not all infections are infectious simultaneously. The relationship between $\overline{R}$ and c has not yet been properly explored in the field or theoretically. Nevertheless, estimates for c from field studies are available based on the number of one or two-locus haplotypes in the blood (Carter and McGregor 1973; Conway et al., 1991; Babiker et al., 1994; Hill and Babiker, 1995; Hill et al., 1995; Ntoumi et al., 1995; Paul et al., 1995), or from studies on the amount of heterozygosity among oocysts formed in mosquitoes. In a high transmission area in Tanzania the estimated number of clones per host was 3.5 (Hill et al., 1995; Hill and Babiker, 1995) and in a low transmission area in New Guinea was 1.1 (Paul et al., 1995).

6.3.3. Drug resistance genes

Now assume that the parasite has two loci at which there are two allelic forms - one the wild-type allele, and the other allele encoding resistance to a given drug which would otherwise kill all parasites not carrying the mutant allele. Denote these alleles as A and a for mutant and wild-type alleles, respectively, for the locus encoding resistance against drug α and similarly B and b for alleles for the locus encoding resistance against drug β. Thus there are four relevant genotypes, AB, Ab, aB and ab. The vector of fitnesses, which determine the relative frequencies of haplotypes within the host, in the presence of both drugs is represented by a vector, $W_T=[1\ 0\ 0\ 0]$, and in the absence of drugs by the vector $W_U=[w^2\ \ w\ \ w\ \ 1]$. These reduce to $W_T=[1\ 0]$ and $W_U=[w\ \ 1]$ when only one drug is in use in which case the second locus is irrelevant. This latter case is called the 'one-locus' model. It is important to note that fitness is defined here as the relative number of gametocytes with the two-locus haplotype at the time of transmission, rather than the number of distinct clones in the host's blood. If a double mutation has arisen at the beginning of

an infection in a host which carries c independent infections, the relative frequencies of the two-locus haplotypes will be

$$W_U = \frac{1}{w^2 + 2c - 1}[w^2 \quad 0 \quad 0 \quad 2c-1] \qquad (6.2a)$$

For example, if a host has one infection ($c=1$) and a double mutation (*AB*) occurs which has equal fitness to the wild-type haplotype (*ab*), then its frequency in the host is ½. If the host is infected twice more ($c=3$) with non-mutant parasites, this frequency is reduced to $\frac{1}{6}$. If the recombinant meiotic products (*Ab* and *aB*) are also in the host, as may occur in subsequent generations if all four meiotic products are transmitted in the same inoculum, the relative frequencies in the host will be:

$$W_U = \frac{1}{w^2 + 2w + 4c - 3}[w^2 \quad w \quad w \quad 4c-3] \qquad (6.2b)$$

6.3.4. Transmission cycle

The basic model of transmission assumes that each host receives one or several independent infections from separate transmission events and that the parasites from these infections co-exist in the blood of the host. The relative frequencies of the haplotypes in these infections are adjusted during the course of the infection according to their relative natural fitnesses by multiplying with W_U and re-scaling. In a proportion of the host population, T, the frequencies are adjusted for drug selection by multiplying with W_T and re-scaling, i.e. the frequencies of mutant parasites in treated hosts are set at unity, and non-mutant parasites at zero. The parasites then form gametocytes which are taken up by a mosquito in a blood meal and undergo self or cross-fertilization during the zygote stage. The frequencies of the diploid genotypes in the mosquito are assumed to reflect random mating among the gametocytes within the host from which the mosquito took the blood meal. These diploid genotypes then undergo recombination and frequencies among the haploid meiotic products are adjusted accordingly. The meiotic products, or a subset of them, are transmitted to a new host to initiate a new infection.

6.4. Survival probability of a new mutant

6.4.1. General introduction to branching process theory

The following section describes the branching process theory used to predict the ultimate survival probability of a single copy of a mutant. This theory enables prediction of the probability that a single replicating particle (in this case a mutant allele) which is subject to stochastic processes during its replicative cycle ultimately survives in the population, i.e. is still in the population after many generations. This probability can be predicted from the distribution of the number of 'offspring' each particle produces each generation. The distribution of the number of offspring can be summarised by a single function called the probability generating function (pgf, denoted $\Phi(s)$ where s is a dummy variable). For example, the pgf for a Poisson variable is represented by $\Phi(s) = e^{-\lambda(1-s)} = \sum_{k=0}^{\infty} \frac{e^{-\lambda}(\lambda s)^k}{k!}$. Probability generating functions are useful because the value of s which solves the equation:

$$\Phi(s) = s \qquad (6.3)$$

gives the ultimate extinction probability, and hence the ultimate survival probability (denoted usp) is given by 1-s. If the mean of the distribution described by $\Phi(s)$ is less than one, the solution to Eq. 6.3 is 1-s=0. Thus the particle will not survive if the mean number of offspring is less than one (because it does not on average replace itself). If the mean number of offspring is greater than one, the particle has a finite, but less than perfect probability of surviving, as given in Eq. 6.3.

A further property of branching process theory is that if there are several distinct processes during the replication cycle, each with different pgf's, then the pgf used in Eq. 6.3 is the compounded distribution of the individual pgf's involved in the process. Note that Eq. 6.3 only holds if the distribution of the number of offspring is the same in all generations. If, however, environmental fluctuations cause a change in the pgf over time, it is necessary to compound over the different distributions from the generations to obtain the overall pgf. Note also that it is assumed that the population size is infinite or very large such that there is a zero

probability that two identical mutants will meet and produce offspring together. A digestible review of branching process theory is given by Schaffer (1970).

6.4.2. Application to malaria mutants

In malaria, the replicative cycle ('generation') is a transmission from one host to the next which involves the transfer of parasites to mosquitoes, meiosis in the mosquito and then transfer to a new host. This process can be broken down into three stochastic processes each with their probability distributions. One distribution is for the number of transmissions from one infected host to a number of new hosts, assumed to have a negative binomial form. The second distribution is for segregation during meiosis, and the third is for survival to sexual maturity in the new host: these last two distributions both have a binomial distribution. The combination of these distributions will determine how many copies of a single mutant are left in the population after one transmission cycle. The parameters of these distributions depend on whether the host has been treated with drugs or not. The case of no drug treatment is given first, and the case of drug treatment is then derived from it.

6.4.2.1. Probability generating functions for untreated hosts

(i) If the host is not treated with drugs, the pgf for the negative binomial distribution representing the number of transmissions to new hosts is given by

$$\Phi_{NB}(s) = \left[\frac{p}{1-(1-p)s}\right]^k \qquad (6.4)$$

(ii) The binomial pgf for meiosis is:

$$\Phi_B(s) = 1 - \rho + \rho s \qquad (6.5)$$

with mean ρ and variance $\rho(1-\rho)$. Here the parameter ρ represents the probability that the mutant haplotype (A in the one-locus case and AB in the two-locus case), when undergoing meiosis with non-resistant haplotypes is represented among the transmitted meiotic products following a mating between a resistant and susceptible haplotype. This probability depends on the relative numbers of each haplotype among the c clones represented in the gametocytes of the mutant-carrying host as given by Eq. 6.2.

Table 6.1 gives the equations for ρ for the first generation of the mutant when there are no recombinant haplotypes (*Ab* or *aB*) present, and for later generations where these recombinant haplotypes may or may not be co-transmitted with the *AB* haplotype. However, if only one meiotic product is transmitted per infection, then these sister haplotypes are extremely unlikely to co-exist within the one host. As it is not known how many haplotypes are on average transmitted per new infection, equations are given for when all four meiotic products are transmitted and when only one is transmitted (Table 6.1).

Table 6.1. Values of the replacement rate ρ under different assumptions about co-transmission of meiotic products. Values for the special cases of $c=1$ and $w=1$ are shown to illustrate the effects of the assumptions.

	One meiotic product transmitted			All meiotic products transmitted		
	General	w=1	c=1	General	w=1	c=1
1 locus						
Generation 1	$\frac{cw}{w+2c-1}$	$\frac{1}{2}$	$\frac{w}{w+1}$	$\frac{2cw}{w+2c-1}$	1	$\frac{2w}{w+1}$
Generation >1	$\frac{cw}{w+c-1}$	1	1	$\frac{2cw}{w+2c-1}$	1	$\frac{2w}{w+1}$
2 loci						
Generation 1	$\frac{cw^2[w^2+\frac{1}{2}(2c-1)]}{[w^2+2c-1]^2}$	$\frac{1}{4}+\frac{1}{8c}$	$\frac{w^2}{w^2+1}$	$\frac{2cw^2[w^2+\frac{1}{2}(2c-1)]}{[w^2+2c-1]^2}$	$\frac{1}{2}+\frac{1}{4c}$	$\frac{2w^2}{w^2+1}$
Generation >1	$\frac{cw^2[w^2+\frac{1}{2}(c-1)]}{[w^2+c-1]^2}$	$\frac{1}{2}+\frac{1}{2c}$	1	$\frac{4cw^2[w^2+2w+2c-1]}{[w^2+2w+4c-3]^2}$	$\frac{1}{2}+\frac{1}{2c}$	$\frac{4w^2}{[w+1]^2}$

These equations show that in the one-locus case, when the mutant is equally as fit as the wild type, the probability that the mutant is among the meiotic products is one. (This is not true in the first generation when, if it has equal fitness to the wild-type from which it arose, and assuming that it arose early in the infection, it will be at a frequency of 0.5 among the gametocytes, in which case its probability that it is transmitted when only one meiotic product survives is 0.5). In the two-locus case, this probability is $\rho=\frac{1}{2}+\frac{1}{4c}$ in the first generation and $\rho=\frac{1}{2}+\frac{1}{2c}$ in later

generations. This is greater than the $\frac{1}{2}$ expected from random mating (where $c = \infty$) because sometimes the parasites self-fertilise. Thus fewer clones per host leads to inbreeding and less recombination breakdown of resistance combinations and therefore higher probabilities of surviving meiosis.

(*iii*) The binomial pgf for survival to sexual maturity is:

$$\Phi_B(s) = 1 - \pi + \pi s \qquad (6.6)$$

where π is the probability that the transmitted parasite successfully establishes itself in the new host and produces transmission forms. In the case where malaria is stable, in the absence of drug pressure, this probability is expected to be $1/\overline{R}$, i.e. out of $\overline{R}$ transmissions to $\overline{R}$ different hosts, only one of these infections will survive to sexual maturity. This is equivalent to assuming that, in a stable population, a neutral allele has an average of one copy of itself in the next generation (transmission cycle), or an average number of offspring of one.

The situation changes when drugs are used in the population in which case two alternative scenarios are envisaged. The first is that drugs have only been recently been introduced to the population when the new mutation occurs: the mutation may have arisen many times before but not survived because of the lack of drug pressure. In this case, if the host is not treated with drugs, the replacement rate is $\pi\overline{R} = 1$, i.e. as in the time before drugs were introduced. This scenario is termed the 'naive' (to drugs) or 'pre-stabilisation' case. The second scenario (the 'exposed' or 'post-stabilisation' case) is that drugs have been in use for enough time for the reproductive rate of the parasites to have increased sufficiently to overcome the decrease in population size due to drug pressure. In this case, parasites in untreated hosts are expected to have a replacement rate of $1/(1-T)$ (Hastings and Mackinnon, submitted) where T is the proportion of hosts treated with drugs. This is because in order to maintain stable malaria in the long-term, the parasites from untreated hosts must be replacing the niches left available by parasites eliminated from treated hosts. Without this replacement at a rate of $1/(1-T)$, the population size continually decreases in the presence of drugs because every generation a proportion T are eliminated by drugs.

The pgf's for the three stochastic processes (Eqs. 6.4, 6.5 and 6.6) can now be compounded into a single pgf. The compounded pgf is denoted as $\Phi_{B,B,NB} = \Phi_{NB}(\Phi_B(\Phi_B(s)))$ and can be shown to be distributed as negative binomial with a mean of $\pi\rho\overline{R}$ and a variance of $\pi\rho\overline{R}(1-\pi\rho+\pi\rho\sigma_R^2/\overline{R})$ (Kojima and Kelleher, 1962). This means that the pgf of the compounded distribution for the untreated host is given by:

$$\Phi^U(s) = \left[\frac{p^*}{1-(1-p^*)s}\right]^{k^*} \tag{6.7}$$

where

$$p^* = \frac{p}{p-\pi\rho p+\pi\rho}$$

$$k^* = k$$

6.4.2.2. Probability generating function for treated hosts

If a host is treated with drugs, the probability that the double mutant is among the meiotic products and survives is $\pi\rho = 1$, i.e. all transmitted mutants are *AB* haplotypes. The relative number of transmissions from a treated host compared with an untreated host may be altered by drugs by the factor t. Thus the pgf if the mutant arises in a drug treated host is negative binomial with mean $t\overline{R}$, and variance is $t^2\sigma_R^2$:

$$\Phi^T(s) = \left[\frac{p^*}{1-(1-p^*)s}\right]^{k^*} \tag{6.8}$$

where

$$p^* = \frac{p}{t}$$

$$k^* = \frac{k(1-p)}{t-p}.$$

The combined pgf over untreated and treated hosts is the sum of the pgf's for untreated and treated hosts weighted by the probabilities that the mutant finds itself in a treated or untreated host:

$$\Phi(s) = (1-T)\Phi^U(s) + T\Phi^T(s) \qquad (6.9)$$

To take into account the different probability of the mutant surviving meiosis in the first generation compared to subsequent generations, the above distribution with substitutions of ρ appropriate for the first generation is compounded with this distribution with ρ appropriate for subsequent generations (Table 6.1).

Eq. 6.9 was used to evaluate numerically the survival probabilities as functions of drug treatment rate (T), drug effect on transmission (t), relative fitness (w), mean transmission rate ($\overline{R}$) and variability in transmission rate (σ_R^2) for the four cases of one or two loci in each of either naive or exposed populations. Parameters which were not variable in these analyses were fixed at the following standard values: T=0.2, t=1, w=1, $\overline{R}$ =3, σ_R^2=15.

6.4.3. When does the mutant have a chance of surviving?

In addition to computing the magnitude of survival probabilities, the threshold set of parameters which determine whether a mutant has a zero or non-zero chance of survival were found, thus establishing some useful targets for management of drug resistance. These thresholds are defined below as a function of drug pressure, transmission rate, and the effect of the drug on transmission for the two cases of a drug-naive and drug-exposed population. Natural selection is ignored for this exercise.

Survival probability is non-zero when the mean of the distribution is greater than one. In a naive population the mean number of copies which the mutant transmits to the next generation is

$$(1-T)\rho\pi + Ttc \qquad (6.10a)$$

and in the exposed population is

$$\rho\pi + \frac{Ttc}{(1-T)}. \qquad (6.10b)$$

Substituting c for $\bar{R}$ and $\pi\rho = 1$ for the one-locus case and $\pi\rho = \frac{1}{2} + \frac{1}{2c}$ for the two-locus case, the conditions under which the mutant has a non-zero chance of surviving are:

(i) One locus, naive population $T > 0, \quad t > \frac{1}{c}$ (6.11a)

(ii) One locus, exposed population $T > 0, \quad t > 0$ (6.11b)

(iii) Two loci, naive population

$$T > \frac{c-1}{2tc^2 - 1 - c}, \quad \frac{1}{c(2-c)} > t > \frac{1}{c}, \quad c < 2 \quad (6.11c)$$

$$T > \frac{c-1}{2tc^2 + 1}, \quad t > \frac{1}{c(2-c)}, \quad c < 2$$

$$T > \frac{c-1}{2tc^2 - 1 - c}, \quad t > \frac{1}{c}, \quad c > 2$$

(iv) Two loci, exposed population

$$T > \frac{c-1}{2t^2 - 1 + c}, \quad t > 0, \quad c > 1 \quad (6.11d)$$

In the first three cases, when the mean number of offspring is greater than one, as transmission rate increases, i.e. c increases, survival probability also increases. Thus increased transmission rate under these circumstances is always expected to promote drug resistance. However, in the two-locus, exposed population, there is a range of values for T and t for a given c for which the mutant has a non-zero survival probability which decreases as transmission rate increases, i.e. decreasing transmission promotes drug resistance. The conditions under which this occurs are when:

$$\frac{1}{2tc^2 + 1} > T > \frac{c-1}{2tc^2 - 1 - c}, \quad t > 0, \quad c < 2 \quad (6.11e)$$

Note that this window of values of T and t for which this occurs is restricted to cases when $1 < c < 2$, and that this window becomes narrower as c approaches 2.

The critical values of T and t were plotted to delineate the areas of the parameter space in which survival probability is zero, increasing or decreasing with changes in transmission rate from a given value of c.

6.5. Results

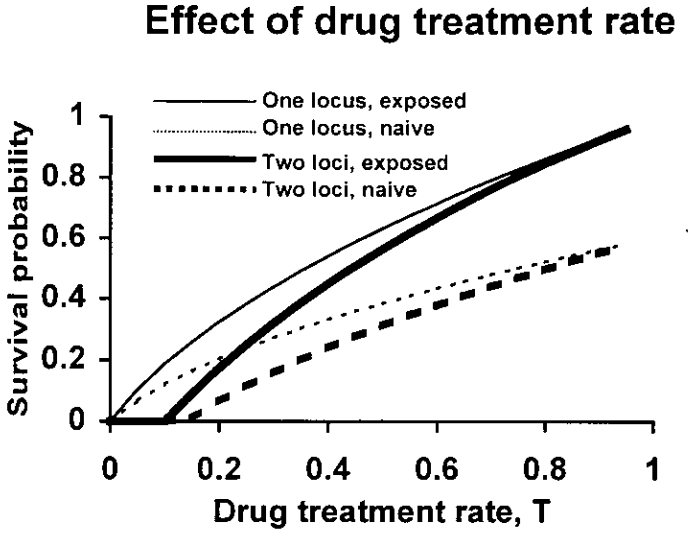
Figure 6.1 shows how the survival probabilities change as each of the parameters of the model is varied. One-locus mutants (thin lines) had higher survival rates than two-locus mutants (thick lines) because recombination breakdown occurs in the latter. Exposed populations (solid lines) had higher survival rates than naive populations (broken lines) because of the relative increase (by a factor of $1/(1-T)$) in transmission rate once the population has stabilised to the point where parasites from untreated hosts compensate in their reproductive output for those killed in treated hosts.

Drug treatment rate (T) had the strongest influence on survival probabilities illustrating that drug pressure is the primary force governing the evolution of drug resistance (Figure 6.1a). The effect of the drug on transmission (t) also had an influence: the higher the transmission from hosts carrying a mutant, the greater the number of copies of the mutant transmitted to the next generation (Figure 6.1b). Thus the fitness advantage of a mutant depends on not only its survival in the presence of the drug, but also on its transmission to the next host. Natural selection against the mutant reduced its survival rate (Figure 6.1c) and, if high enough, was sufficient to prevent the mutant from surviving because natural selection and drug selection act in opposite directions. Survival increased as mean transmission rate increased when $T=0.2$ (Figure 6.1d). In some cases, at low transmission rates ($c<1.5$) and lower drug treatment rates (around $T=0.1$) survival probability decreases as c increases (see later). Increasing survival probabilities as transmission rate rises occur because mutants from treated hosts are transmitted in higher copy number to the next generation when transmission is high, and this effect is stronger than the recombination breakdown effect. The opposite can occur, however, when drug pressure is weak and recombination breakdown is infrequent: in such cases, recombination is a stronger force than drug pressure.

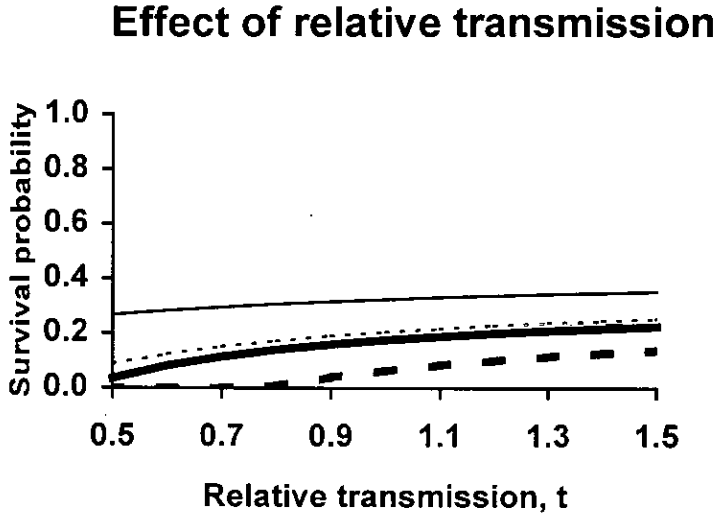
Higher variability of transmission reduced survival probability (Figure 6.1e) reflecting the greater degree of risk to mutants in not being transmitted, and hence lower survival, when transmission is unreliable.

Figure 6.2 shows the values of drug treatment rate (T) and effect of the drug on transmission (t) which will allow a mutant to survive when $c=1.5$. In the one-locus case (Figures 6.2a,b), the mutant will survive whenever there is any drug pressure. In the naive population (Figure 6.2a), the additional qualification for this to be true is that the drug does not reduce transmission from treated hosts by a factor greater than the natural transmission rate, i.e. tc must be greater than one. Thus whenever drugs confer a total fitness advantage on the mutant, which is a combination of survival and transmission rate, it has a chance to survive. In the two-locus case (Figure 6.2c,d) this fitness advantage must be balanced against effective recombination, which, is also a function of transmission rate. In the naive population, whenever the outcome of this balance is positive, i.e. the mutant can survive, then it will continue to increase its chances as transmission rate increases. Thus, as transmission rates continue to increase, the relative advantage of higher copy number afforded by higher transmission rates becomes stronger than the concomitant recombination breakdown. An exception occurs in exposed populations (Figure 6.2d) at low levels of transmission ($c<2$): for a limited range of values of T and t, survival probability decreases as transmission rate increases. Thus in only one of the four cases examined here – that of two loci in an exposed population – increases in transmission rate lead to decreases in survival probability if transmission is already low and drug pressure is within a given range spanning approximately 20%. In all other cases, survival probability either remains zero or increases as transmission rate increases.

(a)



(b)



(c)

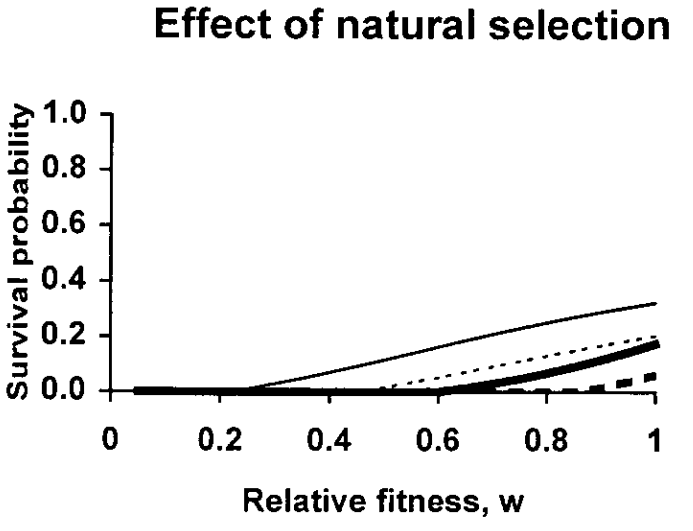
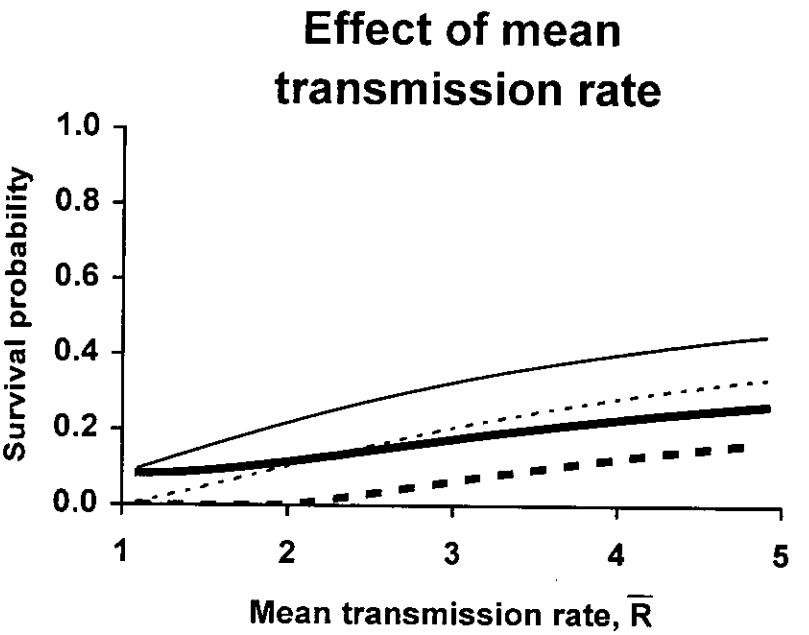


Figure 6.1. Effects of parameters on survival probability of one-locus (light lines) and two-locus mutants (bold lines) in naive (broken lines) or pre-exposed (solid lines) populations. (Continued overleaf).

(d)



(e)

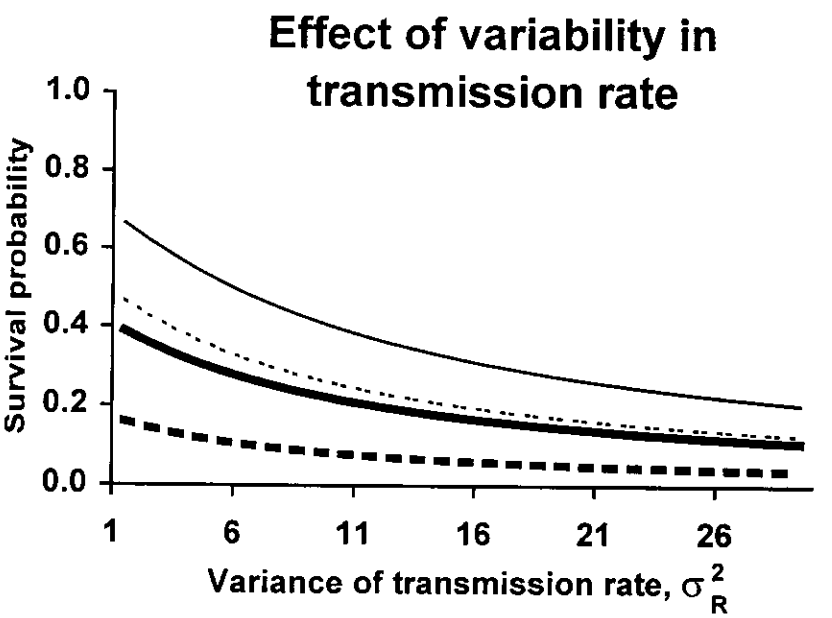


Figure 6.1. Effects of parameters on survival probability of one (light lines) and two-locus (bold lines) mutants in naive (broken lines) or exposed (solid lines) populations. (Continued from previous page).

