The ecology and evolutionary implications of malaria parasite virulence in mosquito vectors

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Declaration

I declare that the research described within this thesis is my own work except where stated, and that the thesis is my own composition

Acknowledgments:

I spent my first day as a student at Edinburgh University standing in Andrew Read's office, nodding my head nervously while pretending to know what the 'oocyst' thing he kept mentioning was. I'll never know if he realized the full extent of my ignorance at that time (and all the times since), but over the last three years he has done an exceptional job of dispelling it. I have never felt so motivated, intrigued and enthusiastic about science as I have while working in his group. Andrew made evolutionary biology come alive to me, and equipped me with the skills to explore it. I don't know how to thank him for this; hopefully numerous pints will go some way towards it.

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Why is the effect of malaria parasites on mosquito survival still unresolved? 2002. H. M. Ferguson and A. F. Read. Trends in Parasitology 18: 256-261

Genetic and environmental determinants of malaria parasite virulence in mosquitoes. 2002. H. M. Ferguson and A. F. Read. Proceedings of the Royal Society of London Series B – Biological Sciences 269: 1217-1224

A note on publications arising from this work

The results chapters of this thesis (Chapter 2-6) have been written as self-contained papers. Two of these papers have been published (Appendix I), and the remaining three are about to be submitted. Details on the authorship of these papers are as follows:

Chapter 2 **Why is the effect of malaria parasites on mosquito survival still unresolved?** 2002. H. M. Ferguson and A. F. Read. Trends in Parasitology 18: 256-261

Chapter 3 **Genetic and environmental determinants of malaria parasite virulence in mosquitoes.** 2002. H. M. Ferguson and A. F. Read. Proceedings of the Royal Society of London Series B – Biological Sciences 269: 1217-1224

Chapter 4: For submission as: **The influence of malaria parasite genetic diversity on mosquito feeding and fecundity.** H. M. Ferguson, A. Rivero and A. F. Read, to Parasitology. A. Rivero assisted with experimental concept and procedures.

Chapter 5: For submission as: ***Plasmodium* virulence and energy uptake in mosquito vectors.** A. Rivero and H. M. Ferguson, to Proceedings of the Royal Society of London Series B – Biological Sciences. H.M.F. and A.R. planned and jointly conducted the experiments and their statistical analysis. Both A.R. and H.M.F made substantial contributions to the writing.

Chapter 6: For submission as: **Mosquito mortality and the evolution of malaria virulence.** H. M. Ferguson, M. Mackinnon, B. H. K. Chan and A. F. Read, to Evolution. M. Mackinnon laid foundation for virulence experiments in mice and demonstrated experimental techniques to HF, and B.H.K. provided experimental assistance.

Abstract:

Vector-borne diseases are amongst the most deadly known to man. Generally, the insect vectors that carry these diseases are assumed to suffer no detrimental consequences from infection, as any such virulence would be maladaptive for the parasite. It is possible, however, that some reduction of vector fitness is an unavoidable consequence of infection. If the magnitude by which infection reduced vector fitness varied between parasite genotypes, vectors could be an important agent of selection on parasite populations.

In this thesis, I tested whether malaria parasites (*Plasmodium* sp.) are virulent to their anopheline mosquito vectors, and using a rodent model of malaria, examined whether virulence in the vector was influenced by parasite genotype, parasite infection diversity, and/or vector nutrition. Additionally, I examined whether there was any genetic or phenotypic correlation between malaria parasite virulence in their vertebrate and vector hosts.

A meta-analysis of published accounts of *Plasmodium*-vector interactions indicated that there is an overall negative effect of parasites on mosquito survival. However, virulence appears to be stronger in studies that use non-natural vector-parasite associations, and in those that extend at least until the sporozoite stage of mosquito infection.