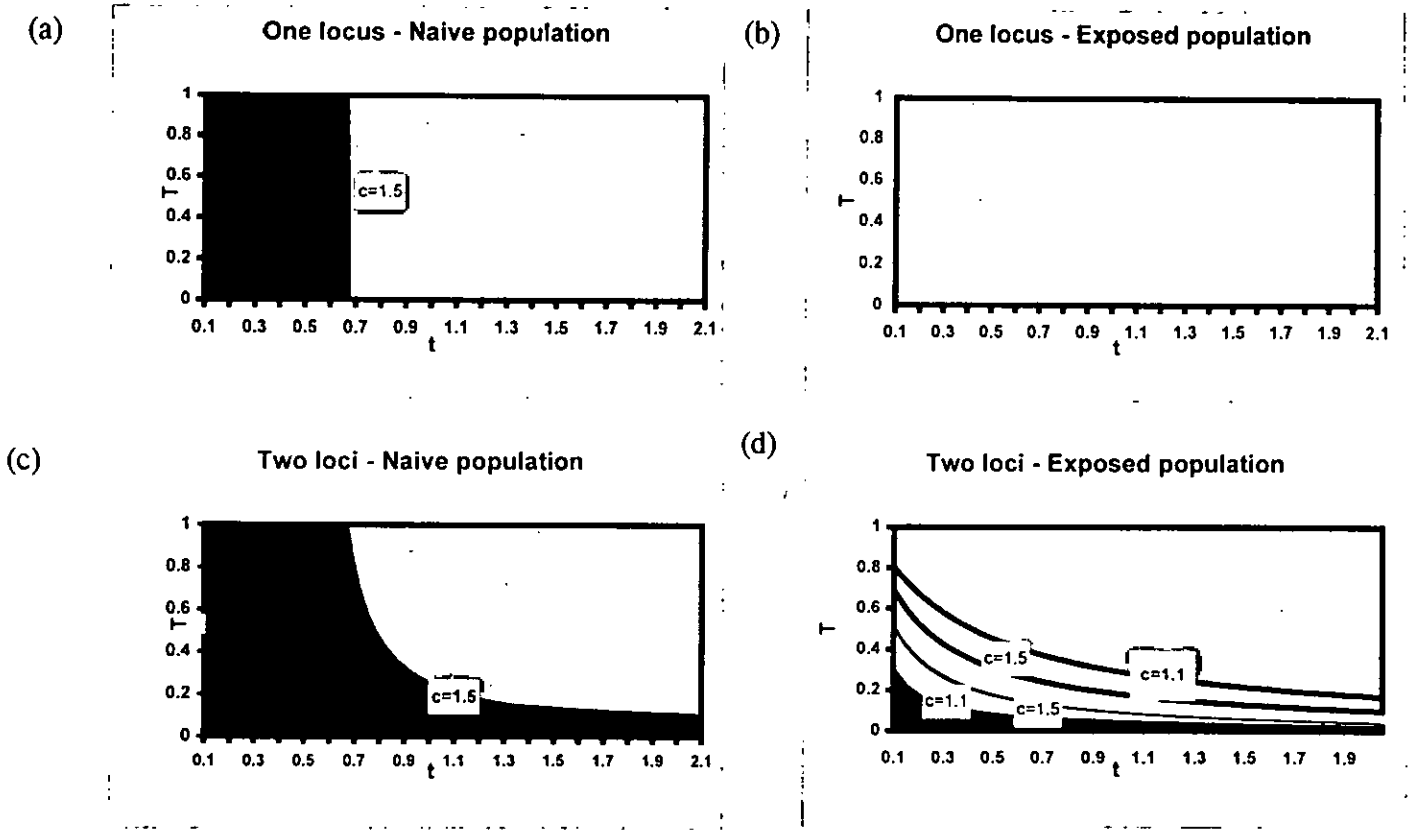


Figure 6.2. Values of drug treatment rate (T) and effect of drug on transmission (t) for which a drug resistant mutant will or will not spread in an area with transmission rate c in the one or two-locus cases where the population is either naive or pre-exposed to drugs. Dark areas indicate when the mutant's survival probability is zero, light grey areas indicate when the mutant's survival probability decreases as transmission rate decreases, and white areas indicate when survival probability increases as transmission rate decreases.

6.6. Discussion

This study demonstrates that the fate of a newly arisen drug resistant mutant is primarily determined by whether drugs are in use in the host population, and the rate of transmission of the parasite. The reason why these two factors jointly determine whether a mutant survives is because the mutants only have a selective advantage when they encounter the drug. This advantage is realised best if high transmission rates assist the mutant to transmit to the next generation. In this study there were two separate effects of transmission under consideration: one was the prevailing transmission rate which determines how many transmissions each infected host typically makes, whether treated or not, and the second was the effect of the drug on reducing or curbing transmission from treated hosts. Both of these are important (and different) targets for restricting the development of drug resistance, and thus provide two new possibilities for avoiding drug resistance which could be implemented alongside the current major goal for avoiding drug resistance which is to minimise drug usage, a goal which is not always practicable or ethical. These targets are to reduce overall transmission rate and to reduce transmission from treated hosts. As these effects act in a synergistic way they should be implemented jointly with minimisation of drug usage. These targets are also compatible with those recommended for slowing the spread of an already established mutant (Mackinnon and Hastings, 1998; Chapter 5).

There is one caveat, however. In very low transmission areas and at low drug pressures, if resistance to two drugs is encoded by two or more loci, and drugs have been in use for some time such that the population has adjusted its equilibrium reproductive rate to accommodate the killing of parasites by drugs, mutant survival will be aided by attempts to cut transmission rate further (Figure 6.2d, Hastings and Mackinnon, submitted). This is because, under these conditions, the eroding force of recombination on doubly resistant gene combinations is approximately equal to the positive force of drug selection: reducing the negative force of recombination by decreasing transmission allows the positive force of drug selection to dominate. It is fortunate that reducing transmission rate is usually of low priority in low transmission

areas (unless the aim is to eradicate the disease). In such circumstances, the objective should be to reduce the transmission rate from individual treated hosts, but not in the overall population. At high levels of drug pressure these may not be compatible objectives.

Predictions from this model are dependent on the assumptions. The effect of the assumption of whether the population is naive or exposed to drugs, and its effect at low transmission rates is just one example of this. Which of these (if any) is the most valid assumption is difficult to decide and likely to depend on the level of endemicity in the area. While it is clear that parasite populations in endemic areas eventually restabilise to a new equilibrium after control measures are introduced, it is not well understood how long this process takes, or how it occurs, or whether it incurs a reduction in the number of genotypes per host. Factors such as transmission pattern, vector density, host immunity and superinfection will all interact to determine this process. What matters is whether the time-frame for a new mutant to become 'safe' is within the pre-stabilisation phase or largely in the post-stabilisation phase. It is not possible to answer this question here.

Another assumption of the model was that drugs are taken randomly by the infected host population and that the frequency of treatment is independent of transmission rate. This allowed the conclusion to be reached that effective selection on the mutant is directly related to both drug pressure and transmission rate. However, if hosts only take drugs when they are sick and the frequency of being sick depends on transmission rates (e.g. due to the number of previous infections) then the effect of transmission rate in the field may be somewhat less than predicted here.

It was also assumed that the mutant had reached a significant frequency among the gametocytes of the host in which it had arisen. This might occur if the mutant arose very early in the infection. Given that most mutations will occur when populations sizes are large, the chance of this event occurring is itself small. Thus the calculations in this study, because they are conditional on the mutation having already established within the first host, are gross overestimates of the actual probability that a mutant arises and survives across the host population. Nevertheless, the results are of a comparative nature and are not invalidated by this assumption. To estimate the

overall probability of a new mutant surviving, the survival probabilities predicted in this study would be multiplied by the probability of the mutant reaching high frequency among the gametocytes in the host in which it first arose.

A key assumption made in this study is that malaria is stable, i.e. the net change in the average number of parasite infections per host does not change over the generations. Clearly this is unlikely to be true in malaria parasite populations which typically fluctuate in size due to seasonal factors. During expansion phases, most mutants, even neutral ones, will survive because the mean number of offspring is greater than one. During contraction phases, few will survive. Thus the fate of new mutations under selection very much depends on the prevailing population dynamics when the mutant arises. However, such fluctuations are only likely to affect the conclusions from this study in a quantitative, but not qualitative way because comparisons of the effects of drug pressure, transmission rate and natural selection were performed under the same conditions.

Chapter 7

General discussion and future directions

There are several interesting findings in this thesis which deserve further discussion and may stimulate new research goals. These are now discussed, simultaneously drawing on the findings of all the studies presented here.

7.1 Relative importance of between- versus within-host selection in malaria

One of the common themes in the theoretical literature on the evolution of virulence is the relative importance of within- and between-host selection (Bull, 1994). Within-host evolution is driven by competition between genetically different parasites occupying the same host, the winner depending on its within-host replication rate and survival relative to the loser. Between-host evolution is driven by natural selection for increased transmission which is presumed to be a function of replication rate. Thus direct selection is assumed to operate on different traits in the two cases. Importantly, virulence is an expected consequence of both. However, the optimum level of virulence is likely to differ for the two cases. This is because (a) direct selection intensities will differ, (b) correlated selection pressure on virulence will differ (Lande, 1982), and (c) the likelihood of host death constrains the virulence caused by between-host, but not within-host selection. As a result, within- and between-host selection are often thought of as competing processes which the parasite has to optimise. The relative importance of each process at the optimum will depend on the particular parasite system and trait in question. The results presented in this thesis suggest that both processes are important in determining the optimum virulence in malaria parasites.

7.1.1 Between-host selection

In Chapter 2, it was shown that (a) malaria parasite genotypes differed widely in their virulence and transmission potential and that these differences were stable;

and (b) replication rate was the link between these traits. The first of these observations, combined with similar observations in *P. falciparum* and *P. vivax* in parasite lines used to treat neurosyphilis in the 1930's (James et al, 1932), suggests that between-clone selection on virulence has some genetic variation to work with. This is because, for between-host selection to be effective, parasite genotypes have to retain their advantage from one generation to the next. The second observation underpins the main hypothesis for the evolution of virulence by between-host selection – the adaptive trade-off hypothesis. Thus there are strong arguments that between-host selection could play an important role in malaria parasite evolution.

One major objection to this claim might be that the observations were made in a laboratory mouse, mosquito and environment (none of them natural to the parasite) and therefore may not be relevant to the field situation. However, it is argued that because these parasite clones were recently isolated from the field, the biological basis for the relationship between virulence and transmission is likely to be unchanged, even though the expression of it may have been exaggerated by the artificial conditions. In this respect, the observations in these studies are likely to be more meaningful than from other studies where such relationships between virulence and transmission have been generated by selection in an artificial environment. Even the widely cited example of the myxoma virus suffers from such criticism (Bull, 1994). For example, in *P. falciparum*, the ability to produce gametocytes and to cytoadhere was lost simultaneously in a line maintained *in vitro* in the laboratory: the loss was found to be due to a subtelomeric deletion on chromosome 9 (Day et al., 1993). In *P. gallinaceum*, lines selected for the ability to replicate asexually in the presence of drugs showed correlated increases in gametocyte numbers (Bishop, 1954). In contrast, in *P. berghei*, Dearsly et al. (1990) reported a loss of gametocyte production and increase in asexual parasitaemia over generations of lines maintained by serial passage, and cite other similar examples in *P. vivax* and *P. berghei*. All of these studies have been quoted in discussions of the relationship between virulence and transmission (Bull, 1994; Ewald, 1994; Gupta and Day, 1996; Lipsitch and Moxon, 1997), but may be misleading (Bull, 1994) because of the artificial environment in which these

relationships were generated.

The high level of variability between clones deserves further comment because it is expected that if virulence and transmission were optimised in the wild, then genetic variation for these traits would have been exhausted. Two arguments and one observation are offered, viz: (a) the unnatural host environment exposed variation in the traits because expression of parasite genes is highly dependent on host physiology, and the interactions between them are new, (b) much parasite variation is maintained in the wild because of host variability, and (c) fitness traits measured at the quantitative phenotypic level always show some level of genetic variation (Mousseau and Roff, 1987).

7.1.2 Within-host selection

The study in Chapter 3 showed that within-host evolution may also be significant in malaria parasites. Between-host selection for high versus low replication rate, as indirectly measured by virulence, was ineffective. Instead, unintentional selection for parasites present on Day 12 of the infection generated a permanent genetic change in two parasite lines. Thus it seems that within-host evolution, either through mutation or altered phenotypic expression of virulence factors, could significantly alter the level of virulence if selection conditions were appropriate. Theoretical studies could be done on the probability that new mutations survive and increase in frequency given the within-host population dynamics and method of transmission. Branching process methodology, incorporating the effects of fluctuating population size (Kimura and Ohta, 1974; Otto and Whitlock, 1997) could be employed for this exercise.

It is not known, however, whether such evolution would be maintained when subjected to regular mosquito transmission during which mutations may be purged by DNA repair, by selection on variation created by sexual recombination, or by population bottlenecks. The ability to produce gametocytes (even in higher numbers), and the ability to infect mosquitoes in all four lines after twelve generations of selection suggests that this would not be a constraint in the short-term, e.g. each transmission cycle. However, if mosquito transmission was to re-

set the expression of variant antigens, then there would be no long-term benefit of within-host evolution.

A more important issue might be whether there is a conflict between within-host and between-host evolution, as predicted by many theoretical models, and how selection resolves the obvious trade-offs between within-host replication (growth) and between-host transmission (reproduction), and increased host mortality. For example, selection may act to reduce the conflict between these processes. As there was no increase in host mortality in these experiments, and some indication that maximum virulence was constrained by an unidentified host mechanism, it would appear that there is not a strong trade-off between short and far-sighted evolution in this system. The idea that malaria parasites have evolved to minimise this trade-off is consistent with the phenotypic observations of Taylor et al (1997a,b, in press) showing that within-host competition increases both virulence and transmission, and the field observation that mixed genotype infections in hosts are very common. It also fits well with the phenomenon of antigenic variation which may allow parasites to maintain chronic parasitaemias and transmission without imposing much damage on the host. Mechanisms for managing the potentially fierce within-host competition during the rapid replication phase may be an alternation of sequestration patterns by competing genotypes (Farnbert et al., 1997), or by maintenance of across-host population variation in surface antigens. Thus at present, it is possible only to conclude that malaria parasites apparently have evolutionary strategies which are both short- and far-sighted. In this respect, the virulence models predicting otherwise, which are based on simple relationships between transmission rate per contact, rate of contact, and rate of host death may not hold true because details of within-host dynamics are ignored (Bull, 1994).

7.1.3 Malaria and the Red Queen

While these studies have shown that malaria parasites have the ability to evolve, the question remains as to whether they succeed in doing so in the field. In Chapter 4 it was observed that parasites face much host variation. This variation seems to be derived largely from non-genetic factors relating to previous immune

exposure, sex, age and other unexplored attributes. Much effort has been given to determining whether host variation is maintained by parasites and *vice versa* (reviewed by Lively and Apanius, 1995). This is the Red Queen hypothesis, so named because it conjures up a picture of Alice and the Red Queen (parasite and host) running hard to keep up with each other, but not actually gaining ground. Data support the hypothesis that hosts maintain diversity in immune function genes in order to mount responses to genetically variable pathogens (Hurst and Peck, 1996; Apanius, 1997). Specific examples of host variability for protection against malaria include the associations between the highly polymorphic human lymphocyte antigens (Hill et al., 1991; 1994). These associations appear to vary across populations suggesting that there are interactions between host variants and parasite variants, and such interactions may be one reason why the host genetic component was found to be generally low in the Sri Lankan study. On the parasite side, there appears to have been selection for diversity in the circumsporozoite protein and in other surface antigens (Hughes and Hughes, 1995; Conway, 1997). The Red Queen hypothesis also encompasses the rapid somatic evolution within the vertebrate host immune system which, with its ability to target specific parasite genotypes, may be a strong factor retarding between-host evolution of parasites, while promoting their within-host evolution.

An alternative hypothesis to the Red Queen is that between-host variation is large for environmental reasons (e.g. ecological factors relating to vector density, stochasticity in the transmission process, variation in nutritional status of hosts) or for genetic reasons, but that the parasite has no selective impact on the host's variation. Thus this hypothesis differs from the Red Queen hypothesis in that it involves parasite evolution only, as compared with co-evolution of both parasite and host. In malaria, given the stochasticity in the transmission system, it is feasible that environmental or host genetic noise ruins the efficacy of optimising selection in the parasite, i.e. limits its rate of evolution.

What do the data from the studies described here contribute to the question of whether the Red Queen hypothesis is important for malaria? Ebert and Hamilton (1996) suggest some empirical tests for establishing whether the Red Queen

hypothesis is operating in nature. First, it is expected that when host variability is removed, parasites evolve a higher level of virulence. This is because selection on the parasite by the host is uniform and therefore more effective in bringing about genetic change in the parasite to match the host's defences. This test was supported by the results from Chapter 3 where the parasite underwent genetic increases in virulence when introduced to a uniform host. However, this host was also a novel host. A second test is that the parasite should be less virulent in a novel host than its natural host. The high levels of virulence of *P. chabaudi* observed in the experiments presented here appeared to counter this prediction, although a direct comparison was not made with the natural host. Thus it is not clear whether the observed evolution to higher levels of virulence was due to the removal of host variation or to adaptation to a novel host. Additionally, the field data from Sri Lanka (Chapter 4) suggest that host variability in immune experience and status is very high and has a large impact on the parasite's success, at least as measured by overall virulence. However, there was some evidence of a genetic component in the host's capacity to develop an infection and so this may be a limiting factor for the parasite.

In summary, it is likely that host variation has a limiting effect on the rate of malaria parasite evolution, but it remains an open question as to whether this involves co-evolution with the host or not. One way to begin to address this issue is to evaluate a panel of parasite genotypes for virulence and transmission, as in Chapter 2, in a range of different host genotypes and determine whether host-parasite genetic interactions are strong.

7.2 Mechanisms of virulence in malaria

Most studies on virulence in malaria have been concerned with molecular mechanisms and associations between parasite variants and disease severity across the population of hosts (Carlson et al., 1990; Hill et al., 1991; Allan et al., 1993; Carlson et al., 1994; Rowe et al., 1995). This contrasts with the studies presented here which took a phenotypic, quantitative and within-host approach to the problem. In so doing, it is hoped that a better understanding of the causes of

virulence was achieved, and in a way which complements what is known at the molecular level. For example, it was shown that asexual parasitaemia is strongly related to the degree of virulence experienced by the host. This might not have been the case, e.g. levels of circulating TNF, irrespective of parasite density, may have been the cause of virulence.

This points to the importance of factors relating to population growth and maintenance, such as the degree of sequestration and avoidance of splenic clearance, the number of merozoites produced per schizont, the success of red blood cell invasion by merozoites, the efficacy of antibody-mediated responses, and the ability to vary surface antigens. Of these, the most likely explanation (though there are probably many), because of its parsimony with other information about virulence, is that the ability to sequester is an important means by which the parasite can achieve maximum asexual growth early in the infection in order to survive the ensuing onslaught by the immune system. Later in the infection, the variant antigens, which are expressed on the same molecule which allows cytoadherence, are probably important for maintaining asexual parasite numbers for extended periods from which gametocytes for transmission can be generated. The association between excessive cytoadherence and high rates of host mortality due to cerebral malaria fits in with this hypothesis, as do the high levels of anaemia and cytokine release in the other virulent class of malaria, severe malaria. Also, the apparent adaptation to a new host observed in Chapter 3 may reflect an interaction between host receptor types and parasite ligands involved in cytoadherence.

Another interesting possibility for a mechanism which regulates parasite growth rate is one which decreases the deformability of the *uninfected* red blood cell either to increase sequestration (Dondorp et al., 1997) or to surround the infected cell with uninfected cells (rosetting) for the emerging merozoites to invade. Further studies on parasite variation in virulence, along the lines of those conducted here, can now be done to determine whether the ability to sequester is a key factor in achieving high asexual parasitaemias. Similarly, the ability to maintain infections after crisis needs to be studied in relation to cytoadherence

properties and antigenic variation. By conducting these in mice with a variety of endothelial receptor genotypes, it may be possible to demonstrate if virulence is highly dependent on the specific combination of host-parasite molecules.

It is also suggested that the wide range in virulence observed, which has been classified into mild, severe and cerebral malaria, is largely a reflection of some continuous variation in parasite growth rate, but expressed in a non-linear way. This does not rule out the possibility that some single genes encoding a 'virulence factor', such as that for rosetting, may be found in some populations, but it is likely that there are many genes contributing to the virulence phenotype, and so a more quantitative approach than has hitherto been taken would seem to be justified.

Finally, it is an intriguing possibility that virulence in malaria is caused by an infectious organism which is not *Plasmodium* itself, i.e. virulence may not be encoded for by the parasite's own genome, but by a horizontally transmitted agent such as a virus. Examples of highly virulent pathogens which have their virulence determined by acquired organisms, such as bacteriophages or viruses, are common (reviewed by Levin and Tauxe, 1996). For example, cholera bacteria became virulent only when invaded by a bacteriophage (Waldor and Mekalanos, 1996), and the bacterium causing the bubonic plague became virulent only when two mutations - one encoded by a bacterial chromosome, but the other coded by a plasmid carried by the bacterium - occurred (Rosqvist et al., 1988). Viruses are carried by many of the major protozoal species such as *Giardia*, *Leishmania, Trichomonas, Eimeria* and *Babesia* spp. (Wang and Wang, 1991), although none have been found in *Plasmodium*. Some of these affect the parasite's growth rate, others are rarely transmitted horizontally, and in *Trichomonas vaginalis* one such virus causes the expression of an antigen on the infected cell membrane which undergoes phenotypic variation! (Wang et al., 1987). Thus a virus of low transmissibility, which survives mosquito transfer (Alger et al., 1971), or a foreign genetic element in the plasmid-like extrachromosomal element of the malaria genome (Wilson et al., 1991), may conceivably be a virulence determinant which gives the appearance of being stably inherited. The question of conflict in selection between and within hosts would then have to be expanded to include other genomes.

7.3 Parasite evolution in relation to population structure

The theoretical studies in this thesis highlighted a number of factors relating to population structure which should influence parasite evolution. The first is recombination and outbreeding which determines how well gene combinations affecting fitness are held together, and is important only for multi-locus traits. At very low levels of outcrossing, outbreeding can successfully counteract strong selection, such as that imposed by drugs. In cases of weak selection, it can successfully erode population structure (Hastings and Wedgwood-Oppenheim, 1997) such as might arise if immune selection against different parasite antigens, or antigen combinations were to produce 'strain-structuring' of the population, i.e. the dividing of the population into immunodominant strains which are transmitted independently (Gupta et al, 1994a,b,c, 1996).

The second issue relating to population structure is that of selection. When the selecting unit is the individual host, and hosts vary in the nature of their selection on the parasites, then it matters to the overall effectiveness of selection how the parasite population is distributed through their hosts (Chapters 5 and 6). This is for two reasons: (a) the between-host fitness of the parasite depends on the number of other genotypes with which it competes, and (b) parasite genotypes with low (but not rare) frequencies have a greater probability of encountering the least common selective agent when they are more dispersed through the host population. In the studies presented here, the effect of the mean number of parasites per host was studied and was shown to influence the rate of change in frequency of a selected mutant. It is envisaged that variation in the number of parasite genotypes per host is also important to the rate of parasite evolution. For example, if most of the parasite population was transmitted from a small proportion of the hosts, then if a selective agent was applied at random to a portion of the population, most of the parasite population would not be exposed to it, and changes in frequency of the relevant parasite gene as a result of this selection would be small. This selective agent may be artificial, e.g. a drug or vaccine, or may be natural such as immunity to a rare parasite strain. The effects on rate of parasite evolution may be even more exaggerated if there is a non-random association between parasite genotype

and the number of parasite genotypes per host, such as might occur by concentration of the more virulent (and more transmissible) parasite genotypes in the hosts carrying the bulk of the parasite population. Thus where host selection is heterogeneous, and parasite populations are genetically variable, the details of population structure could be important in parasite evolution.

This population genetics example seems to be analogous to the interaction between host heterogeneity in the number of parasites carried per host and spatial variation in contact rates which together determine the total fitness of the parasite population. There is much recent interest in the effects of spatial variation on epidemiological processes (Mollison and Levin, 1995; Bolker et al., 1995) and so models incorporating spatial structure (e.g. Barlow, 1991; Becker and Dietz, 1995) may be able to be adapted to incorporate population genetics. More information on the structure of malaria parasite populations, particularly the distribution of the number of clones per host and the distribution of parasite genotypes across the host population is thus required. This information may be found directly from the field by genotyping parasites in hosts (as already done in a limited way), and may also be modelled as a birth-death process which allows for superinfection (as described by Bailey, 1982; Aron and May, 1982; McLean, 1995) and short-term immunity.

If the impact of host heterogeneity on population genetics was well defined, it might be possible to apply control measures in a way which maximises their effect. One simple example of this might be the use of a variety of drugs across a population, each host receiving a single drug (i.e. not a cocktail), and adjacent hosts receiving different drugs. If resistance to one drug arose in one host, and this host transmitted to a new host which was treated with a different drug, then the new mutant would not survive. This is in contrast to the case of using drug cocktails (i.e. each treated host receives all drugs), where a mutant arising from a host treated with two drugs would also be resistant in its next host. It should also be contrasted with the strategy in which different drugs are used sequentially, i.e. only one drug is used in the population for a period, followed by replacement with another drug. This latter strategy has been shown to spread resistance faster than always using cocktails (Curtis and Otoo, 1986). The success of this 'spatial

strategy' will depend on the initial frequencies of the mutants, the spatial distribution of drugs in relation to the network of transmissions, and the distribution of parasite genotypes through the host population. The model presented in Chapter 5 could be modified to evaluate the impact of such an approach.

A spatial strategy to limit the rate of pathogen evolution (Barrett, 1980) has been successfully used in the field (Wolfe and Barrett, 1980). Powdery mildew, a fungus of barley normally causes severe loss of yield. The pathogen cannot attack a given host genotype unless it has the corresponding virulence gene. In order to limit the pathogen's success, mixed genotype stands of barley were planted. It was found that there was a 6.5% increase in production. Note that this strategy is an example of the Red Queen idea that host variability confounds the pathogen's evolution by disruptive selection. This strategy is also dependent on gene-for-gene interactions between pathogen and host (as drugs are to resistant mutants) and so works best when specific host-parasite interactions operate strongly to determine fitness. Cross-virulence reduces the effectiveness of this strategy, in a manner analogous to high cross-reactivity between antigens in a polyvalent vaccine leading to a higher rate of breakthrough, (i.e. vaccine resistance) (Gupta et al., 1997). In malaria, interactions between host endothelial receptors and parasite cytoadherence molecules, and parasite genotype-specific immune responses, may be good examples of specificity in host-parasite associations that limit parasite success. It is hoped that the combination of population genetics and epidemiology, drawing on the lessons of evolutionary biology (e.g. the Red Queen hypothesis), will generate many insights into parasite-host interactions in the coming years.

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Appendix

Publications generated during this study period

Chapters 5 and 6 have been published. An erratum to the paper from Chapter 6 was also published. Reprints of these are attached.

I was also the second author on two other papers, now in press, by virtue of my contribution to the statistical analysis and/or design of the experiments. These do not relate to the work in this thesis but manuscripts are also attached.

Four other senior author papers relating to animal breeding were published during the course of study. These are not included here.

A list of the attached publications is given below.

Publications

Mackinnon, M.J. (1997) Survival probability of drug resistant mutants in malaria parasites. *Proceedings of the Royal Society of London, Series B.* **264:** 53-59.

Erratum to Mackinnon (1997). *Proceedings of the Royal Society of London, Series B.* **264:** 1849-1849.

Mackinnon, M.J. and Hastings, I.M. The evolution of multiple drug resistance in malaria parasites. *Transactions of the Royal Society of Tropical Medicine and Hygiene.* **92**: 188-195.

Carlton, J., Mackinnon,M.J. and Walliker, D. (in press) A chloroquine resistance locus in the rodent malaria parasite. *Molecular and Biochemical Parasitology*

Taylor, L.H., Mackinnon, M.J. and Read, A.F. (in press) Virulence of mixed-clone and single-clone infections of the rodent malaria *Plasmodium chabaudi. Evolution*

Survival probability of drug resistant mutants in malaria parasites

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SUMMARY

This study predicts the ultimate probability of survival of a newly arisen drug resistant mutant in a population of malaria parasites, with a view to understanding what conditions favour the evolution of drug resistance. Using branching process theory and a population genetics transmission model, the probabilities of survival of one- and two-locus new mutants are calculated as functions of the degree of drug pressure, the mean and variation in transmission rate, and the degree of natural selection against the mutant. Probability of survival increases approximately linearly with drug pressure, the slope of the line increasing with mean transmission rate. Thus increased drug pressure, especially in combination with high transmission rates, strongly favours the evolution of drug resistance. These conclusions also hold for the case of multiple drug resistance where it is coded for by two unlinked loci: the greater effective recombination breakdown in high transmission areas is counteracted by greater effective selection so that the net effect of higher transmission rates is to favour the evolution of multiple drug resistance. High variability in transmission rate and natural selection against the mutants are unfavourable to mutant survival, though these are relatively weak forces.

1. INTRODUCTION

Malaria parasites have a remarkable ability to develop resistance to drugs. This has created an urgent problem because resistance to all of the available drugs has arisen at least once (Bjorkman & Phillips-Howard 1990), and the development of new drugs has virtually stalled. Evidence from field and theoretical studies indicates that resistance continues to spread as long as there is any drug pressure, and that beyond a certain frequency, the rate of spread is very rapid (Curtis & Otoo 1986; Dye 1991; Wernsdorfer 1991). It therefore seems wise to try and prevent or delay the development of resistance in the first place.

The fate of a new drug resistance mutant, or set of mutants, in a malaria parasite depends on the relative forces of selection by drugs, natural selection (presumably unfavourable), recombination between resistance loci in the case of multi-locus resistance, and the probability that the mutant is transmitted. While it may be easy to predict the qualitative effect of each of these forces in isolation, it is their relative strengths and the interactions among these forces which determine the final outcome. Due to the structuring of the parasite population into hosts, and the heterogeneity of selection of the parasites by the hosts (i.e. hosts are either treated or untreated with drugs), prediction of the outcome of, say, a combination of drug pressure and transmission rate is not straightforward. For example, Paul *et al.* (1995) suggest that where drug resistance is coded for by more than one locus, higher levels of transmission will hinder the evolution of drug resistance because the associated increase in the degree of outbreeding will lead to greater effective recombination breakdown between the resistance loci. They do not examine this hypothesis in a quantitative way. On the other hand, simulations by Dye 1991 indicate that high transmission rates favour the spread of multi-locus drug resistance because when parasites are dispersed more widely at higher levels of transmission they encounter drugs more often, and so effective selection by drugs is stronger. Such contrasting conclusions demonstrate the need for studies which properly account for the two major effects of parasite population structure on gene flow, namely inbreeding due to limited numbers of genetically different haplotypes per host, and heterogeneous selection in the parasite population due to variable selection pressures by hosts. Dye & Williams (1997) account for the effects of inbreeding by relating it to the rate of recombination breakdown between resistance loci, but they ignore the effects of structuring on the intensity of selection by drugs. In the present study, a population genetics-transmission model is built which accounts for both factors.

Specifically, this study addresses the first of two relevant issues regarding the evolution of drug resistance. The first issue is how easily resistance can develop in the first place, i.e. from new mutations. Here the model is used to predict the probability that a newly arisen mutant survives the first few rounds of transmission until selection by drugs can bring it to a 'safe' frequency, i.e. beyond the risk of being lost to the population due to chance events during transmission.

Thus it addresses the issue of how readily a drug resistance problem can arise from a new mutation event, or from the arrival of a new migrant parasite into a resistance-free population, and therefore has implications for limiting the further evolution of drug resistance. The second issue is how rapidly the frequency of an established mutant will increase over generations of transmission under continuous drug pressure. This question will be addressed elsewhere.

2. THE BASIC MODEL AND ASSUMPTIONS

In the following section the assumptions made in the model about the epidemiology, transmission cycle, drug selection and relative fitness of mutants are given.

(*a*) *Distribution of number of transmissions per host*

Transmission of malaria is characterized by its variability due to a multitude of host, vector and parasite-related factors. Though transmission rate is usually described in terms of a single parameter, R_0, which is often thought of as a constant because it represents the average number of transmissions from one infected host to other hosts, it is recognized that there is variability around R_0, i.e. in the number of transmissions from individual hosts (Koella 1991). It is this variability which makes new mutants vulnerable to loss during the first few rounds of transmission. If the average R_0 is one, then on average the total parasite population replaces itself each generation. However, individual parasites with distinct multi-locus haplotypes may not always replace themselves due to sampling variation in the transmission process. Thus when considering the population genetics of parasites, it is necessary to account for the uneven redistribution of genes from one generation to the next.

In the model presented here the transmission rate is modelled by a variable, R, which is distributed as a negative binomial with a mean of $\bar{R}$ and variance of σ_R^2. The parameters of the negative binomial distribution, p, and k, are related to the mean and variance in the following way:

$$\bar{R} = \frac{k(1-p)}{p}, \quad \sigma_R^2 = \frac{k(1-p)}{p^2}. \tag{1}$$

The parameter k can be thought of as the aggregation or shape parameter: as k increases, the less 'clumped' are the data and when $k = \infty$, the distribution is Poisson. The parameter p can be thought of as the zero probability parameter: the frequency of the zero class is given by p^k so that the mean increases as p decreases.

(*b*) *Number of clones per host*

It is assumed that a host carries an average of c independent infections. It is also assumed that c is the number of haplotypes which simultaneously have sexual forms of the parasite (gametocytes) in the blood and therefore are potential mates during fertilization after the mosquito has taken a blood meal. Here c is assumed to be constant even though these c infections come from a variable number of transmissions. In this study c is defined as a function of $\bar{R}$ in the following way, although such a relationship has not yet been properly explored in the field. The conditional mean number of successful (i.e. non-zero) transmissions from one host to others is $\bar{R}/(1-p^k)$. If malaria is stable it follows that the average rate of loss of infections is equal to the average rate of acquisition of infections. However, depending on levels of immunity, the average number of infections per host could be between zero and $\bar{R}/(1-p^k)$. In this study it is assumed that the average number of infections is halfway between these extremes, i.e.

$$c = \frac{1}{2}\frac{\bar{R}}{1-p^k} \tag{2}$$

though in the remaining theory c is modelled independently of $\bar{R}$ and σ_R^2 to keep the model general. Values of c from the field have been inferred from the observed number of one or two-locus haplotypes in the blood (Carter & McGregor 1973; Conway *et al.* 1991; Babiker *et al.* 1994; Hill & Babiker 1995; Hill *et al.* 1995; Ntoumi *et al.* 1995; Paul *et al.* 1995), or from studies on the amount of heterozygosity among oocysts formed in mosquitoes. In a high transmission area in Tanzania the estimated number of clones per host was 3.5 (Hill *et al.* 1995; Hill & Babiker 1995) and in a low transmission area in New Guinea was 1.1 (Paul *et al.* 1995). As an example, some realistic values of $\bar{R}$ and σ_R^2 to correspond to $c = 3.5$ and $c = 1.1$ are

$$(\bar{R} = 5: \quad \sigma_R^2 = 50) \quad \text{and} \quad (\bar{R} = 1.5: \quad \sigma_R^2 = 3.0).$$

(*c*) *Drug resistance genes*

Now assume that the parasite has two loci at which there are two allelic forms – one the wild-type allele, and the other allele encoding resistance to a given drug which would otherwise kill all parasites not carrying the mutant allele. Denote these alleles as A and a for mutant and wild-type alleles, repectively, for the locus encoding resistance against drug α and similarly B and b for alleles for the locus encoding resistance against drug β. Thus there are four relevant genotypes, AB, Ab, aB and ab. The fitnesses, which determine the relative frequencies of haplotypes within the host, in the presence of both drugs, is represented by a vector, $\boldsymbol{W}_T = [1 \quad 0 \quad 0 \quad 0]$, and in the absence of drugs by the vector $\boldsymbol{W}_U = [w^2 \quad w \quad w \quad 1]$. These reduce to $\boldsymbol{W}_T = [1 \quad 0]$ and $\boldsymbol{W}_U = [w \quad 1]$ when only one drug is in use, in which case the second locus is irrelevant. It is important to note that fitness is defined here as the relative number of gametocytes with the two-locus haplotype at the time of transmission, rather than the number of distinct clones in the host's blood. If a double mutation has arisen at the beginning of an infection in a host which carries c independent infections, the relative frequencies of the two-locus haplotypes will be

$$\boldsymbol{W}_T = \frac{1}{w^2+2c-1}[w^2 \quad 0 \quad 0 \quad 2c-1]. \tag{3a}$$

For example, if a host has one infection ($c = 1$) and a double mutation (AB) occurs which has equal fitness to the wild-type haplotype (ab), then its frequency in the host is $\frac{1}{2}$. If the host is infected twice more ($c = 3$) with non-mutant parasites, this frequency is reduced to $\frac{1}{6}$. If the recombinant meiotic products (Ab and aB) are also in the host, as may occur in subsequent generations, the relative frequencies in the host will be

$$\boldsymbol{W}_{\mathrm{U}} = \frac{1}{w^2+2w+4c-3}\begin{bmatrix} w^2 & w & w & 4c-3 \end{bmatrix}. \qquad (3b)$$

(*d*) *Transmission-genetics cycle*

The basic model of transmission assumes that each host receives c independent infections from separate transmission events and that the parasites from these infections co-exist in the blood of the host. The relative frequencies of the haplotypes in these infections are adjusted during the course of the infection according to their relative natural fitnesses by multiplying with $\boldsymbol{W}_{\mathrm{U}}$ and re-scaling. In a proportion of the host population, T, the frequencies are adjusted for drug selection by multiplying with $\boldsymbol{W}_{\mathrm{T}}$ and rescaling i.e. the frequency of mutant parasites in treated hosts are set at unity, and non-mutant parasites at zero. The parasites then form gametocytes which are taken up by a mosquito in a blood meal and undergo self or cross fertilization during the zygote stage. The frequencies of the diploid genotypes in the mosquito are assumed to reflect random mating among the gametocytes within the host from which the mosquito took the blood meal. These diploid genotypes then undergo recombination and the frequencies among the haploid meiotic products are adjusted accordingly. The meiotic products, or a subset of them are transmitted to a new host to initiate a new infection.

3. SURVIVAL PROBABILITY OF A NEW MUTANT

(*a*) *General introduction to branching process theory*

The following section describes the branching process theory used to predict the ultimate survival probability of a single copy of a mutant. This theory enables prediction of the probability that a single replicating particle (in this case a mutant allele) which is subject to stochastic processes during its replicative cycle ultimately survives in the population, i.e. it is still in the population after many generations. This probability can be predicted from the distribution of the number of 'offspring' each particle produces each generation. The distribution of the number of offspring can be summarized by a single function called the probability generating function (pgf, denoted $\phi(s)$ where s is a dummy variable). For example, the pgf for a Poisson variable is represented by

$$f(s) = \mathrm{e}^{-\lambda(1-s)} = \sum_{k=0}^{\infty} \mathrm{e}^{-\lambda}(\lambda s)^k/k!.$$

Probability generating functions are useful because the value of s which solves the equation

$$\phi(s) = s \qquad (4)$$

gives the ultimate extinction probability, and hence the ultimate survival probability (denoted usp) is given by $1-s$. If the mean of the distribution described by $\phi(s)$ is less than one, the solution to equation (4) is $1-s = 0$. Thus the particle will not survive if the mean number of offspring is less than one (because it does not on average replace itself). If the mean number of offspring is greater than one, the particle has a finite, but less than perfect probability of surviving, as given in equation (4). A further property of branching process theory is that if there are several processes during the replication cycle, each with different pgfs, then the pgf used in equation (4) is the compounded distribution of the individual pgfs involved in the process. Note that equation (4) only holds if the distribution of the number of offspring is the same in all generations. If, however, environmental fluctuations cause a change in the pgf over time, it is necessary to compound over the different distributions from the generations to obtain the overall pgf. Note also that it is assumed that the population size is infinite or very large such that there is a zero probability that two identical mutants will meet and produce offspring together. A digestible review of branching process theory is given by Schaffer (1970).

(*b*) *Application to malaria mutants*

In malaria, the replicative cycle is a transmission from one host to the next which involves the transfer of parasites to mosquitoes, meiosis in the mosquito and then transfer to a new host. This process can be broken down into two stochastic processes each with their probability distributions. One distribution is for the number of transmissions from one infected host to a number of new hosts, assumed to have a negative binomial form. The second distribution is for segregation during meiosis which has a binomial distribution. The combination of these distributions will determine how many copies of a single mutant are left in the population after one transmission cycle. The parameters of these distributions depend on whether the host has been treated with drugs or not. The case of no drug treatment is given first, and the case of drug treatment is then derived from it.

If the host is not treated with drugs the pgf for the negative binomial distribution, assumed here to represent the number of transmissions to new hosts, is given by

$$\Phi_{\mathrm{NB}}(s) = \left[\frac{p}{1-(1-p)s}\right]^k. \qquad (5)$$

The binomial pgf for the segregation during meiosis is:

$$\Phi_{\mathrm{B}}(s) = 1-\pi+\pi s, \qquad (6)$$

with mean π and variance $\pi(1-\pi)$. Here the parameter π represents the average probability that in each of $\bar{R}$ independent successful transmissions, the mutant haplotype AB is represented among the

transmitted parasites. This probability is worked out from the overall self replacement rate, ρ, of the AB haplotype over $\bar{R}$ transmissions as

$$\pi = \frac{\rho}{\bar{R}}. \tag{7}$$

If malaria is stable, individual alleles with equal fitness should on average replace themselves. Rare two-locus haplotypes should have replacement rates of $\frac{1}{2}$ if the loci are unlinked and the parasites are mating randomly. In the case of unequal fitness, and some inbreeding due to a limited number of genotypes per host, the replacement rate is calculated from the relative fitnesses among the gametocytes in the host (3*a*) as

$$\rho = \frac{2c[w^2 + \frac{1}{2}w^2(2c-1)]}{(w^2+2c-1)^2}. \tag{8a}$$

The first and second terms within the brackets of the numerator represent the probabilities of getting an AB meiotic product from $AB \times AB$ and $AB \times ab$ matings, respectively, and the denominator is the appropriate scaling factor. The factor of $2c$ in the numerator ensures that the replacement rate for equally fit alleles is unity and reflects the fact that for each transmission and meiosis, two gametes are sampled. Note that equation (8*a*) reduces to $\rho = 1/2 + 1/4c$ when the mutant has equal fitness to the wild-type allele. This is greater than the value of $\frac{1}{2}$ expected from random mating (where $c = \infty$) because sometimes the parasites self-fertilize. In subsequent generations, when the recombinant meiotic products may also be within a host (3*b*), the replacement rate is

$$\rho = \frac{4c[2w^2(w+c)]}{(w^2+2w+4c-3)^2}, \tag{8b}$$

and the replacement rate when $w = 1$ is

$$\rho = \frac{1}{2} + \frac{1}{2c}.$$

Now that the two component probability distributions determining the overall probability of the mutant surviving the transmission have been defined, they can be compounded into a single probability distribution by using their pgfs. The compounded pgf is denoted as $\Phi_{B.NB} = \Phi_{NB}(\Phi_B(s))$ and can be shown to be distributed as negative binomial with a mean of $\pi\bar{R}$ and a variance of $\pi\bar{R}(1-\pi+\pi\sigma_k^2/\bar{R})$ (Kojima & Kelleher 1962). This means that the pgf of the compounded distribution is given by:

$$\Phi_{B.NB} = \left[\frac{p^*}{1-(1-p^*)x}\right]^{k^*}, \tag{9a}$$

where

$$p^* = \frac{p}{p-\pi p+\pi}, \mathrm{k}^* = \mathrm{k}. \tag{9b}$$

If a host is treated with drugs, the probability that the double mutant is among the meiotic products is $\pi = 1$. Thus the pgf, if the mutant arises in a drug treated host, is negative binomial with mean $\bar{R}$ and variance σ_R^2, i.e. the same as the transmission distribution (5).

Because the mutant may arise in a treated or untreated host and will be transmitted to either treated or untreated hosts in each subsequent generation, the overall probability of survival will depend on the sequence of treatment in the hosts encountered by the mutant during the first few generations. For example, the pgf for the number of offspring for a mutant which arises in a treated host and then is transmitted to an untreated host is not the same as if the mutant arose in an untreated host and then transmitted to a treated host, i.e. $\Phi^T(\Phi^U(s))$ is not the same as $\Phi^U(\Phi^T(s))$ where Φ^U and Φ^T denote the pgfs in the cases where the host is untreated and drug treated respectively. Thus to obtain the average probability of survival over all possible sequences of treatment, the usps for all of the 32 possible combinations of sequence of treatments in the first five generations (i.e. $\{U, U, U, U, U\}$, $\{U, U, U, U, T\}, \ldots \{T, T, T, T, T\}$) were found after compounding five times and then solving the treated and untreated generating functions in sequence (i.e. $\Phi^{UUUUU}(s) = s$, $\Phi^{UUUUT}(s) = s, \ldots \Phi^{TTTTT}(s) = s$). These usps were weighted by their binomial probabilities of occurrence assuming that all possible sequences are equally likely (i.e. $(1-T)^5$, $(1-T)^4 T, \ldots, T^5$) and then summed to obtain the average usp. For the first generation of each series, it was assumed that there were no recombinant haplotypes (Ab and aB) in the host and so equation (8*a*) was used, though for subsequent generations they were assumed to be present and so equation (8*b*) was used.

(*c*) *Numerical evaluation*

The procedure described above was used to evaluate survival probabilities of single-locus and two-locus mutants as functions of T for three levels of natural selection ($w = 1, 0.9$ and 0.5) and three levels of transmission – one with low mean transmission number and low variability ($\bar{R} = 1.5; c = 1.1; \sigma_R^2 = 3$), one with high mean transmission and high variability ($\bar{R} = 5; c = 3.5; \sigma_R^2 = 50$), and one with high mean and low variability ($\bar{R} = 5; c = 2.6; \sigma_R^2 = 10$). The corresponding parameters for the negative binomial in these three cases are ($p = 0.5; k = 1.5$), ($p = 0.1; k = 0.56$) and ($p = 0.5, k = 5$), respectively. The same calculations were also performed over a wide range of values of all the parameters ($T = 0$ to 1, $w = 0.1$ to 1, $\bar{R} = 1$ to 15, $p = 0.1$ to 0.9) with the restriction that $1 \leqslant \sigma_R^2 \leqslant 50$ (considered to be the bounds of reality). This was done in order to generate data to which a linear regression model was fitted, to enable a first order approximation of the influence of the main factors affecting survival probability to be obtained. The regression terms fitted were the interactions between T and $\bar{R}$, σ_R^2 and w (or w^2 for the two-locus case). By fitting interactions with T rather than main effects, it was ensured that the intercepts at $T = 0$ were always zero since mutants are only expected to survive if they have above average

fitness. Quadratic terms were also fitted to test whether there was an improvement in fit of the model by allowing for some curvilinearity in the relationship.

4. RESULTS

Figure 1 shows the probability distributions for the three transmission levels. It illustrates that more hosts produce zero transmissions when the mean level of transmission is low, and for the same mean level, when the variability is high. Thus a new mutant is at higher risk in more variable transmission environments.

Figure 2 shows the survival probabilities as a function of drug treatment rate. It illustrates the following five qualitative conclusions demonstrated by the model.

1. The strongest influence is that of drug pressure: survival probability approximately linearly increases with drug treatment rate (figures 2*a–f*). Even though the probability that an individual mutant survives is low when drug pressure is low (figures 2*c–f*), if there are ten separate mutation events, then the probability that at least one survives (which is all that is required for drug resistance to become established when drug pressure continues) is moderate (figures 2*a*, *b*).

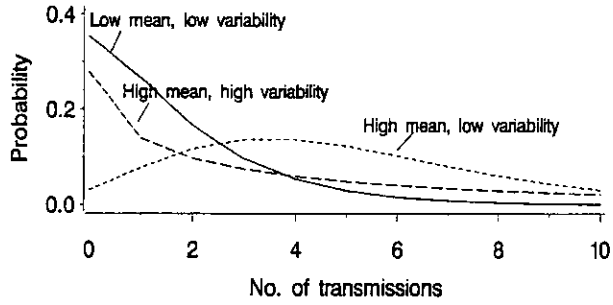

Figure 1. Probability distribution of number of transmissions per host for transmission areas with low mean and low variability (solid line, $\bar{R} = 1.5$; $c = 1.1$; $\sigma_R^2 = 3$), high mean with high variability (long dash, $\bar{R} = 5$; $c = 3.5$; $\sigma_R^2 = 50$) and high mean with low variability (short dash, $\bar{R} = 5$; $c = 2.6$; $\sigma_R^2 = 10$).

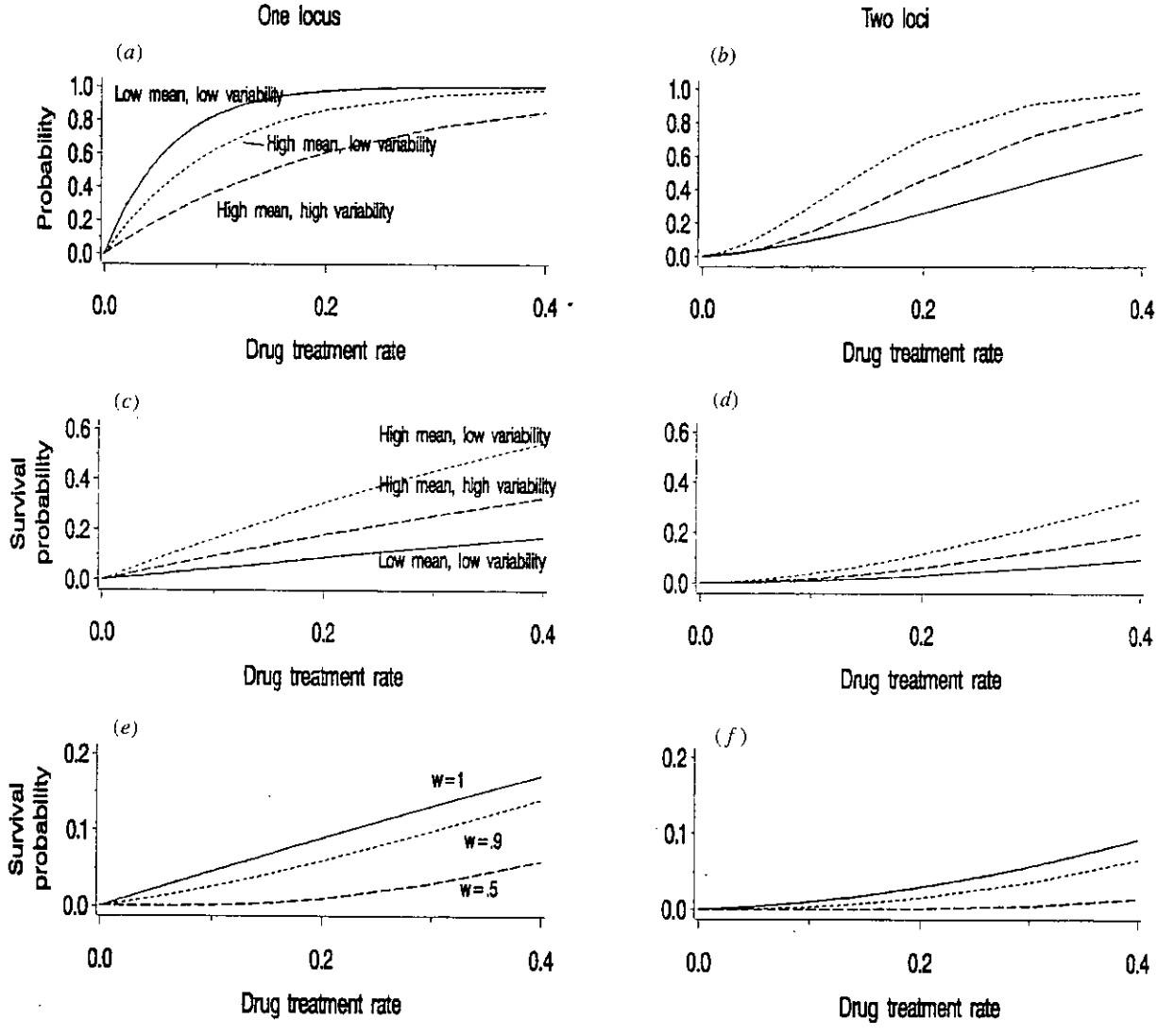

Figure 2. Survival probabilities as functions of drug treatment rates for one (left) and two-locus mutants (right). (*a*) and (*b*) Probability that at least one out of ten survives for three different transmission intensities. (*c*) and (*d*) Probability of survival of an individual mutant for three different transmission intensities. (*e*) and (*f*) Probability of survival of an individual mutant for three different levels of natural selection. Labels of line types are consistent within each row of the figure.