A laboratory study with the rodent malaria parasite *P. chabaudi* and *A. stephensi* vector indicated that mosquito mortality varied with parasite genotype, infection diversity and nutrient availability. In standard conditions, mixed clone infections were the most lethal, but when glucose water was limited, mortality was highest in mosquitoes infected with one of two single clone infections. A second

experiment showed that under standard conditions, mixed infections also had the greatest impact on vector fecundity. The virulence of mixed infections could not be explained by parasite load, nor their rate of resource uptake by parasites within the mosquitoes. During the parasite development period, infected mosquitoes had the same amount of three key physiological resources (lipids, glycogen, proteins), as those that were uninfected. Furthermore, mosquitoes infected with the most virulent parasite genotypes had an increased abundance of glucose relative to the controls. This is consistent with *Plasmodium* manipulating mosquito sugar-feeding behaviour in order to increase its own transmission.

Several laboratory studies of malaria parasites and some field observations suggest that *Plasmodium* virulence in vertebrates is positively correlated with transmission to mosquitoes. A final experiment was undertaken to test whether the transmission advantage of infections that are virulent to vertebrates could be offset by an increased probability of causing death to their vectors. Mice were infected with one of seven distinct genetic clones of *P. chabaudi* that are known to vary in virulence. Infection virulence in mice (weight loss and anaemia) was positively correlated with mosquito infection rate but not with mosquito survival. Vector survival was influenced only by parasite clone and oocyst burden (negative association). These results suggest that vector fitness should not place an upper limit on malaria virulence.

Overall, this research demonstrates that *Plasmodium* can be virulent to its vector, and that the magnitude of virulence is dependent on parasite genotype, infection diversity and environmental conditions. Although *P. chabaudi* virulence in vectors was not correlated with virulence in vertebrates, parasite genetic differences

do impact vector fitness. Thus differential vector-mortality could play an important role in determining the genetic composition of *Plasmodium* populations.

Chapter 1: General Introduction

1.1 Why study parasites?

Benjamin Franklin is attributed with the oft-quoted phrase : ' . . .in this world nothing can be said to be certain, except death and taxes'. Were a similar truism to be derived for all forms of life, the most apt summary would include only the certainty of death, and of parasites. Parasites are organisms that obtain their nutrients from one or several hosts, a process that causes harm and/or a reduction in host fitness but, unlike predation, not immediate death (Begon *et al.* 1996). This strategy has thrived, and is the most ubiquitous lifestyle on earth. Parasites comprise up to 50% of all known animal species (Poulin and Morand 2000). Probably the majority of species have parasites (Esch and Fernandez 1993), with all participating in parasitic interactions either as a host or parasite (deMeeus *et al.* 1998). The success of parasitism is underlined by the fact that this strategy evolved independently at least 60 times, a much higher frequency than known for strategies such as predation (Poulin and Morand 2000). The inclusion of parasites in food webs dramatically alters their shape (Huxham *et al.* 1995), and challenges notions about the efficiency with which resources can be transferred from one trophic level to another. The study of parasites, thus, is the study of how to live successfully on earth.

1.2 Importance of parasites in nature

In addition to their well documented effects on human health and agriculture, parasites have profound effects in natural ecosystems. Theory suggests that they are capable of driving their hosts into decline and/or extinction (Boots and Sasaki 2002),

and there are several examples where this appears to have occurred (e.g. Ginsberg *et al.* 1995; Gulland 1995; Daszak *et al.* 1999). Other ecological effects of parasites include the ability to alter competitive dynamics between species (Hudson and Greenman 1998; Tompkins *et al.* 2001), induce population cycles (Hudson *et al.* 1998), regulate population dynamics (Kohler and Hoiland 2001) and interfere with mutualisms (Currie 2001; Yu 2001). These population-level effects may be either the direct result of parasite-imposed resource drains on individuals, or changes in host behaviour (e.g. Poulin 1995; Webster 2001), resource provisioning (Buechler *et al.* 2002) and mate choice (Able 1996) that are known to accompany parasitism. In any case, the effects of parasites on their host species are diverse, widespread, and sizeable.

1.3 Parasites and evolution

The ecological implications of parasites may be exceeded by their central role in evolution. Parasites have been suggested as a driving force behind the speciation of their hosts (Poulin and Morand 2000), and provide the best evidence for alternate modes of speciation that do not require allopatry (deMeeus *et al.* 1998). There is good reason to believe that parasites experience more intense selection than free-living organisms (deMeeus *et al.* 1998): their environment can actively change to expel them. As a consequence, parasitism itself may intrinsically foster diversity (e.g. Mitter *et al.* 1988).