2. One-locus mutants have a higher chance of survival than two-locus mutants (figures 2*a*, *c*, *e* vs figures 2*b*, *d*, *f*). This is because recombination breaks down the *AB* haplotypes at each meiosis if the parasite does not mate with the same haplotype as itself.

3. The mean transmission rate increases the probability that a new mutant survives. The effect of increasing mean transmission rate from 1.5 to 5 while keeping the mean to variance ratio the same ($p = 0.5$) is shown by comparing the 'low mean, low variability' vs 'high mean, low variability' lines in figures 2*c*, *d*.

4. An increase in the variance of transmission while keeping the mean the same decreases survival probability. This is shown by comparing the 'high mean, low variability' and 'high mean, high variability' lines in figures 2*c*, *d*. Much of this difference is due to the greater proportion of hosts which produce zero transmissions (figure 1).

5. Natural selection against the mutant decreases survival probability (figures 2*e*, *f*).

The above qualitative conclusions can be described more generally and quantitatively using results from the regression analysis. The best fit to the data were obtained using the following equations in the one-locus and two-locus cases,

$$\begin{aligned} \mathrm{usp}_1 &= T(0.196\bar{R} - 0.008\bar{R}^2 - 0.008\sigma_R^2 + 0.217w), \\ \mathrm{usp}_2 &= T(0.191\bar{R} - 0.008\bar{R}^2 - 0.007\sigma_R^2 + 0.127w^2), \end{aligned} \quad (10)$$

where the subscripts on usp denote the number of loci involved. Thus while T had a linear effect on usp, the slope of this line increased with increasing $\bar{R}$ with a plateau effect at values of around $\bar{R} > 10$. The slope also increased with increasing w (or w^2 in the two-locus case) and with decreasing σ_R^2. As $\bar{R}$ accounted for 52% and 58% of the variation in usp_1 and usp_2 respectively, and $\bar{R}^2$ for a further 22% and 23%, transmission rate, over and above the effect of drug pressure, was the major influence on survival probability. The influences of natural selection and variation in transmission rate were much weaker, with w and σ_R^2 each explaining less than 5% of the variation in *usp*.

5. DISCUSSION

This study demonstrates that the fate of a newly arisen drug resistant mutant is primarily determined by whether drugs are in use in the host population, and the rate of transmission of the parasite. The reason why these two factors jointly determine whether a mutant survives is because the mutants only have a selective advantage when they encounter the drug. If transmission rates are high, the new mutant has a greater chance of being transmitted to at least one host which is treated with drugs and therefore subjected to selection by drugs. Low transmission rates reduce the probability that a copy of the mutant is exposed to the drug.

The study also shows that even when resistance is coded for by two mutant alleles at unlinked loci, the frequent breakdown of the double mutant haplotype under conditions of high transmission is not sufficient to prevent the double mutant surviving because selection by drugs is a more powerful force to keep the mutant in the population. Thus the suggestion by Paul *et al.* (1995) that high transmission rates are unfavourable for the evolution of multi-locus drug resistance because of greater recombination breakdown is not supported, and the results of Dye (1991) are. In other words, this study shows that effective selection and effective recombination are both increased by high transmission rates, but selection wins. This conclusion was reached by taking a population genetics approach in which the two major effects of population structuring of the parasite into hosts were delineated – namely, mating structure (or degree of inbreeding), and selection structure (heterogeneous selection by hosts). As both of these factors determine the rate of gene flow through populations in compartmentalized populations typical of many parasites, the model developed here is likely to have wider applicability to theories on parasite evolution.

In the present study it was assumed that the drug was completely effective in killing parasites. In practice, improper administration of the drug (e.g. through underdosing) will mean that selection pressure on drug resistance mutants is not as strong as assumed here and, therefore, that drug resistance in the field will evolve less easily than predicted here. This is not true, however, in the case when drug resistance (to one or more drugs) is controlled by more than one locus: partial killing will cause more resistant alleles to be maintained in the population so that the chances of formation of a doubly resistant haplotype are greater. Thus for the same proportion of hosts treated with drugs, incomplete efficacy will favour faster spread of multiple drug resistance, but not single drug resistance. This does not mean, however, that intermediate drug treatment levels (proportion of hosts treated) will increase the rate of spread of resistance.

It was also assumed that drugs are taken randomly by the infected host population and that the frequency of treatment is independent of transmission rate. This allowed the conclusion to be reached that effective selection on the mutant is directly related to both drug pressure and transmission rate. However, if hosts only take drugs when they are sick and the frequency of being sick depends on transmission rates (e.g. due to the number of previous infections), then the effect of transmission rate in the field may be somewhat less than predicted here.

A key assumption made in this study is that malaria is stable, i.e. the net change in the average number of parasite infections per host does not change over the generations. Clearly this is unlikely to be true in malaria parasite populations which typically fluctuate in size due to seasonal factors. During expansion phases, most mutants, even neutral ones, will survive because the mean number of offspring is greater than one. During contraction phases, few will survive. Thus the fate of new mutations under selection very much depends on the prevailing population dynamics when the mutant arises. However, such fluctuations are only likely to affect the conclusions from this study in a quantitative, but not qualitative way because com-

parisons of the effects of drug pressure, transmission rate and natural selection are performed under the same conditions (i.e. stable malaria).

As the model described here shows, variation in transmission rate can have considerable consequences to the fate of new mutants, and probably any low frequency allele or multi-locus haplotype under selection. If variation in transmission rate is high, unique genetic entities have low probabilities of surviving because stochastic forces can easily eliminate them before they can multiply. In the case of drug resistance, high variability in transmission rate is detrimental to the mutants' survival because of the greater risk involved in transmission. Thus if the stability of transmission rate, as well as the mean transmission rate, is reduced by control strategies such as bednets, vaccines or drugs, such interventions may further inhibit the parasite's adaptation to these strategies. The importance of variation in transmission rate on the persistence of 'strains' (multi-locus haplotypes) in a population has been illustrated by Gupta *et al.* (1994) and has implications as to how rapidly hosts acquire specific immunity. More generally, the role of variation among parasites and hosts in transmissibility is relatively unexplored, although there is a growing awareness that its effect on the epidemiology of parasitic diseases can be profound (Anderson & May 1991; Read *et al.* 1995).

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ERRATA

Proc. R. Soc. Lond. B **264**, 53–59 (January issue)

Survival probability of drug resistant mutants in malaria parasites

BY MARGARET J. MACKINNON

On p. 56, equation (8*a*), which predicts the within-host fitness of the doubly resistant mutant in the first generation should read

$$\frac{2cw^2[w^2+\frac{1}{2}(2c-1)]}{[w^2+2c-1]^2}$$

and equation (8*b*), which predicts fitness in subsequent generations should read

$$\frac{4cw^2[w^2+2w+2c-1]}{[w^2+2w+4c-3]^2}$$

This error made no appreciable difference to the predicted survival probabilities.

In addition, the average probability generating function for the variable environment of drug selection (i.e. treated or untreated) was obtained by compounding *over* generations and weighting by the probability of the series of treatment events. This assumes that the entire population is subjected to either drug treatment or no drug treatment, but not a mixture of the two. It is more likely, however, that in each generation some mutants find themselves in treated hosts, others in untreated hosts, and so the generating functions should be averaged *within* generation rather than across generations. Doing so gives the average probability-generating function as

$$\Phi(s) = T\Phi^T(s) + (1-T)\Phi^U(s).$$

This error leads to an underestimation of survival probability of up to 20% in the single-locus case and up to 50% in the two-locus case, as well as a prediction of non-zero probabilities of survival when they should have been zero. The qualitative conclusions of the paper do not change.

Proc. R. Soc. Lond. B **264**, 181–190 (February issue)

Enigmatic phylogeny of skuas (Aves: Stercorariidae)

BY B. L. COHEN, A. J. BAKER, K. BLECHSCHMIDT, D. L. DITTMANN, R. W. FURNESS, J. A. GERWIN, A. J. HELBIG, J. DE KORTE, H. D. MARSHALL, R. L. PALMA, H.-U. PETER, R. RAMLI, I. SIEBOLD, M. S. WILLCOX, R. H. WILSON AND R. M. ZINK

On p. 186, the taxa of *Quadraceps* and *Saemundssonia* lice associated with *Stercorarius parasiticus* and *S. longicaudus* were interchanged in table 3. A corrected version of the table is given below.

Table 3. *Feather-louse taxon distribution on skuas and gulls*

		louse taxa		
hosts	*Haffneria grandis*	*Austromenopon* spp.	*Quadraceps normifer* subspp.	*Saemundssonia* spp.
Stercorarius longicaudus	present (Emerson 1972)	*fuscofasciatum*	*parvopallidus*	*inexspectata*
parasiticus	rare (1/80+)	*fuscofasciatum*	*normifer*	*cephalus*
pomarinus	abundant	*fuscofasciatum*	*stellaepolaris*	*stresemanni*
Catharacta skua	abundant	absent (0/53)	*stellaepolaris*	*stresemanni*
antarctica	abundant	absent (0/78)	*alpha*	*stresemanni*
chilensis	abundant	*fuscofasciatum*	*alpha*	*stresemanni*
hamiltoni	abundant	*fuscofasciatum*	absent (0/26)	*stresemanni*
lonnbergi	abundant	*fuscofasciatum*	absent (0/29)	*stresemanni*
maccormicki	abundant	*fuscofasciatum*	*alpha*	*stresemanni*
Larus spp.	absent (0/tens)	*transversum*	absent (0/tens)	*lari*

(For taxa that were rare or not found, the number of lice found and the number of hosts examined are indicated in parentheses.)

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The evolution of multiple drug resistance in malaria parasites

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Abstract

Forces determining the rate of spread of drug resistance in malaria were explored using a genetics transmission model which took account of the strong population structure of these parasites. The rate of change of frequency of drug resistant mutants in the parasite population is primarily a function of the proportion of hosts treated with drugs, and parasite transmission rates. With high transmission rates, selection by drugs is more effective than with lower rates because the resistant mutant passes on more copies of itself to the next generation of hosts. Thus reducing transmission rates, either at the overall population level or from drug-treated individuals, should be effective in curbing the spread of resistance. An exception to this is when 2 unlinked genes act jointly (not independently) to confer resistance, when the prevailing transmission rate is already low, drug use is minimal, and resistance genes are rare. Reductions in fitness of the mutant in the absence of drugs (i.e., a fitness cost to resistance) and the degree of epistasis and the mode of gene action of the drugs do not alter these conclusions.

Keywords: malaria, *Plasmodium* spp., drug resistance, population structure, genetics

Introduction

Drug resistance, especially multiple drug resistance, is disastrous for treatment and control of malaria. Resistance to almost all available drugs, most notably chloroquine, mefloquine and pyrimethamine, continues to spread throughout the tropical world, and the need for new drugs and a strategy to prevent further spread is urgent (PETERS, 1987; WARHURST, 1989; WERNSDORFER, 1991; SCHAPIRA *et al.*, 1993). In an influential study, CURTIS & OTOO (1986) showed that the prevalence of drug use was the most important factor in determining the rate of spread of resistance, and that resistance to 2 drugs would spread more slowly than resistance to one drug. Thus they were able to make strong recommendations on how to prolong the useful life of drugs.

The model used to reach this conclusion was simple: it assumed that parasites randomly mate with each other, as they might, for instance, if the entire parasite population were to be found in a single large volume of blood just before mating. However, this is unrealistic because malaria parasite populations are strongly substructured into subpopulations in individual hosts. This has the effect of severely restricting the pool of sexual partners during mating and therefore often forcing the parasite to self-mate (inbreed). Indeed, one of the major advances in the study of malaria in the last decade has been the realization of how much inbreeding does occur in natural parasite populations. It is now clear that each host typically carries between one and 4 genetically distinct parasite types at any one time, the number per host tending to be higher in high transmission areas (CARTER & MCGREGOR, 1973; JOSHI *et al.*, 1989; CONWAY & MCBRIDE, 1991; BABIKER *et al.*, 1994; HILL & BABIKER, 1995; HILL *et al.*, 1995; PAUL *et al.*, 1995). So how does this new information on population structure influence our understanding of how drug resistance spreads?

Several authors (CURTIS & OTOO, 1986; DYE, 1991; DYE, 1994; PAUL *et al.*, 1995; DYE & WILLIAMS, 1997; HASTINGS, 1997) have pointed out that, in more outbred populations such as occur in high transmission areas, there is greater effective recombination breakdown between the 2 or more loci encoding resistance. It has therefore been suggested that multiple drug resistance develops more slowly in such areas (PAUL *et al.*, 1995). Analytical examination of this hypothesis has indeed shown that outbreeding can slow the spread of multiple drug resistance, though only in a limited set of conditions, namely, when none of the resistance genes confers significant protection on its own, when the genes are rare, and when selection pressure is low (DYE, 1994; DYE & WILLIAMS, 1997; HASTINGS, 1997). However, this issue of recombination has somewhat diverted attention from the main force driving frequency increases in resistance genes—that of drug selection itself—and so in this study we also considered how transmission rate influences the effectiveness of this selection. Using a deterministic simulation model to pitch the force of effective selection against the force of effective recombination as determined by population structure, we predicted the rate of spread of drug resistance.

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The basic model and assumptions

The following paragraphs describe the model. First the basic transmission-genetics cycle is described, followed by a description of the assumptions made about the transmission process, epidemiology, population structuring, drug pressure and natural selection. Finally, the equations describing the changes in frequencies of resistance genes each generation are given. The Table summarizes the notation used.

Transmission cycle and population structuring

As shown in Fig. 1, in each generation the parasite population flows through vertebrate hosts (boxes) and mosquitoes (circles) undergoing a series of events which alter the genetic composition of the population. At stage 1, the parasite is transmitted to new hosts as sporozoites. Each transmission event is represented by a small box within each host: in this diagram there are 3 independent inoculations to each host. Each inoculum may contain a mixture of genetically different parasites which have probably been derived from a mosquito bite on just one infectious host. The couplets of letters (AB, etc.) each represent a parasite's two-locus haploid genotype (haplotype) which is distinct with respect to resistance or susceptibility to 2 drugs denoted α and β. Haplotype AB is resistant to both drugs, Ab is resistant to only drug α, aB is resistant to only drug β, and ab is susceptible to both drugs. The resistance loci are assumed to be genetically unlinked with a recombination fraction $r=0{\cdot}5$. At stage 2, the frequencies of the haplotypes within the host are adjusted according to how fit the parasites with mutant genes are relative to their susceptible counterparts. At stage 3, drugs are administered to some of the infected hosts. (In this diagram, 2 drugs are administered together, but the model allows for a single drug to be used.) If the host is carrying a resistant parasite, all the susceptible parasites are eliminated leaving only resistant parasites. At stage 4, pairs of parasite gametes form diploid zygotes in the gut of the mosquito (left side of

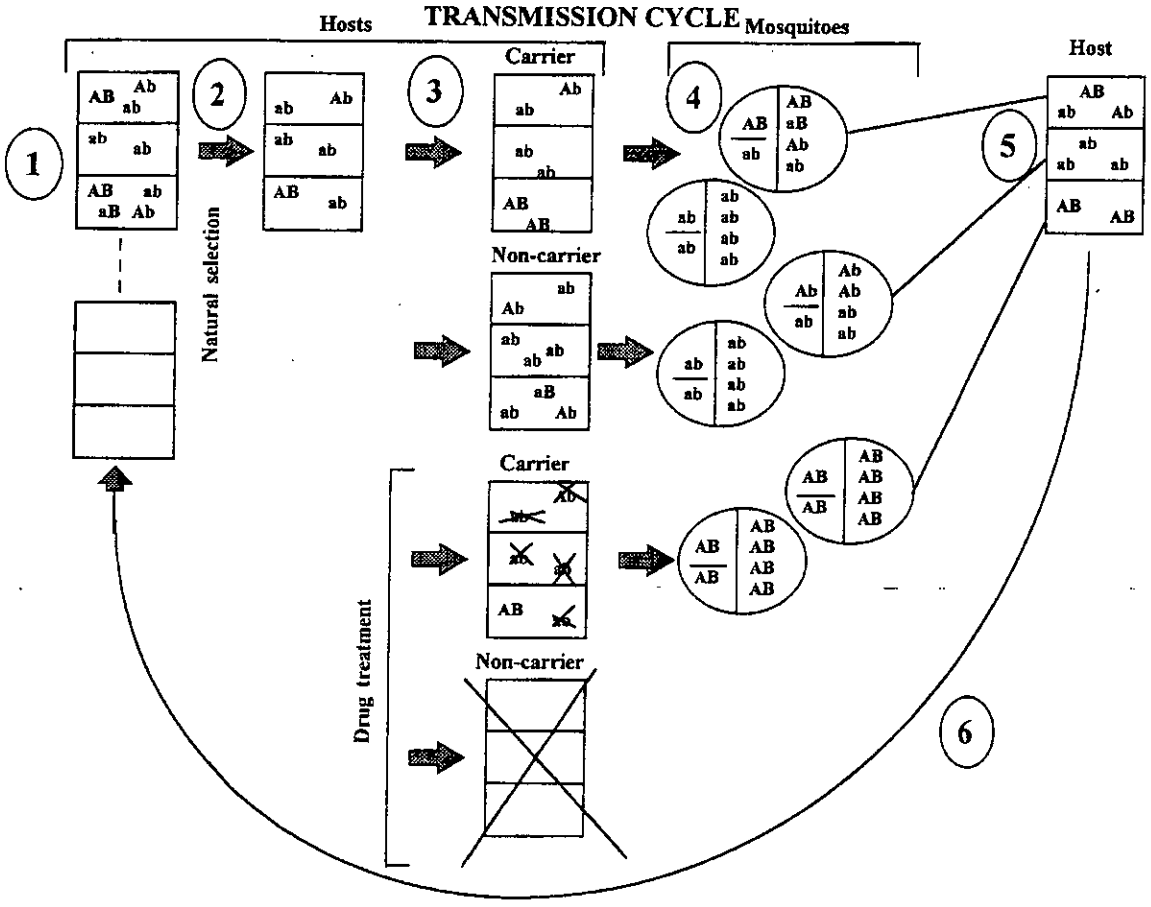

Fig. 1. Transmission cycle of malaria parasites showing the processes of selection and recombination contributing to gene frequency changes. 'Host(s)'=vertebrate host(s); see text for details. Circled numbers denote the stages represented by the mathematical model described in Fig. 2.

Table. Notation and typical values used in the model

Symbol	Meaning	Values used
Transmission		
c	Mean number of independent infections received by each host	1·1,3
M	Mean number of transmitted meiotic products per oocyst	1,4
z	Mean number of oocysts formed per mosquito	1
m	Mean number of genetically distinct haplotypes among gametocytes	1·1,3
S	Proportion of parasites which self-fertilize	$1/m$
I	Proportion of parasites which inbreed	$1/c$
Genes and frequencies		
α, β	Two drugs which kill malaria parasites	
A, B	Mutant alleles encoding resistance to drugs α and β, respectively	
a, b	Wild-type alleles to A and B which are susceptible to drugs α and β	
r	Recombination fraction between the two loci	0·5
$P=[p_{AB}\ p_{Ab}\ p_{aB}\ p_{ab}]$	Vector of population frequencies of two-locus haplotypes	
$P=[p_A\ p_a]$	Vector of population frequencies in the one-locus case	
P^T, P^U	Vectors of haplotype frequencies within treated and untreated hosts	
Σ	Sum of the elements in a vector of frequencies used for re-scaling	
Σp_i	Sum of frequencies of carrier (resistant) haplotypes	
C	Proportion of hosts which carry drug-resistant parasites ('carriers')	
D	Population disequilibrium between the two loci	
d	Disequilibrium as a proportion of the maximum disequilibrium	
Selection		
T	Proportion of the host population treated with drugs	0·05,0·1,0·2
t	No. of transmissions from a treated host relative to an untreated host	1,0·2,0·33
W^T	Vector of fitnesses of the haplotypes in the presence of drugs	
	For the two-locus, two-drug (*2–2*) case $W^T=[1\ 0\ 0\ 0]$	
	For the two-locus, one-drug (*2–1*) case $W^T=[1\ w_T\ w_T\ 0]$	w_T=0·5
	For the one-locus, one-drug (*1–1*) case $W^T=[1\ 0]$	
W^U	Vector of relative fitnesses of the haplotypes in the absence of drugs	
	For the two-locus cases $W^U=[w_U^2\ w_U\ w_U\ 1]$	w_U=1,0·9
	For the one-locus, one-drug case $W^U=[w_U\ 1]$	

the circles) and then undergo meiosis to produce 4 haploid meiotic products per zygote (right side). If the mosquito has bitten a treated carrier host, all the meiotic products are doubly resistant. If the donor host has not been drug-treated, there is recombination breakdown of the doubly resistant haplotypes which reduces their frequency. At stage 5, some of these meiotic products are transmitted in the form of sporozoites to new hosts which become superinfected (stage 6) as in stage 1.

Assumptions and notation

The mathematical model is based on the following assumptions.

(i) Malaria is stable, i.e. the population of infected hosts and parasites remains large enough through time for changes in total parasite population size not to affect the changes in frequency of the mutant genes.

(ii) Each host receives exactly c independent infections each of which may comprise up to Mz (M=1...4) meiotic products or distinct haplotypes among the sporozoites inoculated into the host from a mosquito which has formed z zygotes (oocysts). These c infections give rise to m haplotypes which simultaneously have sexual forms of the parasite (gametocytes) in the blood and $m \leq Mzc$.

(iii) If frequencies of the haplotypes in the blood are equal, the frequency of selfing (mating between identical parasites) is $S=1/m$, the frequency of inbreeding (mating between parasites derived from the same mosquito, including selfing) is $I=1/c$, and the frequency of outbreeding is $1-I$.

(iv) In the presence of drugs, the vector of fitnesses of the haplotypes (AB Ab aB ab) is $W^T=[1\ w_T\ w_T\ 0]$. This notation can represent 2 possible cases: either 2 different drugs are used which both kill parasites carrying the wild-type alleles and only parasites carrying both mutant alleles survive (the two-locus, two-drug case with $w_T=0$, denoted the *2–2* case or 'the epistatic model'); or a single drug is used (i.e. α is the same as β) and each of the mutant alleles confers partial resistance to this drug so that a proportion, w_T, of the parasites carrying a single mutant allele survive after drug treatment (the two-locus, one-drug case denoted the *2–1* case or 'the additive model'). A third case, in which only one locus is involved and only one drug is used (denoted the *1–1* case), is also considered here. The one-locus vector of fitness for haplotypes A and a in the presence of the drug a is represented by the vector $W^T=[1\ 0]$.

(v) In the absence of drugs, the mutant allele confers a lower fitness on the parasite so that its fitness is reduced to a fraction, w_U, where $w_U<1$, of the fitness of parasites carrying the wild-type allele. Fitness is assumed to act multiplicatively so that the vector of fitnesses without drugs is represented by $W^U=[w_U^2\ w_U\ w_U\ 1]$ in the two-drug case. For the one-locus case, $W^U=[w_U\ 1]$.

(vi) A proportion of the host population, T, is treated with drugs and the drugs are fully effective.

(vii) The number of transmissions from a treated host relative to an untreated host is t; i.e. if $t<1$ then drug treatment not only reduces asexual parasite numbers but also reduces the infectiousness of the host to mosquitoes. If $t>1$, then the drugs promote transmission.

Rate of change in frequency of drug resistant mutants

The equations in Fig. 2 describe the change in frequency of the haplotypes from one generation to the next using the process described above and in Fig. 1. It has been written in the most general form which is the *2–1* case, but the *1–1* and *2–2* cases can easily be recovered by substituting in $r=0$ and $w_T=0$, respectively. The method requires keeping track of the haplotype frequencies in the parasite population which are stored in a vector $P=[p_{AB}\ p_{Ab}\ p_{aB}\ p_{ab}]$ which at some stages is split into 2 separate vectors for frequencies among treated and untreated hosts, P^T and P^U. These haplotype frequencies can also be represented by the frequencies of the individual alleles, p_A and p_B, and disequilibrium, D, which represents the deviation of haplotype frequencies from their expected frequencies if the population is randomly mating and not subjected to selection. For example, $p_{AB}=p_Ap_B+D$ where $p_A=p_{AB}+p_{Ab}$ *and* $p_B=p_{AB}+p_{aB}$. Disequilibrium after meiosis is calculated as:

$$D^* = \frac{D}{2}\left[1(-r) + \frac{S}{2} + \sqrt{\left(1 - r + \frac{S}{2}\right)^2 - 2S(1-2r)}\right]$$

(WEIR *et al.*, 1972).

In this study it is assumed that the 2 resistance loci are unlinked, i.e. $r=0{\cdot}5$, in which case disequilibrium changes can be calculated as $D^*={}^1/_2D(1+S)$ if parasites self and $D^*={}^1/_2D(1+I)$ if parasites inbreed. As the equation for the case of mixed selfing, inbreeding and random mating has not yet been derived, the equation for selfing is used in the algorithm because it yields the maximum rate of recombination breakdown and therefore is the least conservative with respect to the recombination effect. In treated hosts, the within-host haplotype frequencies become uneven due to the drug and so selfing rate is not $1/m$ as for untreated hosts, but is increased by a factor of $(1+2w_T^2)/(1+2w_T)^2$.