By definition, parasites harm their hosts, often reducing their fitness (Forbes 1993; Gulland 1995). I refer to these reductions in host fitness as virulence. Such reductions impose selection on hosts for resistance, a phenomenon which garners an

opposite response on the part of parasites for increased virulence. Such antagonism has the potential to drive coevolution in which new parasite virulence and host resistance genes are constantly evolving to evade one another. These interactions are predicted to generate genetic polymorphisms in both parasites and hosts (Sasaki 2000), and favour the spread of other phenomena that also promote genetic diversity such as mutation, recombination, and sexual reproduction (Valen 1973; Hamilton 1980; Hamilton *et al.* 1990; Frank 1993).

Certainly there is a great deal of diversity for virulence and resistance in populations of parasites and their hosts (Dybdahl and Lively 1995; deMeeus *et al.* 1998; Burdon *et al.* 1999; Little and Ebert 2000; Koskela *et al.* 2002; Little 2002). Indeed, almost all medically-important pathogens and parasites of humans are characterized by extreme genetic diversity (Wakelin and Blackwell 1988; Myler 1993; Minchella *et al.* 1995; Barral *et al.* 1996; Robert *et al.* 1996; Fitch *et al.* 1997; Walliker *et al.* 1998; Hutchinson 2001). The rapid emergence of drug and insecticide resistance testifies that host resistance can significantly alter parasite gene frequencies. Evidence for parasite-imposed selection on host gene frequencies is less pervasive (Little 2002), but has been documented (Burdon 1987; Dybdahl and Lively 1998). Thus overall, the parasitic association engenders perhaps the most intense arena of evolutionary conflict and change.

1.4 Evolution of virulence

Of all the evolutionary implications of host-parasite associations, the emergence and maintenance of virulence is perhaps the one that has commanded the most attention (Ewald 1994; Stearns 1999). Presumably this intrigue is derived from

our quest to understand and control infectious disease in humans and our food. Historically, biologists reasoned that parasites should always evolve towards avirulence in their hosts (Ewald 1983), as by doing so they would decrease the chance their host would die before transmission and thus maximise their own fitness.

There are two reasons to doubt this hypothesis. First, there are numerous examples of diseases that appear to have stable levels of intermediate or high virulence in nature (Read 1994). Second, over the last twenty years a substantial body of evolutionary theory has shown that natural selection can favour the evolution of increased pathogen virulence when there is a positive genetic correlation between disease severity and transmission success (Levin and Pimentel 1981; Anderson 1982; Bremermann and Pickering 1983; Anderson and May 1991; Sasaki and Iwasa 1991; Frank 1992; Bull 1994; Read 1994; Ebert and Herre 1996; Frank 1996; Gandon *et al.* 2002). Empirical investigations have shown that positive links between virulence and transmission, the necessary prerequisite for virulence maintenance, are found in most parasite groups that have been studied (Diffley *et al.* 1987; Lipsitch and Moxon 1997; Ebert 1998; Mackinnon and Read 1999a; Mackinnon and Read 1999b; Messenger *et al.* 1999).

Given this preponderance of virulence-associated transmission benefits, the question arises as to what limits the continual evolution towards higher virulence? This issue is usually explained by invoking the existence of a trade-off between parasite virulence and host death (Ewald 1983). However, host death is not a necessary limit to virulence. Natural selection acts to maximise parasite transmission, and if this can be achieved with high host mortality then virulence will increase (Read and Schrag 1991).