Predictions from the model

Predictions of the change in frequency of resistance over the generations are shown in Fig. 3 for the situation when drug resistance is controlled by one or 2 loci and when one or 2 drugs are used. The results for a set of conditions ($T=0{\cdot}1$, $t=1$, $w_U=1$, $c=3$, $M=1$ and $r=0{\cdot}5$) are shown in Fig.3A and the effects of changing any one of these parameters are shown in Fig. 3B–F. The following sections discuss the results.

General considerations

Frequency changes are very slow at low frequencies (not shown), thus giving a long 'lead-in' time (hundreds of generations) before drug selection brings a new mutant to a detectable frequency (ANDERSON & MAY, 1991; DYE & WILLIAMS, 1997). However, once the frequency reaches about 10%, the rate of increase is extremely rapid and, if drug pressure is uninterrupted, it can reach a frequency of 90% within 20 to 60 generations (approximately 2 to 5 years in areas of high transmission, depending on the average generation interval which is expected to decrease as transmission increases). Thus strategies to prevent a drug resistance problem increasing further should be implemented as soon as possible after the problem is detected.

Genetic mechanism of resistance

Two-locus mutants always increase at a slower rate than one-locus mutants because they are continuously broken down by recombination. Therefore it is better to use multiple drugs simultaneously rather than sequentially, as is well established (CURTIS & OTOO, 1986; DYE, 1991, 1994). A further result is that when only one drug is used but is under the control of 2 loci, if single mutants confer partial resistance (i.e. when two-locus resistance is partly additive), resistance increases faster than if both mutant alleles are required for any degree of resistance (i.e. when two-locus resistance is epistatic). DYE & WILLIAMS (1997) examined this in more detail, and noted that the effect of recombination in retarding multi-locus resistance (see below) is eliminated by additivity. This is relevant to chloroquine resistance in which probably several genes are required to confer resistance to a single drug (FOOTE *et al.*, 1990; WELLEMS *et al.*, 1991), but it is not clear whether these genes act in concert (epistatically) or independently and additively.

Stage 1. Set initial values

$$P = [p_{AB} \quad p_{Ab} \quad p_{aB} \quad p_{ab}]$$
$$C = \Sigma p_i$$

Stage 2. Natural selection within blood of hosts

$$P^* = [p_{AB} w_U^2 \quad p_{Ab} w_U \quad p_{aB} w_U \quad p_{ab}] / \Sigma$$

Stage 3. Drug selection

Untreated hosts	Treated hosts
$P^{*U} = P^*$	$P^{*T} = [p^*_{AB} \quad p^*_{Ab} w_T \quad p^*_{aB} w_T \quad 0] / \Sigma$

Stage 4. Meiosis in the mosquito

4a) Degree of selfing

$$S^U = \frac{1}{m} \qquad S^T = \frac{1 + 2 w_T^2}{(1 + 2 w_T)^2}$$

4b) Recombination breakdown

$$D^U = p^{*U}_{AB} p^{*U}_{ab} - p^{*U}_{Ab} p^{*U}_{aB} \qquad D^T = p^{*T}_{AB} p^{*T}_{ab} - p^{*T}_{Ab} p^{*T}_{aB}$$

$$D^{*U} = \frac{1}{2} D^U (1 + S^U) \qquad D^{*T} = \frac{1}{2} D^T (1 + S^T)$$

$$p^{*U}_A = p^{*U}_{AB} + p^{*U}_{Ab} \qquad p^{*T}_A = p^{*T}_{AB} + p^{*T}_{Ab}$$

$$p^{*U}_B = p^{*U}_{AB} + p^{*U}_{aB} \qquad p^{*T}_B = p^{*T}_{AB} + p^{*T}_{aB}$$

$$P^{**U} = [p^{*U}_A p^{*U}_B \quad p^{*U}_A p^{*U}_b \quad p^{*U}_a p^{*U}_B \quad p^{*U}_a p^{*U}_b] + [D^{*U} \quad -D^{*U} \quad -D^{*U} \quad D^{*U}]$$

$$P^{**T} = [p^{*T}_A p^{*T}_B \quad p^{*T}_A p^{*T}_b \quad p^{*T}_a p^{*T}_B \quad p^{*T}_a p^{*T}_b] + [D^{*T} \quad -D^{*T} \quad -D^{*T} \quad D^{*T}]$$

Stage 5. Transmission to new hosts

5a) Frequencies in total surviving parasite population

$$P^{***} = \frac{(1 - T) P^{**U} + TCtP^{**T}}{1 - T + TCt}$$

5b) Frequency of carriers in new hosts

$$C^* = 1 - \left[\frac{(1 - T)(1 - \Sigma p_i^{**U})}{1 - T + TCt} \right]^m$$

Stage 6. Re-start cycle

$$P = P^{***}$$
$$C = C^*$$

Fig. 2. Cyclic algorithm for predicting the change in gene frequencies each generation.

Multiple mechanisms of resistance are expected to evolve more easily than joint (interdependent) mechanisms, which strengthens the case for using 2 drugs simultaneously instead of one.

Drug pressure (T)

The most important influence on the rate of spread of resistance is the proportion of hosts treated. If 20% of the host population is treated with drugs (T=0·2), it will take about half as long, or 5, 7 and 13 generations less in the *1–1*, *2–1* and *2–2* cases respectively, for a mutant allele at a frequency of 10% to increase to 50% than if T=0·1 (Fig. 3B vs Fig. 3A). These differences are 10, 11 and 64 generations if the starting frequency is 0·1%. Nevertheless, with any significant level of drug pressure (i.e. T>0·1) the rate of spread is very rapid in all cases. This result is well known but is emphasized here because it dominates all further considerations. In many circumstances, however, drug treatment is essential to prevent mortality, in which case other options besides reducing drug pressure should be used.

Effect of drug on transmission (t)

The second major influence on the rate of spread is the degree of transmission from treated hosts carrying resistant parasites (Fig. 3C vs. Fig. 3A). If the drug does not reduce transmission, the surviving resistant parasites in effect replace susceptible parasites by being

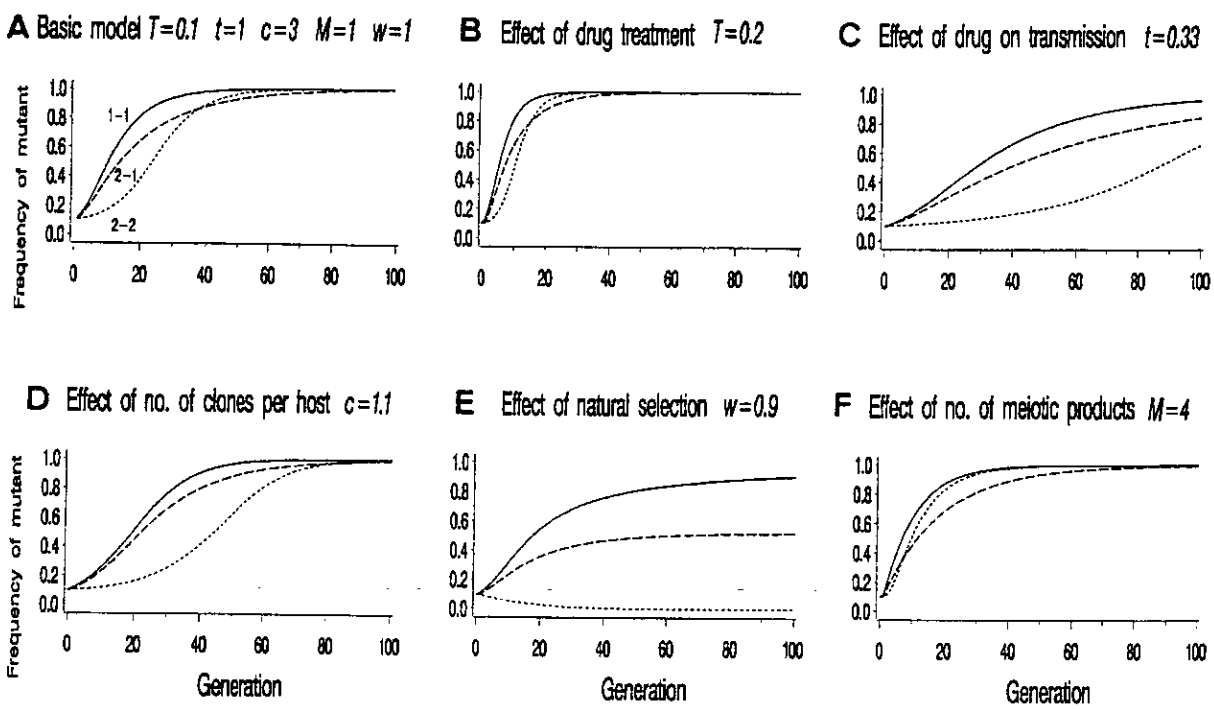

Fig. 3. Change in frequency of mutant alleles (*A* or *B*) over generations for the one-locus, one-drug (*1–1*), two-locus, two-drug (*2–2*) and two-locus, one-drug (*2–1*) cases. A. Parameter values are T=0·1, t=1, c=3, M=1, w_U=1, r=0·5. B–F. Same conditions as in A except for a change in value of one parameter, as shown.

transmitted to the next generation of hosts and thus gain a selective advantage. This advantage is even greater in high transmission areas because the number of copies transmitted is directly related to the average number of infections per host. When the drug does succeed in reducing transmission, however, the spread of resistance is dramatically curbed. This leads to the conclusion that drug life would be significantly prolonged if drug-treated carrier hosts were prevented from transmitting. This might be achieved through using drugs which are effective against transmission stages, or through simultaneous use of other control measures such as bed nets. The adage that 'sub-curative use of drugs generates resistance' is particularly pertinent here because there is some evidence to suggest that chloroquine, a drug which is in widespread use, when applied to resistant parasites or at sub-curative levels, does not impair, and may sometimes promote, transmission (RAMKARAN & PETERS, 1969; WILKINSON *et al.*, 1976; ICHIMORI *et al.*, 1990; HANDUNNETTI *et al.*, 1996; ROBERT *et al.*, 1996; BUCKLING *et al.*, 1997).

If true, overcoming this problem would be of considerable practical benefit in reducing the rate of spread of resistance.

Number of clones per host (c)

Fig. 3D shows that, if there are few haplotypes per host (m=c=1·1, vs. m=c=3 in Fig. 3A), as occurs at low transmission intensities, the rate of increase in the frequency of drug resistance mutants is slower than if there are more haplotypes per host. There are 2 reasons for this which would both contribute to greater effective selection in high transmission areas. The first is that there is greater opportunity for selection: when hosts carry more haplotypes, the chances of the host carrying a drug resistant mutant (i.e. being a carrier) is higher than if each host carries only one haplotype, which means that the mutant is exposed to the drug more often. This effect is important only when frequencies are not low— i.e. above 10%. The second effect is that there is a greater outcome of selection. As described above, if the drug does not impair transmission from a treated carrier host, the resistant mutant transmits more copies to the next host when prevailing transmission rates are high compared with when they are low. This has the effect of amplifying the selective advantage of the resistance genes and thus accelerating the spread of drug resistance in high transmission areas (HASTINGS, 1997). The third and opposite effect of high transmission rates is that there is more effective recombination when there are more clones per host, which retards the rate of spread of resistance when multiple genes are involved (CURTIS & OTOO, 1986; DYE, 1991, 1994; DYE & WILLIAMS, 1997; HASTINGS, 1997). This recombination effect is appreciable only when the number of clones per host is very low (c<1·5) and at low levels of drug pressure (T<0·2). In such cases, it would be detrimental to decrease the number of clones per host in the general population because high levels of inbreeding help to maintain resistance gene combinations. Reducing transmission from individual drug-treated hosts is still beneficial, however: it is only the overall population transmission rate which is relevant to the recombination effect. The issue of recombination versus selection is discussed in more detail in a later section.

Cost of resistance(w_U)

Fig. 3E shows that if, in the absence of drugs, the parasite pays a cost in fitness for harbouring resistance genes, this will retard the spread of resistance. In the case of 2 loci where the cost of each allele is 10%, so that the relative fitness of the double mutant is 81%, this is sufficient to counteract the force of 10% drug pressure in the two-drug cases and to limit the rate of spread in the one-drug case. If 10% of the host population are treated with drugs, reductions in fitness of more than 10%, 6% and 25% for the *1–1*, *2–2* and *2–2* cases respectively are sufficient to prevent the further spread of drug resistance by causing a slow decline in the frequency of the mutants. However, for 20% drug treatment, the mutant would have to be 25%, 18% and 60% less fit to counteract drug selection. In other words, drug selection is far more potent than natural selection. There is no good evidence from the field that

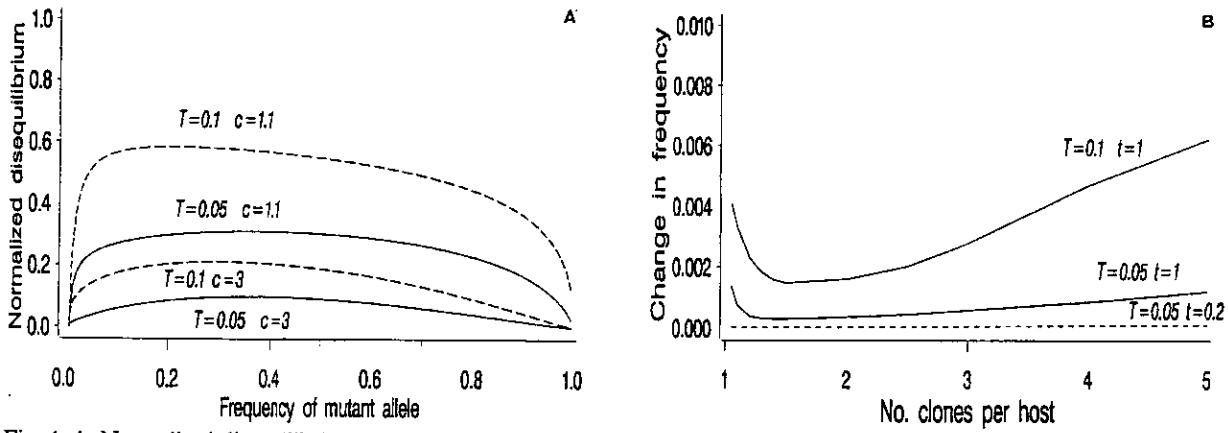

Fig. 4. A. Normalized disequilibrium as a function of mutant allele frequency in the two-locus, two-drug case for 2 levels of drug pressure (T=0·05 and T=0·1) and 2 transmission levels (number of clones per host, c=1·1 or 3). B. Change in frequency of the double mutant haplotype (AB) in the generation when the frequency of both resistance alleles has reached 0·1 for 2 levels of drug pressure (T=0·05 and T=0·1) and when the drug reduces transmission rate from treated carrier hosts to 20% of that from untreated hosts (t=0·2).

there is strong natural selection against any of the drug resistant mutants, and in the laboratory the stability of resistance and persistence of mutants is well established (ROSARIO, 1976; ROSARIO *et al.*, 1978). Further information is required on the fitness costs of drug resistance in order to establish whether complete withdrawal of a drug from a region will result in a decline in the frequency of resistance and ultimately to renewed effectiveness, as seems to have occurred with some anticoccidial drugs used in poultry (CHAPMAN, 1993).

Number of meiotic products per inoculum

Fig. 3F shows the effect of the number of distinct two-locus meiotic products (M=4 cf. M=1 in Fig. 3A) among the sporozoites which successfully infect a new host per transmission event. If many sporozoites successfully infect a new host, so that the probability of getting all 4 meiotic products (from an outbred mating) is high, then the frequency of carriers, and hence the effective selection pressure, is increased. Thus, decreasing the number of sporozoites per mosquito or their establishment in the liver through use of transmission-blocking or liver stage vaccines would help to retard the spread of resistance. Even though this is a relatively unimportant effect, it is, nevertheless, a key parameter for more general population genetics models of malaria parasite gene flow. The lack of data on this question calls for further study.

When does recombination outweigh selection?

It has been postulated that, in high transmission areas, the associated greater amounts of outcrossing will slow the spread of drug resistance compared with low transmission areas (PAUL *et al.*, 1995). This depends on whether the increase in effective recombination more than counteracts the concomitant increase in effective selection. In this section, the conditions under which recombination outweighs selection are described — i.e. when drug resistance spread is promoted by inbreeding. This is done by solving the equation which predicts the change in frequency from one generation to the next:

$$\Delta p_{AB} = p^{***}_{AB} - p^{U}_{AB} = \frac{p^{**U}_{AB}(1-T)+TCt}{1-T+TCt} - p^{U}_{AB} \qquad (1)$$

$$= \frac{(\frac{1}{2}D(1+S)+p_A p_B)(1-T)+TCt}{1-T+TCt} - D - p_A p_B$$

When this is positive drug resistance will be spread: i.e., when

$$\frac{TCt}{1-T}(1-D-p_A p_B) \geq \frac{1}{2}D(1-S) \qquad (2)$$

The left side of equation (2) represents the force of effective selection: this is strongest when drug pressure (T), transmission following drug treatment (t) and the frequency of carriers (C) are high, and when the frequency of the resistance alleles (p_A and p_B) and disequilibrium (D) are low. The right hand side represents effective recombination: it is weakest (i.e. most easily overcome by drug selection) when the selfing rate (inbreeding) is high, which occurs in low transmission areas. However, the important point is that recombination is relevant only when there is some positive disequilibrium. Disequilibrium levels are maintained by a balance between the positive force of selection for the double mutant by drugs and the negative force of recombination breakdown through outcrossing (DYE & WILLIAMS, 1997). Thus the trade-off between effective selection and effective recombination involves disequilibrium. Therefore, in order to predict whether drug resistance will spread, we need to quantify the amount of disequilibrium generated through the selection process. In Fig. 4A the disequilibrium levels generated as the mutant alleles increase in frequency from 0·1 to 1 under drug pressures of T=0·05 and T=0·1 for low transmission (c=1·1) and high transmission (c=3) environments are shown for the two-drug, two-locus case: these were calculated using the algorithm in Fig. 2. The results are expressed in terms of normalized disequilibrium, d, which is disequilibrium as a proportion of its maximum value and is calculated as $D=dp_A(1-p_B)$. It can be seen that higher amounts of disequilibrium are caused by higher drug pressures and fewer clones per host, but these are well below the maximum possible disequilibrium (d=1). This leads to the conclusion that the recombination effect is important only when there are few clones per host: this is illustrated in Fig. 4B where the change in frequency of the double mutant (Δp_{AB}) in the generation immediately after p_{AB} has reached a value of 0·1 is shown for drug pressures of T=0·05 and T=0·10. There is a sharp decline in the rate of change between c=1·05 and c=1·5, after which the rate of change increases with transmission rate. Fig. 4B also shows that this recombination effect is more easily overcome when drug pressure becomes stronger, and virtually disappears when the drug reduces transmission (i.e., t decreases).

In summary, we have shown that the conditions under which decreasing the average number of clones per host promotes double drug resistance are when c<1·5, when drug treatment rates are below 20%, and when the frequency of both mutants, each of which confers no significant protection on its own, is low. These conclusions concur with those of DYE & WILLIAMS (1997), even though we used a quite different model. They are

only slightly quantitatively different from the conclusion of HASTINGS (1997), who assumed that disequilibrium would always be maximal, a condition which results in over-estimation of the recombination effect.

Discussion

Building population structure into models to predict the rate of increase in drug resistance does not alter the following 2 well-established principles regarding the spread of resistance: (i) drug resistance will relentlessly increase to the point of drug treatment failure in the majority of the host population for as long as there is any significant drug use in areas of endemic malaria, and (ii) the use of 2 drugs simultaneously will slow the rate of spread, especially when the mutant alleles are both rare. A more novel conclusion from this study, however, and one which has been the subject of some debate (PAUL *et al.*, 1995; DYE & WILLIAMS, 1997; HASTINGS, 1997; MACKINNON, 1997), is that, in the majority of cases, high transmission rates promote the spread of drug resistance rather than hinder it. This is because selection is more effective in high transmission areas where resistance genes under drug selection are transmitted to the next host in greater copy number than in low transmission areas. This generates 2 new recommendations. The first is that transmission should be minimized from individuals who are treated with drugs. This would be especially effective in areas of high transmission intensity, though it is helpful in all environments. In the short term, this could be achieved through the use of bed nets by drug-treated patients, proper administration of drugs (i.e., avoiding subclinical doses), or, in the longer term, developing drugs which are effective against transmission stages. The second recommendation is that if strategies for managing drug resistance are implemented, these should be accompanied by simultaneous efforts to reduce the overall transmission rate in the population. The exception to this is managing multi-locus resistance in areas where the average number of infections per host is very low (<1·5), drug pressure is also low, and the mutant genes are rare (DYE & WILLIAMS, 1997; I. M. Hastings & M. Mackinnon, paper submitted for publication). Neither of these recommendations is easy to realize: malaria parasites seem to be particularly adept at increasing their transmission output when faced with difficult conditions in the host such as toxic drugs or immunity (CARTER & GRAVES 1988; ALANO & CARTER, 1990; SINDEN *et al.*, 1996; BUCKLING *et al.*, 1997). This is why a better understanding of how the parasite adapts genetically and facultatively in response to host-imposed selection pressures is critical for devising better control strategies. As shown here, population structure can have a profound effect on whether the parasite succeeds in adapting to these control measures and so is an integral part of this understanding.

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Short Report

Quinine sensitivity of isolates of *Plasmodium falciparum* from the coast of Kenya

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Keywords: malaria, *Plasmodium falciparum*, quinine drug-sensitivity, Kenya

Quinine (QN) remains an important drug for the treatment of severe falciparum malaria in Africa. QN resistance in Africa has been reported (JELINEK *et al.*, 1995) but is still uncommon and the drug remains a reliable treatment. However it is important to monitor QN sensitivity to document the extent and distribution of resistance (PASVOL *et al.*, 1992; JELINEK *et al.*, 1995). We wanted to compare the present QN sensitivity of wild isolates of *Plasmodium falciparum* with data obtained by the Kilifi Unit of the Kenya Medical Research Institute in 1989. We decided to modify the World Health Organization microtest (WERNSDORFER & PAYNE, 1988). In the standard test parasites are incubated in a dilution of the patient's blood; however, because the concentration of plasma varies between subjects, the free fraction of QN could be expected to vary between tests (WINSTANLEY *et al.*, 1993, 1994). Given that it is the free (unbound) fraction of the drug which is responsible for its effects (ROWLAND & TOZER, 1989), variability could be expected in the results. We overcame this problem by removing the patient's plasma (after centrifugation) and replacing it with RPMI-1640 medium containing standard serum which was used throughout the experiment.

Patient samples with parasitaemia of over 100 parasites/100 white blood cells (WBC) were examined. Blood samples were centrifuged (3500 *g* for 5 min) and the plasma removed. The pellet was then washed with RPMI-1640 and resuspended in complete medium (RPMI-1640 supplemented with 10% human AB serum) at 5% haematocrit. Fifty mL of the resuspended sample were then placed in the wells of a microtitration plate and incubated in a candle jar at 37°C and high humidity. After 24 h incubation, an aliquot was taken and stained with 10% Giemsa solution. If parasites were still immature, slides were made every 6 h for 48 h to monitor their progress. When the parasites had matured (over 4% of schizonts in the control well) the plate was assayed. Slides were made and stained as described and the number of schizonts was counted. The percentage inhibition of schizont maturation (compared to the control well) was plotted against log QN concentration using a probit scale; 99% and 50% inhibitory concentrations (IC_{99} and IC_{50}) were determined by regression analysis.

Thirty-seven of 60 isolates were successfully examined (62%) (Figure). There were 2 main reasons for assay failure.

(i) Reinvasion by merozoites from schizonts rupturing during the 6 h observation time even when the proportion of schizonts was less than 4%. This occurred in 4 assays which were rejected from the assessment.

(ii) Parasite death (in 19 assays). Parasites were classed as dead if the schizonts had not ruptured after 48 h. Parasite death was significantly increased at high parasitaemias. The reason for this trend was thought to be the increasing concentration of metabolic products of the parasites and poor nutrition.

The mean values (± SD) were: IC_{99}, 3·1 ± 2·6 μM (1·01±0·84 mg/L) and IC_{50}, 0·5±0·26 μM (0·16±0·084 mg/L).

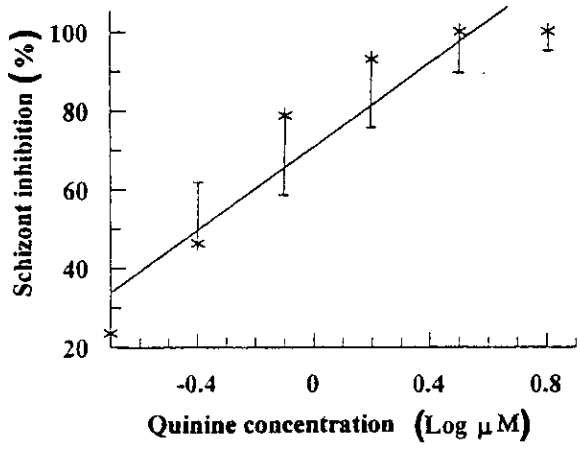

Figure. Regression line resulting from probit analysis of the log dose response to quinine derived from the sensitivity assay of *Plasmodium falciparum*; the points indicate means plus or minus one standard deviation.

No isolate was resistant to QN, and sensitivity had not changed significantly in this area since 1989, when the IC_{99} and IC_{50} values were 2·72 and 0·364 μM, respectively (PASVOL *et al.*, 1992). QN remains the treatment of choice for severe malaria in coastal Kenya but continuous surveillance for the emergence of resistance should be maintained.

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MOLECULAR AND BIOCHEMICAL PARASITOLOGY

A chloroquine resistance locus in the rodent malaria parasite *Plasmodium chabaudi*

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Abstract

We have located a possible chloroquine resistance locus in the genome of the rodent malaria parasite *Plasmodium chabaudi*. Two genetically distinct clones of the parasite were grown in vivo and allowed to undergo genetic crossing. The clones differed from each other in their susceptibility to chloroquine; AS(3CQ) had been selected for a low level of resistance to the drug whereas AJ is chloroquine-sensitive. Independent recombinant progeny (20) were cloned from the products of two crosses, phenotyped for their susceptibility to chloroquine, and genotyped for their inheritance of 46 chromosome-specific markers. No association was found between chloroquine susceptibility and the inheritance of *pcmdr1*, the *P. chabaudi* homologue of the *pfmdr1* multi-drug resistance gene of *P. falciparum*. Also, there was no association between chloroquine susceptibility and the inheritance of a marker linked to a putative chloroquine resistance locus in a *P. falciparum* cross. However, 16 of the progeny clones showed co-segregation of four linked markers on chromosome 11 with their resistance phenotype. This result suggests that a locus for chloroquine resistance exists on this chromosome in *P. chabaudi*.