Alternative hypotheses to explain how virulence could be limited in the absence of host death include the possibility that morbidity itself imposes a cost on transmission (Ewald 1983; Day 2001), and that the transmission benefits of virulence are reduced when the host population is genetically diverse (Gupta and Galvani 1999; Regoes *et al.* 2000). Also, the optimal virulence level for any parasite may be fundamentally determined by its transmission mode. Transmission mode determines the average number of hosts that a parasite will encounter. In comparison to air or waterborne pathogens, transmission opportunities are relatively few for parasites that are transmitted sexually and vertically. These parasites will pay a harsher price for prematurely killing their host than those who have multiple transmission opportunities, and thus should be less virulent (Bull *et al.* 1991; Ebert and Herre 1996; Frank 1996; Sheldon and Read 1997; Agnew and Koella 1999; Koella and Doebeli 1999).

All of the above hypotheses are based on the analysis of virulence evolution in directly transmitted pathogens (use one host species). There has been little theoretical investigation of the evolution of virulence in parasites with complex life cycles (those that must use more than one host species to complete their development), or consideration of whether their evolutionary dynamics should be fundamentally different from those with direct life cycles. As parasites with complex life cycles are common, and are responsible for some of the most deadly human diseases (Ewald 1983), understanding the factors that shape their evolution would be of great value.

1.5 Evolution and epidemiology of vector-borne diseases

Vector-borne diseases are those in which disease-causing micro-organisms are transmitted between vertebrate hosts by a second species (the vector), which is usually an arthropod (McKelvey *et al.* 1981). In most cases, parasites undergo development in both hosts (Marquardt *et al.* 2000), with the vertebrate being the major site of parasite multiplication and the vector primarily responsible for dispersal. Parasite virulence is predicted to evolve in very different directions in each host. Ewald (1983) hypothesised that higher virulence should in evolve in vector-borne diseases than in those that are directly transmitted. This is because diseases that are directly transmitted require their hosts to be healthy enough to be mobile (to infect new hosts), whereas vector-borne diseases do not. As virulence is generally positively correlated with parasite multiplication rate (see above), it should evolve to a higher level in hosts whose morbidity does not influence transmission. To test this prediction, Ewald (1983) conducted a comparative study of diseases with different transmission modes. This analysis indicated that vector-borne are indeed associated with higher case fatality rates than those that are directly transmitted, but whether these differences can be specifically attributed to transmission or social factors is unclear (see Read *et al.* 1999).

In contrast to the vertebrate host, conventional wisdom postulates that natural selection will favour avirulence in vectors (Ewald 1994). This is because parasites require vectors that are healthy enough to fly between hosts, and that survive long enough for the parasite to complete its extrinsic incubation period. In many cases, the time required for a parasite to develop in its vector equals or exceeds the natural longevity of the vector (Anderson 1981). Thus parasites that have even minor effects

on vector survival could seriously reduce their chance of transmission, and should be selected against (Ross 1911).

Consistent with this prediction, there have been numerous demonstrations that parasites do not reduce the survival of their vectors (Chege and Beier 1990; Robert *et al.* 1990; Makumi and Moloo 1991; Yuval 1991; Schaub 1994; Brito *et al.* 1998), and even some indication that parasites may enhance vector longevity (Randolph 1991). There are, however, multiple examples where parasites have been shown to reduce vector survival: filarial worms (Buxton 1935; Wharton 1957; Lavoipierre 1958; Christensen 1978; Vanamail *et al.* 1994; Basanez *et al.* 1996), *Plasmodium*: (Buxton 1935; Klein *et al.* 1982; Anderson *et al.* 2000), schistosomes: (Davies *et al.* 2001; Fernandez and Pieri 2001), trypansomes: (Maudlin *et al.* 1998), and viruses: (Turell 1992; McGaw *et al.* 1998; Scott and Lorenz 1998; Niebylski *et al.* 1999). Parasite-associated reductions in vector fecundity have also been widely documented (Hacker 1971; Hacker and Kilama 1974; Ham and Banya 1984; Elsawaf *et al.* 1994; Renshaw and Hurd 1994; Hogg and Hurd 1995a; Hogg and Hurd 1995b; Hogg and Hurd 1997; Rubinstein and Czosnek 1997). If virulence towards vectors is maladaptive, why are there so many examples of infection-related fitness reductions?