Keywords: Chloroquine; *Plasmodium chabaudi*; Resistance

1. Introduction

Chloroquine resistance in the human malaria parasite *Plasmodium falciparum* arose first in South America and South-east Asia [1,2]. It has now spread to all parts of the world where malaria is endemic and poses a major threat to the elimination of the disease. The genetic basis of this type of resistance is not fully understood, but it seems likely that more than one locus is in-

Abbreviations: PCR, polymerase chain reaction; PFGE, pulsed-field gradient gel electrophoresis; RFLP, restriction fragment length polymorphism.

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volved [3]. Identification of the genes involved is of obvious importance if we are to understand how such resistance arises, and should lead to the development of molecular probes for the diagnosis of resistant parasites in natural infections [4].

In recent years, much attention has been paid to the multi-drug resistance genes *pfmdr1* [5] and *pfmdr2* [6], which code for homologues of the P-glycoprotein implicated in multi-drug resistance in mammalian tumour cells [7]. Similarities were noted between the rapid efflux phenotype shown by chloroquine-resistant *P. falciparum* clones [8], and the multi-drug resistance phenotype seen in the cancer cells, and it was proposed that *pfmdr1* and *pfmdr2* might play a role in chloroquine resistance. Early work claimed that amplification [5,6] and mutations [9] of the *pfmdr1* gene were correlated with chloroquine resistance, but more recently studies on field isolates have given equivocal results [10–13]. A genetic cross between a chloroquine-resistant clone Dd2 and a sensitive clone HB3 showed clearly that neither *pfmdr1* nor *pfmdr2* was the cause of resistance in Dd2, since polymorphisms of these genes did not segregate with the resistance phenotype among the 16 cross progeny [14]. Instead, linkage analysis showed that a locus in a 36 kb segment of chromosome 7 was involved [15]. Several genes have been cloned from this region, including the gene coding for a putative heat shock protein gene *pfhsp86* [16]. More recently, a gene encoding a protein of unknown function denoted *cg2* has been described [17], which not only segregates with the resistance phenotype in the cross, but is also found in naturally occurring chloroquine-resistant isolates of *P. falciparum*.

Genetic studies on *P. falciparum* using classical crossing experiments are difficult because of the problems of maintaining the whole life-cycle in the laboratory. Such work is easier with the rodent malaria species *P. chabaudi*. This species is also a good model for *P. falciparum* because of similarities in their basic biology, such as their preferential invasion of mature erythrocytes, the synchronicity of their asexual blood forms, sequestration of schizont-infected erythrocytes, and gametocyte development late in the infection. Selection of drug-resistant/isogenic mutants of *P. chabaudi* is a more straightforward procedure than in *P. falciparum*, because in vitro culture is not necessary. In early studies, mutants exhibiting low and high-level resistance to chloroquine were produced by exposing sensitive forms to gradually increasing doses of the drug [18,19]. Evidence was obtained that mutant genes at more than one locus additively produced the phenotype of high resistance [19]. This was shown when a parasite clone exhibiting a high level of resistance was crossed with a sensitive parasite, and progeny clones with intermediate levels of resistance were isolated. This result could be explained most satisfactorily by recombination between genes at different loci, each conferring a low degree of resistance. However, the genes involved were not identified at that time.

In this study, we determine the chromosomal location of a gene determining low-level chloroquine resistance in a *P. chabaudi* chloroquine-resistant clone. We show that when this clone is crossed with a drug-sensitive clone, a gene on chromosome 11 segregates with the resistance phenotype in the progeny. This locus is unlinked to the homologue of the *P. falciparum* heat-shock protein gene *pfhsp86*, which co-segregates with a chloroquine resistance locus in that species. Moreover, it is also unlinked to the *P. chabaudi* multi-drug resistance gene *pcmdr1*.

2. Materials and methods

2.1. Parasite lines and maintenance

Two *P. chabaudi* clones denoted AJ and AS(3CQ) were used in this work. Clone AS(3CQ) had previously been selected for resistance to pyrimethamine, and subsequently for a low level of resistance to chloroquine by multiple-step selection [18]. The clones were routinely passaged in outbred laboratory mice as described previously [20]. CBA/Ca male mice, 6–8 weeks old, were used for chloroquine susceptibility tests and for sporozoite-induced infections from mosquitoes. Newly weaned, splenectomised, Wistar rats were used to induce parasite gametocytogenesis [21]. Parasites were transmitted through *Anopheles stephensi* mosquitoes, and maintained as described [20].

2.2. Genetic crossing procedure

Two crosses were made between AS(3CQ) and AJ, the crossing procedure following that of Walliker et al. [22], with minor modifications. Briefly, blood forms of each clone were inoculated separately into mice and allowed to reach a parasitaemia of 20–30%. Equal volumes of blood (0.5 ml) of each clone were then mixed and injected intraperitoneally into a splenectomised rat for production of gametocytes [21]. Inoculations (1 ml) of each clone were injected into other splenectomised rats as controls. Three hundred mosquitoes were fed on each rat 4 days later, when mature gametocytes were present, in order to allow gametes of the mixed clones to undergo cross-fertilisation. Samples of mosquitoes from each cage were examined for oocysts after 10 days; oocysts were found in all cages, the numbers varying between one and ten per midgut. The remaining mosquitoes were allowed to feed on two groups of mice on days 16 and 18 respectively after the original feed. The blood forms which developed in these animals from the mosquitoes which had received mixed gametocytes were termed the progeny of the cross, and were then stored in capillaries under liquid nitrogen. Blood forms from the control animals were treated similarly. For subsequent work, capillaries were thawed and the contents injected intraperitoneally into mice.

To test whether crossing had occurred, the uncloned progeny were first treated with pyrimethamine, and the treated progeny examined for electrophoretic forms of the enzymes lactate dehydrogenase and adenosine deaminase which distinguished each parent clone [22,23]. Clones were then established from the progeny by limiting dilution [22] and examined for genome markers and chloroquine response (see below).

2.3. Test for chloroquine response

Progeny clones were tested for their susceptibility to chloroquine, following the method of Padua [19] but with the following modifications. Infections were established by inoculating 10^6 parasitised blood cells intraperitoneally into groups of five mice. Dilutions of chloroquine sulphate (Nivaquine, May and Baker) were made in distilled water and administered orally to three of the mice at a dose of 3 mg kg^{-1} mouse body weight, 3 h after initiating the infection and at the same time each day for the following 7 days. Blood smears of the two undrugged mice were taken on days 4, 5 or 6 post-inoculation and of drugged mice on days 11, 13 and 15, and parasitaemias counted microscopically. Each clone was tested for its chloroquine response in at least two experimental trials by measuring the parasitaemias reached on days 11, 13 and 15 following chloroquine treatment. AJ and AS(3CQ) parent clones were tested for their chloroquine response on each occasion that progeny clones were examined.

The progeny clones were classified as chloroquine sensitive or resistant relative to their parents by comparing the mean log parasitaemias of each against the corresponding mean for the AS(3CQ) and AJ parents on days 11, 13 and 15, and testing for significant differences between them using a *t*-test. To do this, a model with fixed effects for clone (with levels for AS(3CQ), AJ and the progeny clone) and experimental trial was fitted to the data using least squares techniques.

2.4. Genomic DNA extraction, digestion and Southern hybridisations

Parasites were extracted from host cells by saponin lysis, and DNA was prepared using modifications of the method of Snounou et al. [24]. The DNA was digested with restriction enzymes, fractionated by agarose gel electrophoresis and blotted onto Hybond N+ (Amersham) as recommended by the manufacturer. Plasmid probes and bacteriophage inserts were radiolabelled by random hexamer priming (Boehringer Mannheim High Prime Radiolabelling Kit), and hybridisation and washing were carried out using standard procedures [25].

2.5. Genome markers

Genetic loci exhibiting polymorphisms between AS(3CQ) and AJ were obtained by using three types of DNA marker: (1) known *Plasmodium*

sequences cloned into plasmids or bacteriophage which cross hybridise to *P. chabaudi*; (2) anonymous markers produced through the RAPD-PCR technique (random amplified polymorphic DNA-polymerase chain reaction) of *P. chabaudi* DNA [26]; (3) anonymous probes from a *Sau* 3A *P. chabaudi* genomic DNA library, constructed in pBluescript II (Stratagene) using common procedures [27].

Restriction fragment length polymorphisms (RFLPs) of each marker were identified by hybridising ^{32}P-labelled probes to Southern blots of restricted genomic DNA. RFLP markers were assigned to individual chromosomes by hybridisation to blots of chromosomes separated by pulsed-field gradient gel electrophoresis (PFGE) [28] using a CHEF DR II (BioRad) apparatus. Run conditions were as detailed in [26].

2.6. *P. chabaudi mdr1 gene*

A section of the *P. chabaudi* homologue of *pfmdr1*, called *pcmdr1*, was cloned and sequenced as follows. Briefly, degenerate PCR primers (858S: 5'-GGG GCA TTC GGT/A GAG/A T/ACT/A GGA/T T/AGT GGG/A AAA TC; and 857S: 5'-GGG GCA TGC CCA/T A/GA/G/TG AA/TG AT/GG TA/GG CT/CT C) recognising the nucleotide binding sites of the *P. falciparum pfmdr1* gene [5] were used to amplify *P. chabaudi* DNA during 35 cycles of 94°C 30 s^{-1}, 45°C 60 s^{-1}, 70°C 90 s^{-1}. After cloning into the vector pCRII using the TA Cloning System (Invitrogen) following the manufacturer's instructions, the fragment was sequenced using the dideoxynucleotide chain-termination method [25] and found to show 87% homology at the amino acid level to the carboxyl ATP-binding cassette of *pfmdr1* [30]. *pcmdr1* is on chromosome 12 [29].

2.7. *Linkage analysis*

Linkage was assessed by comparing the inheritance pattern of each RFLP marker with inheritance of the chloroquine phenotype, where phenotype was determined as described in the Test for chloroquine response section above. A convenient measure of linkage is the linkage ratio [15], a ratio of the number of progeny showing linkage of chloroquine susceptibility with a marker to the total number of progeny. Markers exhibiting a ratio of 16/20 or greater were examined further by linkage analysis using the computer programme CRI-MAP [31]. This programme allows construction of the most likely linkage map using maximum likelihood methodology. Significance tests were based on the LOD score (the logarithm of the ratio of the likelihood under the hypothesis being tested, to the likelihood under the null hypothesis) under the standard assumption that twice the natural logarithm of the likelihood ratio is distributed as a χ^2 variable [32].

A second linkage analysis was carried out using the quantitative data on parasitaemia (as opposed to the qualitative chloroquine suscepibility data used above), by estimating the mean difference in log parasitaemias on each of days 11, 13 and 15 between the two groups of progeny clones inheriting alternative parental alleles of each of the 46 markers. To do this, a mixed model with fixed effects for experimental trial and marker allele, a covariate for the mean parasitaemia in the control mice on day 4, 5 or 6 (in order to adjust for any differences between the clones in their early growth rate), and a random effect for clone was fitted by maximum likelihood methods. Marker contrasts were tested for significance against the between-clone variance.

Further analysis was done on the parasitaemia data from days 4 to 6 of the control mice to see whether any of the markers were linked to a gene controlling early parasite growth rate. For this, the same method as that described above for the chloroquine resistance analysis was used, except that the covariate was omitted from the model.

3. Results

3.1. *Identification of genetic markers*

More than 100 DNA markers were tested for RFLP polymorphisms between parent clones AJ and AS(3CQ). Forty six detected useful RFLPs, the majority of these being single copy. Thirty two

of the markers were known genes from other *Plasmodium* species [29], five were anonymous markers obtained by the RAPD-PCR technique [26] and the remainder were anonymous markers isolated from the *Sau* 3A *P. chabaudi* genomic DNA library. A list of the markers is provided in Table 1.

3.2. Identification of recombinant cross progeny

The uncloned, pyrimethamine-treated progeny were shown to contain recombinant parasite clones by the presence of both AJ and AS(3CQ) forms of the enzymes LDH and ADA (data not shown).

In total, 59 clones were obtained by cloning from the progeny of both crosses, 46 from cross I and 13 from cross II. Initially the clones were examined for inheritance of 18 RFLP markers each, in order to exclude genotypically identical progeny and parental types (data not shown). Twenty of the original 59 clones were found to be independent recombinants, 13 from cross I and seven from cross II, and these were used in further linkage analysis studies.

3.3. Chloroquine susceptibility tests.

In the absence of chloroquine treatment, the progeny clones grew rapidly in CBA/Ca mice. Following inoculation of 10^6 blood forms, a parasitaemia of 40% or higher was attained by day 5 (Table 2). Peak parasitaemias were attained around days 6–7, after which a rapid decline in asexual parasite numbers occurred, although gametocytes were normally present until day 18 or later. This course of infection was seen in both AS(3CQ) and AJ, and in all the 20 progeny clones examined here (data not shown), although AS(3CQ) consistently grew a little more slowly than AJ by day 5, and attained a slightly lower peak parasitaemia.

The growth patterns of each progeny clone treated with chloroquine were compared with those of each parent (Table 2). Seven were very similar to AS(3CQ) and were classified as chloroquine-resistant (R), and a further eight were similar to AJ and denoted chloroquine-sensitive (S). The remaining five clones (38/9, 103/6, 63/3, 43/8 and 26/7) showed some variation in their growth patterns following chloroquine treatment; in general, they grew more slowly than AS(3CQ) and attained a lower final level of parasitaemia. These clones were classified as low level chloroquine resistant (LR) clones.

3.4. Linkage analysis

The inheritance patterns of the 46 chromosomal markers and chloroquine response among the 20 recombinant progeny clones are shown in Fig. 1. The strongest linkage of the chloroquine resistance phenotype was to markers on chromosome 11 (Fig. 1(b)), which showed a linkage ratio of 15/19 or greater. For this analysis, R and LR clones were pooled. Markers on all other chromosomes showed a random distribution indicative of normal linkage equilibrium (Fig. 1(a)). These included *pcmdr1*, which produced a linkage ratio of 9/19 (Fig. 1, chromosome 12), and the locus which cross-hybridised to the *P. falciparum* chromosome 7 gene *pfhsp86*, with a linkage ratio of 10/20 (Fig. 1, chromosome 8); these ratios do not differ from those which unlinked markers with an equal chance of being inherited from either parent would be expected to show among the offspring.

With regard to chromosome 11, four tightly linked markers, CRK2, EF-1α, PCNA and OPL-04 showed strongest co-segregation with chloroquine resistance, with linkage ratios of 15/19 or greater. In order to test for linkage statistically, an ordered map of the markers on this chromosome was first constructed using CRIMAP. The best-fit map is shown in Fig. 2(a). This fit was significantly better than the next best-fit map (LOD = 1.90, $P < 0.003$ based on a χ^2 with 1 d.f). The putative chloroquine resistance locus was then incorporated into the map to determine its most likely location (Fig. 2(b)). Two possible positions were found, the most likely being midway between H2A and the CRK2-EF-1α-PCNA-OPL-04 group, and the next most likely (LOD = 0.34, $P = 0.21$) being distal to this group (not shown). These placements were significantly more likely than the possibility that the chloroquine resistance locus was unlinked to any

Table 1
Name and reference of chromosome markers used in this study

No.	Name	Reference/source
1	*P. chabaudi* Ag3020 (uncharacterised antigen gene)	[48]
2	*P yoelii* Ca^{2+} ATPase (calcium ion ATPase gene)	[49]
3	*P. chabaudi* pBS 110 (schizont-specific gene)	W. Deleersnijder, Institute for Molecular Biology, Brussels, Belgium
4	*P. chabaudi* Ag3003A (uncharacterised antigen gene)	[48]
5	P.1 (anonymous)	*P. chabaudi Sau*3A genomic library
6	P.9 (anonymous)	*P. chabaudi Sau*3A genomic library
7	*P. falciparum* αII tubulin gene	[50,51]
8	*P. chabaudi* Ag3035 (uncharacterised antigen gene)	[48]
9	*P. chabaudi* OPL-16	[26]
10	*P. falciparum* DNA pol δ (DNA polymerase δ gene)	[52]
11	P.29 anonymous	*P. chabaudi Sau*3A genomic library
12	*P. chabaudi* Ag3024 (uncharacterised antigen gene)	[48]
13	*P. berghei* 9.2 (anonymous, located on two chromosomes)	[53]
14	P.12 (anonymous)	*P. chabaudi Sau*3A genomic library
15	*P. falciparum* DNA pol α (DNA polymerase α gene)	[52]
16	*P. chabaudi* RESA (ring-infected erythocyte surface antigen gene)	[23]
17	*P. chabaudi* DHFR (dihydrofolate reductase gene)	[54]
18	P.23 (anonymous)	*P. chabaudi Sau*3A genomic library
19	*P. chabaudi* MSP-1 (major surface protein gene)	[55]
20	*P. falciparum* hsp86 (heat shock protein gene)	[16]
21	*P. chabaudi* Ag3027 (uncharacterised antigen gene)	[48]
22	*P. chabaudi* AMA-1(apical membrane antigen-1 gene)	[56]
23	*P. falciparum* ran (GTPase gene)	[57]
24	*P. falciparum* 5S rRNA gene	[58,59]
25	*P. chabaudi* cDNA 121 (uncharacterised antigen gene)	[60,61]
26	*P. falciparum* VAP B (vacuolar ATPase gene)	[62]
27	*P. chabaudi* OPR-14	[26]
28	*P. chabaudi* mdr1 (multi-drug resistant gene)	[30]
29	*P. berghei* aldo-1 (aldolase 1 gene)	[63]
30	*P. chabaudi* Ag3042B/EDGA (exported dense-granule antigen gene)	[48]
31	*P. chabaudi* pcpS590.7	[30]
32	*P. falciparum* RNA pol III (RNA polymerase III gene)	[64]
33	*P. chabaudi* OPL-12	[26]
34	*P. chabaudi* cDNA 148 (37 kDa antigen gene)	[59,60]
35	*P. falciparum* G6PD (glucose 6 phosphate dehydrogenase gene)	[65]
36	*P. chabaudi* OPR-02	[26]
37	*P. chabaudi* Ag3040 (uncharacterised antigen gene)	[48]
38	*P. berghei* CRK2 (yeast CDC-2 homologue)	[66]
39	*P. falciparum* EF-1α (elongation factor 1 gene)	D. Williamson, London
40	*P. falciparum* PCNA (proliferating cell nuclear antigen gene)	[67]
41	*P. chabaudi* OPL-04	[26]
42	*P. falciparum* H2A (histone 2A gene)	[68]
43	P.22 (anonymous)	*P. chabaudi Sau* 3A genomic library
44	*P. falciparum* TBP (TATA binding protein gene)	[69]
45	*P. falciparum* Topo I (topoisomerase I gene)	[70]

Table 2
Chloroquine resistance phenotype of 20 progeny clones from two AS(3CQ)×AJ crosses, as measured by log parasitaemia on days 11, 13 and 15 post-inoculation following administration of chloroquine on days 0–7

Clone	Day 5 control	Day 11 drugged	cf AJ	cf AS	Day 13 drugged	cf AJ	cf AS	Day 15 drugged	cf AJ	cf AS	Phenotype
AJ	−1.14 (0.8)	−4.90 (0.11)			−4.52 (0.11)			−4.80 (0.14)			S
AS	−1.55 (0.8)	−3.15 (0.11)			−2.17 (0.12)			−1.99 (0.14)			R
38/9	−1.7 (0.18)	−4.14 (0.34)	*	*	−3.49 (0.35)	*	***	−2.47 (0.32)	***	n.s	LR
39/2	−1.52 (0.15)	−3.58 (0.29)	***	n.s	−2.74 (0.30)	***	n.s	−2.47 (0.33)	***	n.s	R
72/2	−1.59 (0.15)	−3.47 (0.29)	***	n.s	−2.89 (0.30)	***	*	−2.55 (0.29)	***	n.s	R
103/6	−1.52 (0.15)	−3.24 (0.29)	**	*	−3.04 (0.30)	***	**	−2.65 (0.29)	***	*	LR
39/1	−1.45 (0.15)	−3.38 (0.30)	***	n.s	−2.54 (0.31)	***	n.s	−2.74 (0.29)	***	*	R
291/9	−1.29 (0.20)	−2.65 (0.36)	***	n.s	−2.73 (0.39)	***	n.d	n.d	n.d	n.d	R
253/5	−1.55 (0.15)	−3.45 (0.30)	***	n.s	−2.59 (0.31)	***	n.s	−1.20 (0.36)	***	n.s	R
238/7	−1.34 (0.17)	−2.58 (0.30)	***	n.s	−2.69 (0.31)	***	n.s	−4.79 (0.43)	n.s	***	R
185/9	−1.47 (0.17)	−3.32 (0.30)	***	n.s	−3.39 (0.31)	***	***	−5.22 (0.43)	n.s	***	R
63/3	−1.37 (0.15)	−3.86 (0.30)	**	*	−3.69 (0.31)	*	***	−3.55 (0.31)	***	***	LR
266/6	−1.69 (0.14)	−4.84 (0.24)	n.s	***	−4.19 (0.25)	n.s	***	−4.12 (0.43)	n.s	***	S
43/8	−1.42 (0.15)	−4.36 (0.30)	n.s	***	−3.69 (0.31)	*	***	−3.74 (0.29)	***	***	LR
39/3	−1.37 (0.15)	−4.57 (0.29)	n.s	***	−4.54 (0.30)	n.s	***	−4.85 (0.29)	n.s	***	S
62/8	−1.32 (0.18)	−4.52 (0.34)	n.s	***	−4.69 (0.35)	n.s	***	−5.00 (0.32)	n.s	***	S
62/3	−1.60 (0.15)	−4.71 (0.30)	n.s	***	−4.74 (0.34)	n.s	***	−4.99 (0.31)	n.s	***	S
131/1	−1.45 (0.15)	−4.92 (0.30)	n.s	***	−4.77 (0.31)	n.s	***	−4.73 (0.29)	n.s	***	S
115/9	−1.58 (0.16)	−4.92 (0.30)	n.s	***	−4.77 (0.31)	n.s	***	−3.85 (0.29)	**	***	S
225/1	−1.61 (0.17)	−5.40 (0.30)	n.s	***	−4.44 (0.31)	n.s	***	−4.79 (0.43)	n.s	***	S
266/2	−1.39 (0.17)	−5.83 (0.30)	*	***	−4.00 (0.31)	n.s	***	−2.36 (0.43)	***	n.s	S
267/7	−1.48 (0.17)	−5.83 (0.30)	*	***	−5.30 (0.31)	*	***	−2.82 (0.43)	***	n.s	LR

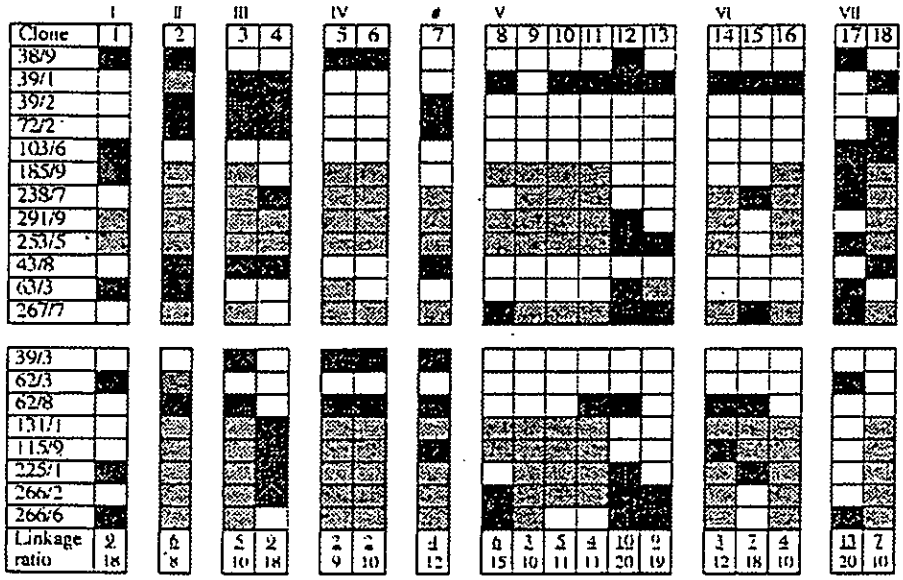
Figures are means of log parasitaemias, from two separate trials, and figures in brackets are standard errors. Columns headed cf AJ and cf AS show whether each clone is significantly different from the parent chloroquine-sensitive AJ and chloroquine-resistant AS(3CQ) clones respectively. R, resistant; S, sensitive; LR, low level resistance.

* $P<0.05$; n.s, not significant, $P>0.05$; n.d, not determined.