Accounts of pathogenicity in vectors could be simply an artefact of the laboratory experiments used to study them, which in some cases may be predisposed towards detecting virulence (Chege and Beier 1990; Randolph and Nuttall 1994; Hogg and Hurd 1997). Alternatively, it could be that as with directly transmitted parasites, some level of virulence in vectors promotes parasite fitness. This scenario could arise in a number of ways. As most parasites develop whilst in their vector (Marquardt *et al.* 2000), there must be a minimum level of resource extraction

necessary for parasites to complete their development. If avirulence could arise only by reducing resource extraction below this threshold, clearly it would not be advantageous. Secondly, parasites that are most lethal to their vectors may develop faster, or induce behavioural changes in their vectors that increase their transmission (e.g. through manipulation of the biting rate -survival trade-off: Anderson *et al.* 1999; Koella 1999; Schwartz and Koella 2001). Finally, it is possible that virulence cannot evolve to zero in vectors because it is positively correlated with parasite fitness in the vertebrate host. Whether such a trade-off exists, and how it might function to limit virulence, has been largely unexplored by empiricists or theoreticians.

1.6 Malaria : natural history and epidemiology

Malaria is a serious public health problem for 40% of the world's population (Beier 1998). Throughout the past 25 years, it has been the most important infectious disease in terms of both hospital admissions and numbers of deaths in several African countries (Petit and Vanginneken 1995). In areas of stable endemic transmission in Africa, it is estimated that there are over 200 million clinical cases of malaria each year, resulting in at least one million deaths (Snow *et al.* 1999; Greenwood and Mutabingwa 2002). Malaria is caused by parasites in the genus *Plasmodium* of the phylum Apicomplexa (Smith *et al.* 2002). Only 4 species of *Plasmodium* can infect humans; the remaining 120 species in this family parasitise rodents, bats, birds and reptiles (Bruce-Chwatt 1985). Human malaria parasites are transmitted between their vertebrate hosts by female mosquitoes of the genus *Anopheles.*

In humans, malaria induces a variety of symptoms ranging from mild (fever, headaches, body pains, cough, diarrhoea, dehydration, hypoglycaemia) to life-threatening (severe anemia, respiratory distress, renal failure, cerebral malaria leading to coma, permanent neurological damage) (Miller *et al.* 1994; Snow *et al.* 1999; Miller *et al.* 2002). Malaria is particularly virulent when immunity is compromised during pregnancy, and has been associated with increased rates of maternal mortality, abortion, still birth and low birth weight (Greenwood and Mutabingwa 2002). In addition to these direct effects, malaria indirectly increases the risk of HIV as the severe anaemia it induces can only be treated by blood transfusion, often from unscreened supplies (Snow *et al.* 1999). These health costs have direct impacts on development and perpetuate poverty in malaria-endemic regions (Sachs and Malaney 2002)

1.6.1 *Plasmodium* life cycle

Vertebrate hosts are infected when mosquitoes inject sporozoites into their blood when feeding. These sporozoites migrate into the liver where they develop into schizonts that produce merozoites. Merozoites are released into the bloodstream six to sixteen days after infection, where they invade red blood cells, absorb haemoglobin and asexually replicate. A cycle of replication takes 1-4 days depending on the species (Bruce-Chwatt 1985). As the percentage of red blood cells that are infected rises, hosts may develop clinical symptoms such as fever, anaemia and weight loss. Where infection is not fatal, parasite numbers begin to fall as immune responses develop and eventually clear parasites.

A small percentage of merozoites differentiate into male and female gametocytes. It is only these sexual forms that can infect mosquitoes. Within an

hour of being ingested by a mosquito, gametocytes differentiate and fertilisation occurs to form an ookinete. Ookinetes penetrate the midgut of the mosquito, coming to rest under the basal lamina approximately one day after infection (Simonetti 1996). The ookinete grows into a round elasticised sphere termed an oocyst, in which between 600-10000 sporozoites develop (Rosenberg and Rungsiwongse 1991; Vaughan *et al.* 1992). Approximately 10-14 days after infection, sporozoites burst out from oocysts and migrate to the mosquito salivary glands where they remain until they are injected into a new vertebrate host or die (Beier 1998).