** $P<0.01$.

*** $P<0.001$.

(a) Chromosomes I-VII



Chromosomes VIII-X, XII-XIV

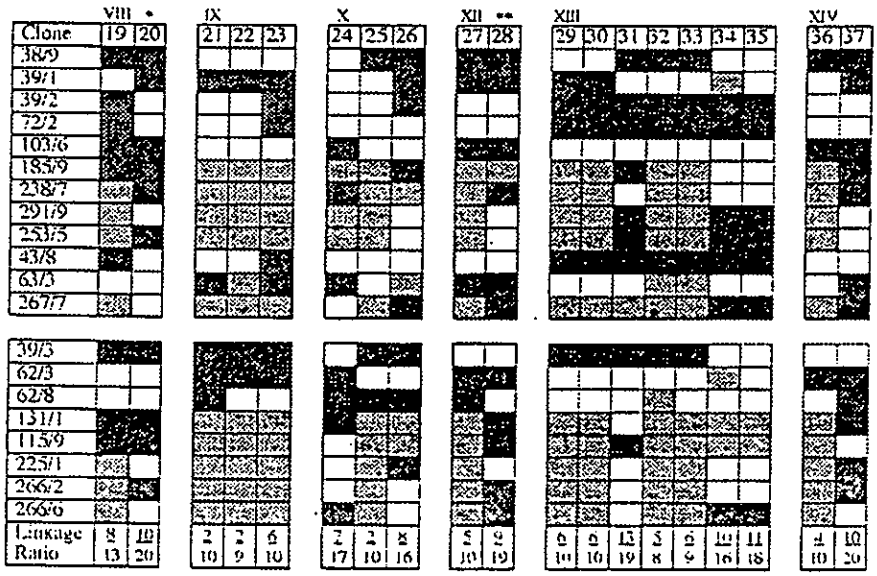
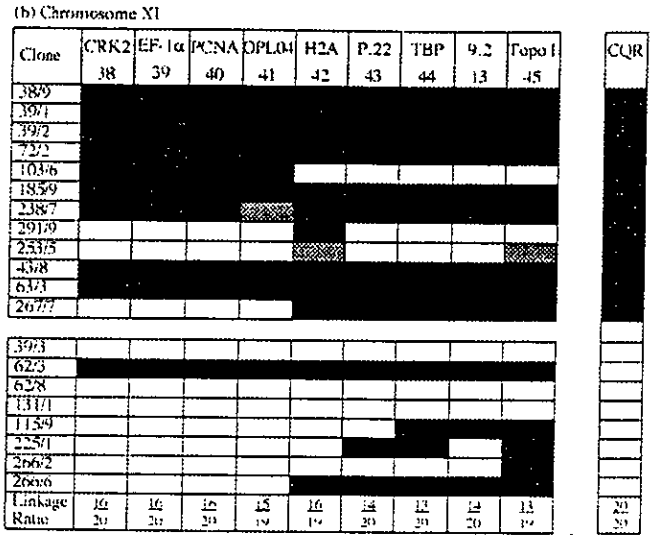


Fig. 1. (a) Inheritance of 37 chromosomal markers among 20 progeny clones from two AJ × AS(3CQ) crosses. Roman numerals refer to the chromosome number: progeny clones are listed vertically. Each chromosome marker has been allocated the same identification number as in Table 1. Open boxes indicate inheritance of AJ-type alleles, closed boxes indicate inheritance of AS(3CQ)-type alleles, and hatched boxes indicate data not available. Markers are ordered so as to minimise the number of crossovers for each of the progeny. Progeny clones are ordered phenotypically: clones above the gap are resistant, those below are sensitive. * Indicates the inheritance profile for *pfhsp86* (chromosome 8), and ** for *pcmdr1* (chromosome 12). # Indicates a marker which is present on either chromosome 4 or 5. Marker 13 is present as two copies in the genome. The linkage ratio for each marker is also shown. (b) Inheritance of chromosome 11 markers among 20 progeny clones. The far right column (CQR) shows the inheritance pattern expected if a primary chloroquine resistance gene (CQR) existed on chromosome 11.



2.04 (P= .002)

Fig. 1. (*Continued*)

of these markers (LOD = 3.08, $P = 0.0002$). When the three progeny which had intermediate resistance were excluded from the analysis, the LOD score was 2.55 ($P = 0.0006$.) Thus, even when accounting for the multiple tests involved in the genome scan using 46 markers, this result provides good evidence [33] that a locus with a very strong effect on chloroquine resistance lies within this chromosomal region.

Results of the quantitative trait data analysis are presented in Table 3. On days 11 and 13, the differences between the mean log parasitaemias of the grouped chloroquine-resistant and chloroquine-sensitive progeny were 1.49 and 1.41 respectively, which is equivalent to an $\approx 30\%$ difference in parasitaemia. Markers which also showed a significant between-allele difference in parasitaemia were the three chromosome 11 markers CRK2, EF-1α and PCNA (Day 13, $0.001 < P < 0.01$, and Day 11, $0.01 < P < 0.05$), showing that these marker alleles segregated with chloroquine resistance. These results thus support the findings of the linkage analysis described above. Other possible loci with less strong significance levels of linkage to chloroquine-resistance assessed by this method were Ag3035 on chromosome 3, an anonymous locus on chromosome 5, and Ag3027 on chromosome 9.

No. Ag 3003A according to Table 1

An additional deduction which can be made from the data in Table 3 concerns genes which may be involved in rates of early growth. In the Days 4–6 data, groups of clones marked by alleles of three loci on chromosome 11 and one on chromosome 14 showed significant marker contrasts. This indicates that these markers may be close to genes which determine different growth rates in the two parental clones.

4. Discussion

In this study, we have examined the inheritance of chloroquine resistance in the malaria parasite *P. chabaudi*. We have shown by linkage analysis of two crosses that a gene determining chloroquine resistance in the AS(3CQ) clone of this species is most probably located on chromosome 11. The *pcmdr1* gene, located on chromosome 12, is not tightly linked to the resistance. This result does not rule out the possibility that *pcmdr1* may be involved in other instances of chloroquine resistance, but it clearly parallels the findings of Wellems et al. [14] that the *pfmdr1* gene of *P. falciparum* is not the cause of chloroquine-resistance in clone Dd2 of that species.

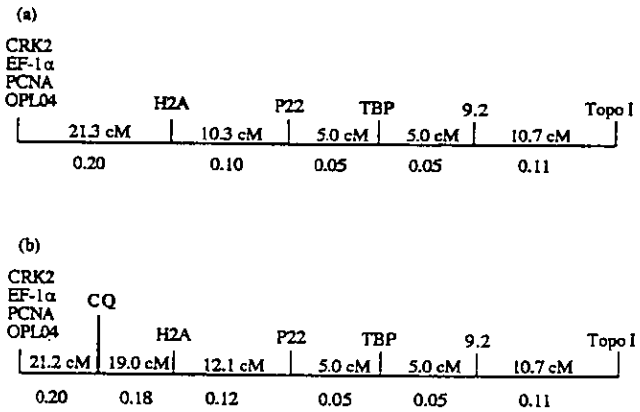


Fig. 2. Best-fit map of a putative chloroquine resistance gene within a region of chromosome 11 delineated by nine markers. (a) Ordered map of chromosome 11 markers. (b) Most likely map position of a putative chloroquine resistance locus. Recombination fractions are shown below the solid line, centiMorgan (cM) distances shown above, and the position of the chloroquine resistance gene is shown in bold type. The maps are not drawn to scale.

As a general rule, micro-organisms are able to adopt a variety of genetic mechanisms to circumvent the action of drugs. Such mechanisms include: (i) decreased uptake of the drug into the target cell; (ii) mechanisms to expel the drug; (iii) structural alterations in the target molecule; (iv) increased expression of the target gene product; (v) mechanisms to detoxify the drug. Each of these mechanisms has been proposed to explain chloroquine resistance in malaria. Most attention has been paid to the possible role of the multidrug resistant genes of *P. falciparum*, especially *pfmdr1* [34]. The protein product of this gene, Pgh1, is found on the surface of the parasite lysosome [35], the organelle where chloroquine is concentrated. Amplification of *pfmdr1* has been noted in certain chloroquine-resistant isolates [5,6,13], which has led some authors to suggest that over-production of Pgh1 may enable the parasite to expel chloroquine and thus cause resistance, although more recent work has suggested an inverse relationship between chloroquine resistance and *pfmdr1* amplification [36–38] and expression [39]. Other studies suggested that certain alleles of *pfmdr1* are associated with resistance [9,40]. However, several surveys in which natural isolates containing these *pfmdr1* alleles have been tested for their chloroquine response have given equivocal results [10,13,41].

The only genetic crossing work completed on chloroquine resistance in *P. falciparum* is that of Wellems et al. [14,15]. In their study, the chloroquine-resistant clone Dd2 was crossed with the sensitive clone HB3. Recombinant progeny (16) from a single cross were typed for chloroquine response. The progeny clones possessed phenotypes of resistance identical to one or other parent; there were no intermediate phenotypes, which would have indicated that the trait was multigenic. The resistance phenotype of each correlated with their ability to efflux chloroquine, and the resistance was reversed by verapamil. RFLP markers of the *pfmdr1* and *pfmdr2* genes did not co-segregate with the resistance phenotype, and in addition there was no correlation between gene copy number of *pfmdr1* and chloroquine resistance among the progeny clones. When the inheritance of 85 RFLP markers was examined, a locus in a 36 kb segment of chromosome 7 was linked to the resistance phenotype. Further mapping and sequencing of this region has resulted in the description of a gene denoted *cg2*, of unknown function but which was present in all of 20 chloroquine-resistant isolates from South-east Asia and Africa [17]. However, this gene was also found in one out of 21 sensitive isolates examined at the same time, showing that by itself it did not inevitably confer resistance,

Table 3
Analysis for linkage between markers and chloroquine resistance

Chromosome	Marker	Day 4–6 undrugged	Day 11 drugged	Day 13 drugged	Day 15 drugged
1	1	−0.14*	−0.13	−0.24	−0.29
2	2	−0.08	0.32	0.48	0.63
3	3	0.14	0.33	0.32	−0.48
	4	0.06	0.81	1.15**	0.41
4	5	−0.01	−0.79	−1.30	−1.59
	6	−0.01	−0.79	−1.30	−1.59
4/5	7	0.15	−0.01	−0.22	−1.01
5	8	−0.01	−0.56	0.01	1.53
	9				
	10	0.01	0.94	1.50	1.30
	11	0.09	0.26	0.07	−0.59
	12	−0.30	−0.71	−0.40	0.63
	13	−0.04	−1.15	−0.40	1.74*
6	14	0.15	0.12	−0.37	−1.10
	15	0.02	0.17	−0.13	−0.85
	16	0.02	0.94	1.50	1.29
7	17	−0.09	−0.03	−0.23	−0.11
	18	−0.03	0.43	0.90	1.15
8	19	−0.08	−0.03	0.54	1.02
	20	0.00	0.38	0.53	0.25
9	21	0.10	−0.25	−0.94	−1.68*
	22	0.04	−0.18	−0.61	−1.09
	23	0.06	0.39	0.29	−0.01
10	24	0.02	0.44	−0.08	0.96
	25	−0.01	−0.79	−1.30	−1.59
	26	0.01	−0.11	−0.27	−0.63
12	27	−0.09	−0.16	−0.27	0.07
	28	−0.13	−0.48	−0.11	1.36
13	29	0.09	0.44	0.72	0.33
	30	0.09	0.44	0.72	0.33
	31	0.01	0.60	0.45	0.18
	32	0.03	−0.12	−0.12	−0.31
	33	−0.01	0.05	0.33	0.55
	34	0.00	0.14	0.20	0.82
	35	0.02	0.14	0.20	0.76
14	36	−0.20*	−0.57	−0.37	0.51
	37	−0.04	−0.38	−0.32	−0.10
11	38	−0.07	1.41*	1.36**	0.60
	39	−0.07	1.41*	1.36**	0.60
	40	−0.07	1.41*	1.36**	0.60
	41	−0.12	1.09	1.28**	1.03
	42	−0.17*	0.12	0.22	0.48
	43	−0.06	1.14	0.83	0.66
	44	−0.12	0.40	0.35	0.72
13	13	−0.15*	0.09	0.18	0.58
	45	−0.18*	−0.43	0.17	1.62*
	CQR	−0.01	1.49**	1.41***	0.713

Figures represent the mean difference between progeny clones inheriting the A1 versus AS(3CQ) allele of each marker. The marker contrast test could not be carried out for marker 9 because no progeny inherited the AS(3CQ) allele of this locus.

* $P<0.5$.
** $P<0.01$.
*** $P<0.001$.

* $P<0.05$

from two *P. chabaudi* crosses. The parent AS(3CQ) clone was deliberately chosen because it exhibits a low level of resistance to chloroquine, and that another locus or loci were involved [17].

In our study, we have examined the inheritance of chloroquine response among 20 progeny clones having been selected from a drug-sensitive clone by exposure to low doses of drug over several blood and mosquito passages [18]. This makes it more likely that resistance is due to changes in only one or a few genes. Moreover, the phenotype of this clone has been studied in detail; verapamil has been shown to reverse the resistance [42], and ultrastructural changes associated with this are similar to those found in chloroquine-resistant *P. falciparum* [43]. Miki et al. [44] have also reported reduced accumulation of chloroquine in AS(3CQ) compared with a sensitive clone, which they attribute to enhanced efflux of the drug in the resistant line. The similarities between AS(3CQ) and chloroquine-resistant *P. falciparum* make the results of our study highly relevant to the mechanism of chloroquine resistance in *P. falciparum*.

The in vivo tests for chloroquine sensitivity were able to distinguish AS(3CQ) from AJ clearly. The sensitivity of the progeny clones was assessed at least 2 × for each clone, and the results were consistent on each occasion. Eight clones were clearly sensitive, and seven clearly resistant, with growth after chloroquine treatment being similar to one or other parent. Five clones were classified as low-level resistant, their growth after chloroquine treatment being slower than in the other seven, and they did not attain equivalent levels of parasitaemia. This could indicate that more than one gene was involved in the resistance of AS(3CQ). For example, it may have been caused by an additive effect of mutant genes at two loci, each able to confer low resistance. If so, the five progeny clones exhibiting low resistance might have contained only one gene, due to meiotic recombination and segregation of alleles at each locus during the cross. A variant of this two-gene hypothesis is that both could be involved in the resistance of AS(3CQ), but that the first gene is epistatic to the second, i.e the latter has no effect in the absence of the former, but increases the resistance level when it is present. In this case, the progeny clones exhibiting parent AS(3CQ)-type resistance would contain both genes, while the three containing the low resistance have only the one epistatic gene, located on chromosome 11. This model fits in with the competent *mdr* model invoked by Foote et al. [9], who postulated that two loci of this type could reconcile the discrepancies between the results of their field surveys of *pfmdr1* alleles and chloroquine resistance in *P. falciparum*, with those of the genetic crossing work of Wellems et al. [14]

Another hypothesis is that only a single gene determines the chloroquine resistance of AS(3CQ), while other genes segregating in the cross influence growth of parasites under drug pressure. The growth characteristics of the parent clones differed, AJ growing slightly faster than AS(3CQ) during routine passage through mice during this experiment. In addition, 18 of the 59 progeny clones isolated were found to be AJ parental types, most probably derived from selfing in mosquitoes, while only two clones of parental AS(3CQ) genotype were found; an excess of AJ parent types was also found by Rosario among the progeny of the original cross using these clones [18]. Nothing is known of genes determining growth rates in malaria parasites, but they are likely to assort independently of those determining drug resistance. Some weak association of growth variations among the clones examined in the present *P. chabaudi* cross with loci on chromosomes 11 and 14 were seen in the quantitative trait analysis described here.

An important aspect of this study was the allocation of 46 polymorphic genomic DNA markers to the 14 *P. chabaudi* chromosomes, and linkage analysis of each marker to chloroquine sensitivity among the 20 progeny clones. In total, 673 RFLPs were examined in this work, most of which exhibited an approximately even distribution in the recombinant progeny—306 observed AS(3CQ) alleles inherited, compared with 336.5 expected. However, there was a skewed inheritance of markers associated with one chromosome, those of chromosome 5 being largely AJ-type, in which 64 AJ markers occurred instead of an expected 43 (86 polymorphic sites sampled).

Similar skewed RFLP distributions were also found in the progeny of the HB3/Dd2 *P. falciparum* cross on four chromosomes [45]. The reasons for this are unknown, but might be due to genes on the chromosomes concerned conferring advantages in proliferation.

All chromosomes analysed for the inheritance of more than two markers during this work were shown to have undergone at least one crossing-over event. With regard to chromosome 11, ten cross-overs were found among the nine chromosome loci studied, indicating that this chromosome was able to recombine freely. Six of the loci are known to be genes which are conserved between rodent malaria species and *P. falciparum* [71] and synteny data (not shown) suggests that they were not sufficiently closely linked to have prevented chromosomal rearrangements in the evolutionary history of these parasites.

Finally, it may be questioned whether we scored enough progeny clones in this work to give a high chance of detecting real linkage. Based on the method of Lander and Botstein [46], we calculate that to achieve $>90\%$ power (probability of detecting a real linkage) using a significance threshold of 5%, the number of clones required if the closest marker is 0, 10 or 20 centiMorgan (cM) away from the gene are 9, 17 and 32 respectively. Thus the 15 clones with clearly resistant or sensitive phenotypes should have been enough to search confidently for a gene at a recombination distance of 0.1. This calculation is based on a test for a single marker only. When 25 independent markers are used, allowance must be made for the increased probability of obtaining a false positive for at least one of the linkage groups. This increases the number of clones required for 90% power to 14, 27 and 52. Thus the power of the experiment for detecting loci other than those tightly linked was not optimal, and some false positives are expected given the multiple markers tested.

This study has shown that the most likely location of a gene determining chloroquine resistance in *P. chabaudi* is on chromosome 11. Future work will be aimed at identifying this gene and determining its relationship, if any, to those involved in chloroquine resistance in *P. falciparum*. Other mutants of *P. chabaudi* have been produced which possess higher levels of resistance than that of AS(3CQ) [19]. In due course, such mutants can be analysed by conventional genetic analysis, as described in this paper, and subsequently by transfection methodology [47], to identify how many genes are involved in this type of resistance and to determine their mechanism of action.

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VIRULENCE OF MIXED-CLONE AND SINGLE-CLONE INFECTIONS OF THE RODENT MALARIA *PLASMODIUM CHABAUDI*

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Abstract.—Most evolutionary models treat virulence as an unavoidable consequence of microparasite replication and have predicted that in mixed-genotype infections, natural selection should favor higher levels of virulence than is optimal in genetically uniform infections. Increased virulence may evolve as a genetically fixed strategy, appropriate for the frequency of mixed infections in the population, or may occur as a conditional response to mixed infection, that is, a facultative strategy. Here we test whether facultative alterations in replication rates in the presence of competing genotypes generate greater virulence. An important alternative, not currently incorporated in models of the evolution of virulence, is that host responses mounted against genetically diverse parasites may be more costly or less effective than those against genetically uniform parasites. If so, mixed clone infections will be more virulent for a given parasite replication rate.

Two groups of mice were infected with one of two clones of *Plasmodium chabaudi* parasites, and three groups of mice were infected with 1:9, 5:5, or 9:1 mixtures of the same two clones. Virulence was assessed by monitoring mouse body weight and red blood cell density. Transmission stage densities were significantly higher in mixed- than in single-clone infections. Within treatment groups, transmission stage production increased with the virulence of the infection, a phenotypic correlation consistent with the genetic correlation assumed by much of the theoretical work on the evolution of virulence. Consistent with theoretical predictions of facultative alterations in virulence, we found that mice infected with both parasite clones lost more weight and had on average lower blood counts than those infected with single-clone infections. However, there was no consistent evidence of the mechanism invoked by evolutionary models that predict this effect. Replication rates and parasite densities were not always higher in mixed-clone infections, and for a given replication rate or parasite density, mixed-clone infections were still more virulent. Instead, prolonged anemia and increased transmission may have occured because genetically diverse infections are less rapidly cleared by hosts. Differences in maximum weight loss occured even when there were comparable parasite densities in mixed- and single-clone infections. We suggest that mounting an immune response against more that one parasite genotype is more costly for hosts, which therefore suffer higher virulence.

Key words.—Malaria, mixed infection, parasite, *Plasmodium chabaudi*, virulence.

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Virulence is the reduction in host fitness caused by parasitic infection. The evolution of virulence has important implications for the control of infectious disease and has stimulated a considerable body of theory (reviewed by Bull 1994; Read 1994; Ewald 1995; Frank 1996; Ebert, in press). However, these ideas have been subjected to remarkably little experimental testing, particularly in the context of vertebrate infectious disease where the utilitarian benefits of the theory have been most stridently advocated (Williams and Nesse 1991; Ewald 1994; Westoby 1994; Futuyma 1995).

Most evolution of virulence models regard virulence as an unavoidable consequence of parasite adaptation. Virulence itself is viewed as detrimental to parasite fitness but genetically correlated to fitness-enhancing traits (reviewed by Bull 1994; Read 1994; Frank 1996; Ebert, in press). Increased virulence (usually modeled as a direct consequence of microparasite replication rate) is assumed to correlate with risk of host death, but also increased transmission stage production. Observed levels of virulence are then said to represent schedules of host exploitation that balance those two factors so as to maximize some measure of parasite fitness. In the simplest models, which consider just one parasite genotype per host, the level of virulence that evolves is that which maximizes the total number of new infections resulting from an infection (e.g., Levin and Pimentel 1981; Anderson and May 1982; Bremermann and Pickering 1983). Optimal virulence may be high if transmission probability is enhanced by host morbidity, or low as prudent parasites attempt to prolong host survival to maximize long-term transmission.

However, many authors have pointed out that where mixed-genotype infections are common, levels of virulence greater than those optimal for single-genotype infections will be favored by natural selection (e.g., Hamilton 1972; Eshel 1977; Axelrod and Hamilton 1981; May and Anderson 1983; Knolle 1989; Frank 1992; Hellriegel 1992; Herre 1993, 1995; Bonhoeffer and Nowak 1994a,b; Nowak and May 1994; van Baalen and Sabelis 1995a,b; Frank 1996; Ebert and Mangin, in press). In the competitive situation of a mixed-genotype infection, parasites that exploit host resources are expected to be outcompeted by those exploiting hosts more rapidly. Thus, optimal levels of virulence are higher in mixed infections, even if this leads to fewer secondary infections than might be otherwise achieved. In the extreme, short-term selection arising from competition within a host can lead to greatly increased levels of virulence, with greatly decreased transmission rates, so-called short-sighted evolution (Levin and Bull 1994).

Where mixed-genotype infections occur, parasites could evolve a genetically fixed strategy appropriate for the frequency of mixed infections in the population. Alternatively,

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a conditional strategy might evolve where parasites facultatively alter their growth rates, and hence virulence, according to the type of infection they find themselves with (Sasaki and Iwasa 1991; Frank 1992; van Baalen and Sabelis 1995a). A conditional response is adaptive if the level of competition experienced is variable and cannot be predicted in advance. Such responses require that parasites are able to detect other parasite genotypes within a host and respond accordingly. One possible mechanism for this is the detection of a host immune response mounted to an antigenically similar, but not identical, parasite. Whatever the underlying mechanism, both genetically fixed and facultative strategies should higher parasite virulence being observed where mixed genotype infections are common.

Whether parasites facultatively alter their within-host growth schedules in the presence of competing genotypes, thus inducing greater virulence in mixed-clone infections, remains to be tested. However, a complexity in such tests is the role of host factors in determining virulence. Although generally ignored in most formal models, virulence must be a consequence not only of what parasites do to hosts but also on the costs and effectiveness of parasite control by hosts. Responses against a more heterogenous parasite population may utilize more host resources. It may also be less effective. It is well known that parasites that create antigenic diversity within infections can evade host immunity that probably prolong the infection (e.g., in malaria and trypansome infections; Wakelin 1996). It therefore seems plausible that genetically heterogeneous infections will be less rapidly cleared by the host. Thus, even in the absence of facultative alterations in parasite growth schedules, differences in host responses could result in mixed clone infections being more virulent. The crucial test for the existence of conditional virulence strategies is to compare both virulence and within-host growth schedules in mixed- and single-clone infections. We did this using the rodent malaria parasite *Plasmodium chabaudi*.

In vertebrate hosts, malaria parasites infect red blood cells and replicate mitotically in the blood stream until the host dies or the infection is controlled by host immunity. For a variety of experimental and ethical reasons, it is not practical to directly assay mortality rates. Instead, we assayed weight loss and anemia as measures of virulence. In the strain of mouse used in our experiments, *P. chabaudi* is usually nonlethal (Stevenson et al. 1982), but it is assumed that in less-resistant host genotypes these parameters are correlated with probability of death. Host responses to anemia have been linked with resistance to infection with *P. chabaudi* (Stevenson et al. 1982; Yap and Stevenson 1994) and both weight loss and anemia induced by single-clone infections of *P. chabaudi* differ repeatably between parasite lines (Mackinnon, unpubl. data; Read and Anwar, unpubl. data).

We infected groups of mice with one of two clones of *P. chabaudi* or mixtures of the two, with all mice receiving the same total number of parasites. Both parasite growth rates and host reponses in mixed infections may be affected by the relative abundance of the parasite clones. Three types of mixed-clone infections were therefore initiated, with inocula composed of 9:1, 5:5, or 1:9 ratios of the two clones. Relative virulence of these three groups is hard to predict a priori: to do so would require detailed information about optimal growth rates and host responses in the three situations, and by how much both could be altered.

These experiments also allow us to test whether virulence is phenotypically correlated with parasite replication rate and transmission. Phenotypic correlations can be indicative of underlying genetic correlations. *Plasmodium chabaudi* parasites replicate synchronously every 24 h in the host blood stream. As all infections were initiated with the same number of parasites, we monitored parasite density early in the infection (before significant parasite mortality due to limiting nutrient or host immunity occur) as a correlate of replication rate. As a measure of transmission rates, peak gametocyte density was assayed. Gametocytes are nonreplicating parasites that develop from the replicating blood stage (asexual) parasites, and the only stages capable of infecting mosquitoes. Previous experiments with *P. chabaudi* have shown that gametocytes are present for only a brief period, during which gametocyte density is correlated with both the proportion of mosquitoes infected and average parasite burdens per mosquito (Taylor et al. 1997a).

Methods

Parasites and Hosts

Two cloned lines of *P. chabaudi chabaudi* denoted CR and ER (Beale et al. 1978), obtained from the WHO Registry of Standard Malaria Parasites, Edinburgh University, were used. Clones are derived from asexual proliferation of a single parasite obtained from wild isolates by serial dilution. Isolates from infected tree rats (*Thamnomys rutilans*) collected in the Central African Republic in 1965 and 1970 were the sources of the CR and ER clones, respectively (Beale et al. 1978; McLean 1986). Since isolation and cloning, the parasites have been maintained for long periods as stabilates in liquid nitrogen. In total, the CR and ER parasites used for this study had been maintained in asexual passage in rodent hosts for 4.5 and 5 months, respectively. The clones have different alleles of the merozoite surface protein 1 distinguishable by genetic or monoclonal antibody techniques (Taylor et al. 1997b). Hosts were male C57BL/6J/Ola mice (Harlan, England). Mice were fed on SDS rat and mouse maintenance diet, and drinking water was supplemented with 0.05% para-amino benzoic acid (PABA) to enhance parasite growth. Artificial illumination was provided from 0530 to 1730 h.

Inoculation of Mice with Standard Numbers of Parasites

Parasite densities in infected donor mice were determined from Giemsa-stained, thin blood smears and red blood cell counts made using flow cytometry (Coulter Electronics). For inoculations, infected blood was diluted in calf serum-ringer solution (50% heat-inactivated calf serum, 50% ringer solution [27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl], 20 units heparin/mL mouse blood) to give separate dilutions of 10^5 parasitised red blood cells per 0.1 mL volume of the two parasite clones.

we understand the abbreviation

Monitoring of Infections and Virulence

Mice were weighed to the nearest 0.01 g on days 0, 2, and 4, then daily until day 22, and on days 24, 28, and 31 post-infection (PI). Red blood cell counts were taken on day 4, then every second day until day 18, and on day 22 PI between 1700 and 1830 h. Thin blood smears were taken at the same time as the blood count measures on days 6, 8, 10, 12, and 16, and used to calculate the asexual parasite density (parasites per mL) on each of these days and gametocyte density (gametocytes per mL) on day 16 PI. Uninfected mice were subjected to the same sampling procedure.

Experimental Design

Mice were weighed and allocated at random to treatment groups. Six treatment groups, each containing four mice, were used: one control (uninfected), two single-clone (CR or ER parasites), and three mixed-clone infection groups (parasite clones in ratios of 1CR:9ER, 5CR:5ER, or 9CR:1ER). All mice allocated to noncontrol groups received a total of 10^5 *P. chabaudi* parasites in a 0.1-mL inoculum intraperitoneally. Control mice received an equivalent number of red blood cells from an uninfected mouse. Mice were inoculated between 1500 and 1700 h. The whole experiment was repeated 13 days later (with different mice from the same cohort) to give two experimental blocks and a grand total of 48 mice in the whole experiment. (One mouse in the ER treatment group of the second block showed an abnormally high parasite density on day 10 and died on day 12 PI. It was excluded from the analysis.)

Statistical Analyses

Weight, weight loss, red blood cell density, and gametocyte density were analyzed using ANOVA to determine the effects of treatment and experimental block. In most cases, there were significant differences between the two experimental blocks, probably because mice in the second block were 13 days older. Block effects were always included in the models where significant, but are of little interest in their own right; only significant interactions between experimental block and other model terms are reported.

Correlations between virulence parameters and measures of asexual parasite densities were carried out using ANCOVA in GLIM (Crawley 1993). The following seven measures of the asexual infection were used: (1–5) parasite densities on days 6, 8, 10, 12, and 16; (6) the difference between day-8 and day-6 parasite density; and (7) an estimate of the total number of parasites during the infection. This last measure was calculated for each mouse individually from the area under the curve of asexual density through time between days 6 and 16 PI. As *P. chabaudi* replicates once every 24 h, this represents a reasonable approximation to the total number of asexuals produced during this period of infection. In all cases, treatment, experimental block, and all interactions with the asexual measure in question were included as predictor variables in a full model, with the virulence measure as the response variable. No interaction terms were significant when removed from these full models. Significance of the remaining effects was assessed from models containing the asexual measure, treatment, and block effects as predictor variables. *F*-ratios were calculated from the change in deviance per degree of freedom divided by the residual mean square deviance as terms were removed from the minimal model (Crawley 1993). The same approach was used to investigate relationships between virulence and transmission, with gametocyte density as the response variable and the virulence measure as the predictor variable.

Results

Most of the weight loss following infection occurred between days 7 and 18 PI, with the lowest weights typically between day 11 and 13 PI (Fig. 1a). However, within and across treatment groups, mice did not lose weight synchronously, so that comparisons of weight loss at specific points in time were not meaningful. Instead, two composite measures of weight loss were used: mean loss (weight on the day of infection minus average weight from days 7–18 PI) and maximum loss (weight on the day of infection minus the average of the two lowest weights subsequently recorded). Red blood cell density fell from day 6 PI to minima on days 10–12 PI (Fig. 1b). Mean blood count over days 4–22 PI, and an average of the two lowest blood counts were calculated. Parasites became detectable in blood films on day 6 PI, densities peaked between days 8 and 10, and had fallen dramatically by day 12 (Fig. 1c). In the later parts of the infection, mixed-clone infections maintained higher asexual densities than single-clone infections, although none were comparable to earlier stages of infection.

The virulence of CR infections was not significantly different from that of ER infections as assessed by mean weight loss, maximum weight loss, mean blood count, or the lowest blood count ($F_{1,11} = 0.27$, $F_{1,11} = 0.26$, $F_{1,11} = 0.53$, $F_{1,11} = 0.28$, respectively; $P > 0.2$ in all cases). Similarly, mixed-clone infections with initial CR:ER parasite ratios of 1:9, 5:5, and 9:1 did not differ significantly in mean or maximum weight loss or mean blood counts ($F_{2,18} = 2.55$, $P > 0.05$; $F_{2,18} = 2.00$, $P > 0.1$; $F_{2,18} = 0.44$, $P > 0.5$, respectively). The effect of the initial ratio on lowest blood count differed significantly between the two experimental blocks (block × treatment interaction, $F_{2,18} = 5.05$, $P < 0.05$), with the rank orders of the three groups different in the two blocks, but there was no main effect of initial ratio ($F_{2,18} = 1.65$, $P > 0.2$). As no consistent differences between the two single-clone or between the three mixed-clone groups could be shown, the remaining analyses compare the virulence of mixed-clone and single-clone infections.

Comparison of Mixed- and Single-Clone Infections

Virulence

Mixed-clone infections were more virulent than single-clone infections. Maximum weight loss was greater following infection with two clones than it was following infection with one ($F_{1,35} = 4.79$, $P < 0.05$). Mice with mixed-clone infections lost about 30% more weight than those with single-clone infections (Fig. 2). Mean weight loss showed a similar pattern ($F_{1,35} = 3.66$, $P = 0.07$). By the end of the experiment (day 31 PI), weights of mice given mixed-clone infections and single-clone infections had returned to weights compa-

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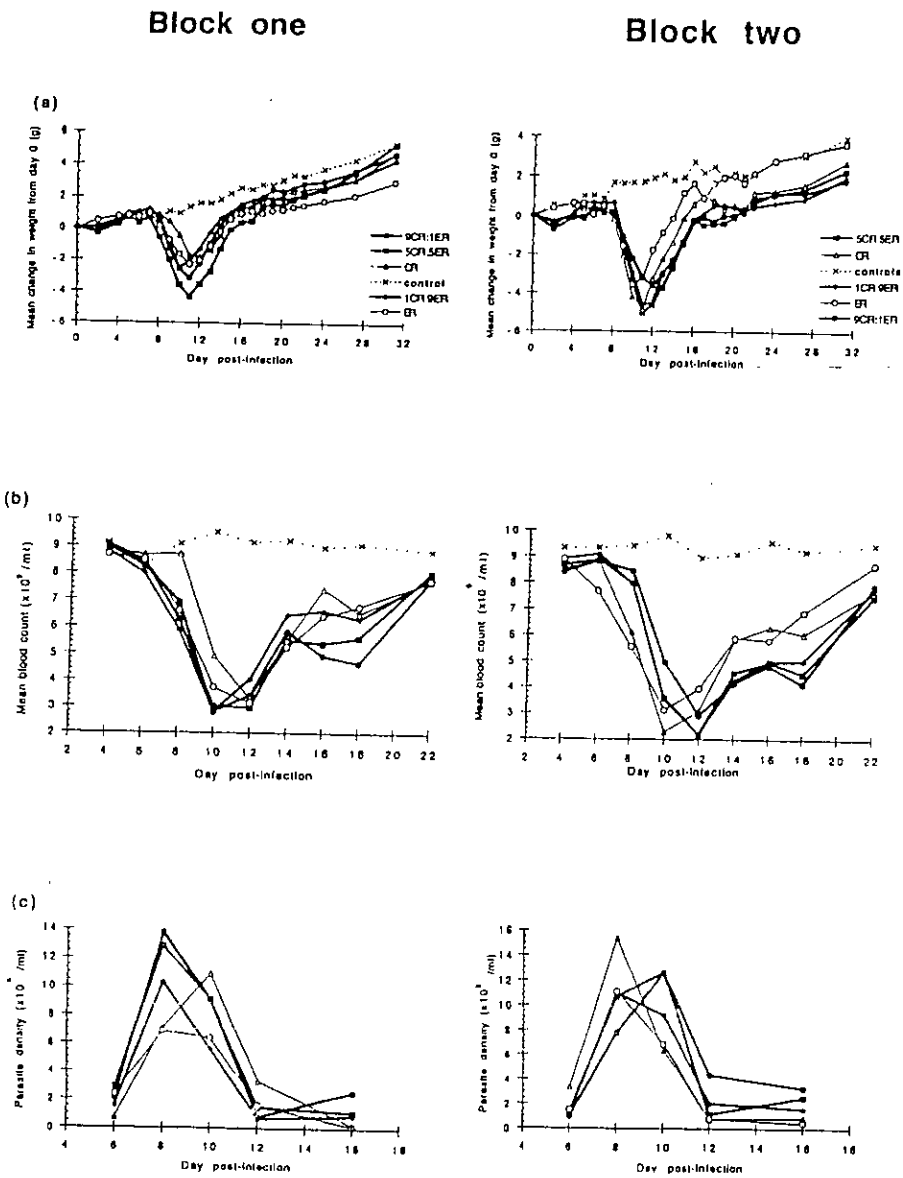

FIG. 1. Mean weight loss (a), mean red blood cell densities (b), and parasite densities (c) of the six groups of mice in experimental blocks one and two during the infections. Day of inoculation = day 0. Each line represents the mean of four mice.

rable to the uninfected controls ($F_{2,41} = 1.53$, $P > 0.2$). The reduction in mean blood count was higher for mixed-clone infections compared to single-clone infections ($F_{1,35} = 6.60$, $P < 0.05$, Fig. 3), with mixed-clone infections suffering a further 5% reduction on average. The average of the two lowest blood counts did not differ significantly between mixed-clone and single-clone infections ($F_{1,35} = 1.86$, $P > 0.1$).

Parasite Densities

The density of parasites achieved by day 6 PI, day 8 PI, and the increase between days 6 and 8 PI, was greater for the mixed-clone infections in block one of the experiment, but greater for the single-clone infections for the second block (treatment × block interactions, $F_{1,36} = 3.39$, $P \approx 0.07$, $F_{1,36} = 9.69$, $P < 0.01$, and $F_{1,36} = 8.04$, $P < 0.01$, respectively). The total number of parasites in mixed-clone and single-clone

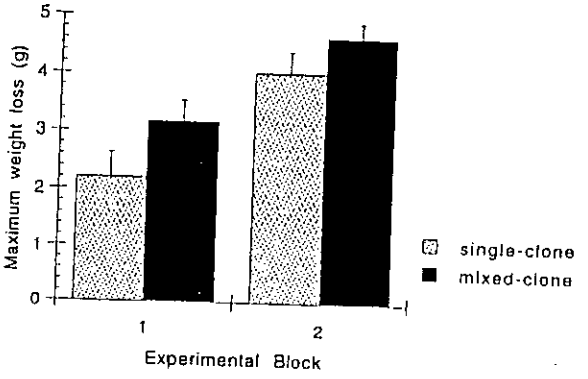

FIG. 2. Maximum weight losses of mixed-clone and single-clone infections, calculated from the mean of the two lowest weights reached. Within each block there were 12 mixed-clone infections and eight single-clone infections. Error bars represent +1 SE.

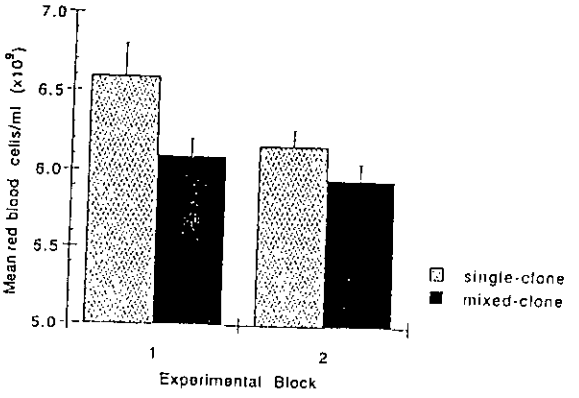

FIG. 3. Mean blood counts of the mixed-clone and single-clone infections over days 4 to 22 of infection. Within each block there were 12 mixed-clone infections and eight single-clone infections. Error bars represent +1 SE.

infections also differed between blocks, being higher for single-clone infections in block one and higher for the mixed-clone infections in block two (treatment × block interaction $F_{1,35} = 5.81$, $P < 0.05$). Thus, there were no consistent differences across blocks in parasite densities or total parasite numbers analogous to those for virulence.

Virulence and Parasite Numbers

Relationships between parasite densities and virulence were investigated using the virulence measures that showed the clearest treatment effects: maximum weight loss and mean blood count. In none of the following analyses were any interactions between the asexual parameter being tested and either block or treatment significant, demonstrating that any relationships between virulence and the asexual infection dynamics did not differ between single-clone and mixed-clone infections.

Of the seven variables relating to the asexual infection analyzed, only asexual densities on day 6 PI, day 8 PI and the difference between them were positively correlated to maximum weight loss, when controlling for treatment effects (Table 1). Parasite densities later in the infection and the total number of parasites during the infection were unrelated to maximum weight loss. Thus, weight loss was greater when parasite density early in the infection was higher, but was unrelated to the parasite burden later in the infection or to the total number of parasites in an infection.

However, differences in parasite densities did not explain why maximum weight loss was greater for mixed-clone than single-clone infections. On average, mixed-clone infections resulted in an additional weight loss of about 0.8 g compared to single-clone infections with the same parasite density on day 6 PI (Table 1; Fig. 4a). This difference was equivalent to about 3% of the weight of uninfected controls of the same age.

Mean blood count was negatively correlated to day 6 PI, day 8 PI, and day 8 PI minus day 6 PI parasite densities (Table 1), showing that higher parasite densities were associated with lower blood counts. Later in the infection, on days 10 and 12 PI, there were positive associations between parasite density and blood count. But as with weight loss, differences in the mean blood count of mice infected with single-clone or mixed-clone infections were not explained by differences in parasite densities early in the infection (Table 1). Mixed-clone infections showed on average 0.4×10^9 fewer red blood cells per mL than single-clone infections for a particular asexual parasite density at each of the points in

TABLE 1. Relationships between virulence measures and asexual infection dynamics. In ANCOVA models, maximum weight loss or mean blood count was the response variable, with treatment (single- or mixed-clone infection), experimental block, and (when fitted) the asexual measure as predictor variables. Tabulated values are parameter estimates from the full models; the statistical significance of their difference from zero was tested by removing each term from the model (Crawley 1993). Thus, significant terms explain additional variation above that explained by the other terms. In no case did inclusion of higher-order interaction terms significantly improve model fit. † $P \approx 0.07$ (marginal), * $P < 0.05$, ** $P < 0.01$, ns = not significant.

Virulence measure	Asexual measure	Slope of regression	Mixed vs. single intercept
Maximum weight loss	none		0.79*
	day 6 pars/mL	0.029*	0.93*
	day 8 pars/mL	0.011*	0.76*
	day 8 − day 6 pars/mL	0.010*	0.72†
	day 10 pars/mL	−0.0039 ns	0.87*
	day 12 pars/mL	−0.016 ns	0.80*
	day 16 pars/mL	−0.0046 ns	0.86 ns
	Total parasites	0.0023 ns	0.62 ns
Mean blood count	none		−0.36*
	day 6 pars/mL	−0.019**	−0.45*
	day 8 pars/mL	−0.0056**	−0.35**
	day 8 − day 6 pars/mL	−0.0046*	−0.33*
	day 10 pars/mL	0.0065**	−0.49**
	day 12 pars/mL	0.0011**	−0.37**
	day 16 pars/mL	−0.00073 ns	−0.35 ns
	Total parasites	0.00096 ns	−0.43*

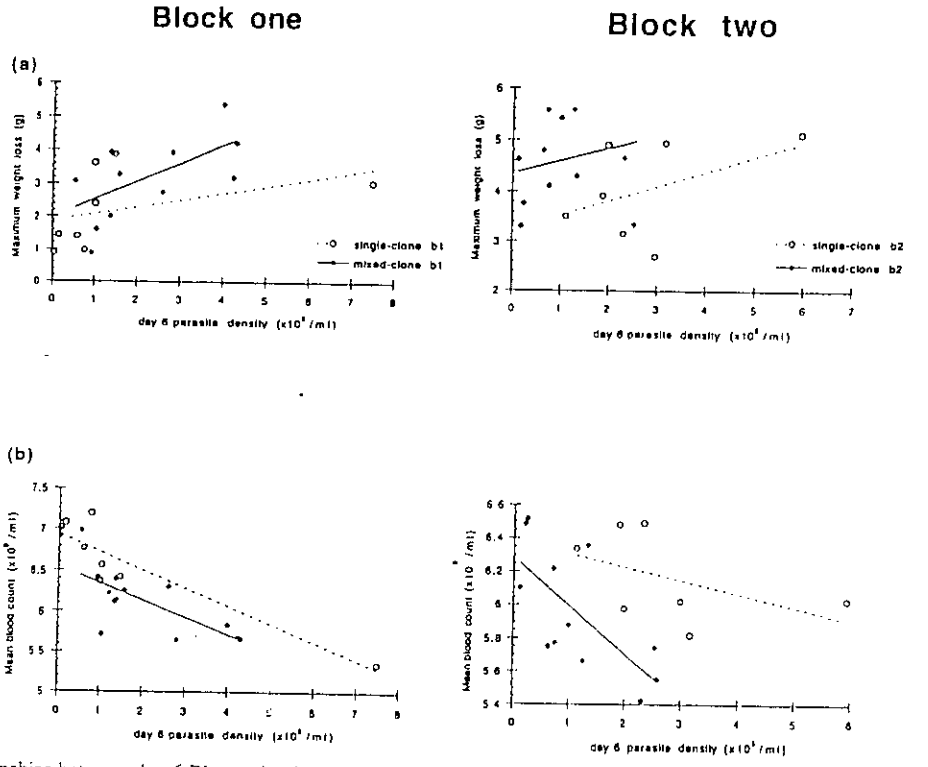

FIG. 4. Relationships between day 6 PI parasite densities and maximum weight loss (a) and mean blood cell count (b) for experimental blocks one and two. OLS regressions were fitted separately for single- and mixed-clone infections. For maximum weight loss, the fitted line for the mixed-clone infections lies above that for the single-clone infections in both cases; for mean blood count, the mixed-clone line lies below that of the single-clone infections in both cases.

time during the infection that were sampled. This difference was equivalent to about 4% of the red blood cell densities of the uninfected control mice (Fig. 4b).

Transmission

In both experimental blocks, gametocyte densities were higher in mixed-clone infections than in single-clone infections ($F_{1,35} = 13.43$, $P < 0.001$, Fig. 5); this difference was larger in the second block (treatment × block interaction; $F_{1,35} = 3.60$, $P \approx 0.07$).

Across all mice, both mean and maximum weight loss were positively correlated with gametocyte density ($F_{1,38} = 5.78$ and $F_{1,38} = 6.09$, respectively, $P < 0.05$ in both cases). In neither case did adding a quadratic weight loss term improve the model fit ($F_{1,37} = 0.49$, $P > 0.2$ for mean weight loss; and $F_{1,37} = 0.40$, $P > 0.2$ for maximum weight loss). Thus gametocyte density was linearly correlated with virulence as measured by weight loss. Using gametocyte density, replicate, treatment, and all interactions as predictor variables in the analysis of weight loss and mean blood count, the minimal model contained only the treatment effect in each case ($F_{1,38} = 8.59$, $P < 0.05$). However, weight loss was still positively correlated with gametocyte densities when the difference between mixed- and single-clone infections was controlled for, although the effects were only marginally significant ($F_{1,37} = 3.48$, $P \approx 0.07$ for mean weight loss; $F_{1,37} = 3.48$, $P \approx 0.07$ for maximum weight loss). Thus mixed-clone infections had higher gametocyte densities and caused greater weight loss. However, within single-clone and within mixed-clone

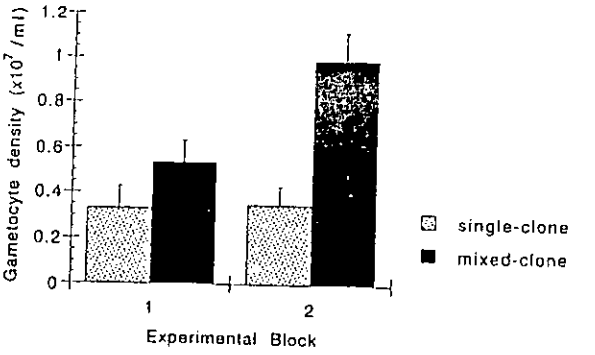

FIG. 5. Gametocyte densities on day 16 PI for the mixed-clone and single-clone infections. Within each block there were 12 mixed-clone infections and eight single-clone infections. Error bars represent +1 SE.

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infections, there was also evidence that gametocyte density positively correlated with weight loss.

Similar analyses were carried out for the blood count measures. For mean blood count there was no significant correlation with gametocyte density ($F_{1,38} = 3.16$, $P > 0.05$), and no additional effect of the quadratic term ($F_{1,37} = 0.14$, $P > 0.5$). For the lowest blood count measure, the pattern was the same with neither the linear term ($F_{1,38} = 3.64$, $P > 0.05$) nor the quadratic term ($F_{1,37} = 0.18$, $P > 0.5$) showing a significant correlation with gametocyte density.

Discussion

Consistent with theory, the virulence of mixed-clone infections was greater than that of single-clone infections, when measured by weight loss and mean blood count. However, there was no consistent evidence that this was due to facultative alterations in within-host growth strategies by parasites in mixed-clone infections. Asexual parasite densities early in the infection of a nonimmune host are correlates of parasite replication rates. Densities later in the infection and the total number of parasites are measures of parasite burden. None of these parasite density measures taken were sufficient to fully explain the observed patterns of virulence. Within treatment groups, more rapid parasite replication resulted in greater virulence, but for a given parasite replication rate, mixed-clone infections were still more virulent. Thus there is a phenotypic correlation between virulence and parasite replication rate, but there is also an additional effect of genetic diversity, independent of parasite density. If parasite replication rate was the only determinant of virulence, the single-clone and mixed-clone infections should fall along the same fitted line in Figure 4, with the mixed-clone infections concentrated in the upper-right-hand quadrant. Instead, there is considerable overlap in the ranges of parasite replication rates for the two groups, and a significant difference in the intercepts of the fitted lines for single- and mixed-clone infections.

The greater virulence of mixed-clone infections for a given number of parasites may be due to facultative alterations in some aspect of parasite life history other than replication rate. One possibility is that parasites engage in direct interference competition by releasing substances, analogous to allelopathic substances in plants (Rice 1984), to which they themselves are immune but that are toxic to both competing genotypes and the host. Alternately, virulence may be a consequence of the way in which the immune response of the host interacts with the parasites. Blood count and weight loss are directly affected by parasite densities and indirectly affected by the host immune response. Both destroy red blood cells and consume host nutrients. Thus, if there are differences in the immune responses to mixed-clone and single-clone infections, this could explain differences in virulence not attributable to parasite density. For example, diverse parasite populations could stimulate a larger number of T- or B-cell clones, and hence a greater immune cascade causing the destruction of more red blood cells, greater consumption of host resources, or higher production of factors such as TNF (tumor necrosis factor), which is associated with many aspects of disease severity (Titus et al. 1991). Under this view, virulence is indeed a side effect of resource extraction from hosts, as assumed by most models of virulence evolution, but with hosts rather than parasites extracting more resources in mixed infections.

Host responses to parasitic infection may play a greater role in determining the virulence of an infection than adaptive parasite evolution (e.g., Ewald 1980; Read 1994; Ebert and Hamilton 1996). The severity of disease incurred by *P. chabaudi* varies greatly with the strain of mouse host (Stevenson et al. 1982), demonstrating that host factors are a major determinant of virulence. Optimality approaches to host responsiveness have yet to be incorporated into theoretical or experimental analyses of the evolution of virulence.

Mixed-clone infections were able to sustain higher parasite densities toward the end of the infection (days 14–16 PI) than single-clone infections (Fig. 1c), as has been observed in previous experiments with *P. chabaudi* (Taylor et al. 1997a). As higher gametocyte densities were also found in mixed infections, this suggests that antigenic diversity in an infection may benefit the parasites in terms of increased transmission from the infections. Genetic analysis of transmission populations in mosquitoes allowed to feed on mixed infections of *P. chabaudi* has shown that both clones were transmitted. Transmission of individual clones was never lower from mixed infections than from single-clone infections, and could be markedly higher (Taylor 1997b). This raises the possibility that mixed infections may have beneficial synergisms rather than being strictly competitive. Immune responses against *P. chabaudi* are known to be at least in part strain-specific (Jarra and Brown 1985; Snounou et al. 1989), and it may be that clones that are numerically dominant early in infections shield rarer clones against the onset of strain-specific immunity. Slower clearance of parasites may also account for the greater anemia in mixed infections. It cannot, however, explain the greater weight loss: when maximal weight loss occurs, parasite densities were comparable in mixed- and single-clone infections.

Only a few field studies relate malaria morbidity to the number of genotypes in an infection. Those few must be interpreted with caution because of the limitations of the monoclonal (Conway et al. 1991) or PCR (Mercereau-Puijalon 1996) techniques used to assess the number of genotypes, and also the problems of accurately defining morbidity attributable to malaria (Gilles 1988). In Gambia, groups of patients with mild and severe malaria did not differ in the number of genotypes they were carrying (Conway et al. 1991). In Senegal, two studies carried out in the same village suggest that symptomatic children have fewer genotypes per infection (mean = 1.4, Contamin et al. 1996) than asymptomatics of the same age group subjected to the same transmission intensity (mean = 4, Ntoumi et al. 1995). The interpretation of these and other studies in the same village is that when a child encounters a novel strain, unrestricted parasite growth leads to symptoms (Mercereau-Puijalon 1996). A study in Papua New Guinea suggested that clinical cases more often involve parasites with a particular family of MSA1 2 alleles than do asymptomatic controls, suggesting that parasite genotypes differed in their pathogenicity (Engelbrecht et al. 1995). The control group contained a higher percentage of mixed-genotype infections (significance not tested), and a

significantly higher proportion of mixed-species infections than were found in the clinical cases. Recent data support these conclusions (Al-Yaman et al. 1997; Beck et al. 1997). Increasingly, data from the field are suggesting that strain-specific immunity is important in developing resistance to malaria, and that multiple infections stimulate protective immunity against a greater range of genotypes. It is therefore likely that in semi-immune hosts, differences in immune status are a crucial determinant of virulence, emphasizing the difficulty of applying current ideas of adaptive parasite evolution to biomedical problems. Nevertheless, comparisons of disease severity in people who contract two novel genotypes and in those contracting a single novel strain would be of great interest.

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