1.6.2 *Anopheles* Life Cycle

Female *Anopheles* require a blood meal to produce eggs. In the wild, females will blood feed once every 2-3 days (Gillies 1953), although they may feed daily or even several times a day if they are disturbed while feeding (Beier 1996). The eggs of *Anopheles* are deposited in or near water, in strings of 100-200 (Bruce-Chwatt 1985). Depending on the temperature, eggs will pass from larvae to adults within 7-20 days (Bruce-Chwatt 1985). Adults copulate a few days after emergence. Females receive enough sperm from one mating to fertilise all subsequent eggs. These eggs develop only if the female has obtained a bloodmeal (1-3 μl in size) prior to laying.

1.6.3 Epidemiology

Ross (1911) was the first to devise a mathematical formulation of malaria transmission. This work was extended by MacDonald (1957) to devise an equation for the basic reproductive rate of malaria infections (R_o). Although more complex formulations of malaria transmission have subsequently been developed (Dietz 1988;

Gandon *et al.* 2002), the Ross-MacDonald model remains widely used because of its generality and simplicity. Under this model, the number of secondary malaria cases that are initiated by one primary case is defined as:

$$R_o = \frac{ma^2bc}{\mu\gamma}$$

In this equation, m is the ratio of vectors to human hosts, a the proportion of vectors that feed on a human host per day, b the proportion of infectious mosquitoes who successfully transmit the parasite, c the proportion of bites by susceptible mosquitoes on infected people that are infectious, μ the per capita rate of mosquito mortality and γ the per capita rate at which infected humans eliminate their infection.

The equation indicates that transmission is sensitive to mosquito survival. Because of the difficulty in recapturing mosquitoes, there are few reliable estimates of their longevity in the wild. However, intensive study of two *Anopheles* species in Tanzania found adult survival to be 77-83% per day (Charlwood *et al.* 1997). Assuming these rates are constant, only 2-15% of mosquitoes are predicted to survive through the 10-14 day *Plasmodium* incubation period. Further reductions in vector survival as a result of infection could markedly decrease transmission. For example, modelling suggests that a parasite strain that causes a 5% reduction in daily vector survival will have only half the prevalence in mosquitoes than a strain that is avirulent (Anderson 1981).

1.7 Evolution of virulence in malaria

Studies of the rodent malaria *P. chabaudi* suggest that in laboratory mice, infection virulence is correlated with parasite replication rate and transmission

success. The virulence of *P. chabaudi* in mice, as measured by weight loss, anaemia, and mortality, varies substantially between parasite genotypes(Mackinnon and Read 1999a; Mackinnon and Read 1999b; Timms *et al.* 2001; Mackinnon *et al.* 2002). Parasite genotype differences in virulence are maintained even when factors such as host genotype (Mackinnon *et al.* 2002), host immune status (Buckling and Read 2001), infective dose (Timms *et al.* 2001), and serial passage history (Mackinnon and Read 1999a) are varied. Generally, there is a positive relationship between infection virulence and measures of parasite growth (maximum parasite density, proportion of red blood cells that are infected) (Taylor *et al.* 1998; Mackinnon and Read 1999a; Mackinnon and Read 1999b; Timms 2001; Timms *et al.* 2001; Mackinnon *et al.* 2002). Infection transmission success, as assessed either by the abundance of transmission stages (gametocytes) in mouse blood, or the proportion of mosquitoes that become infected from feeding on infected mice, is also positively correlated with virulence (Taylor *et al.* 1998; Mackinnon and Read 1999a; Mackinnon and Read 1999b; Timms 2001; Timms *et al.* 2001; Mackinnon *et al.* 2002). Thus in the *P. chabaudi* system, parasites that induce the greatest morbidity in their vertebrate hosts should have the greatest transmission success to mosquitoes.

Comparable studies of human malaria parasites are necessarily lacking, however there is evidence that the multiplication rate of *P. falciparum* in culture, a potential correlate of transmission success, is greater in patients with severe malaria than in those who are asymptomatic (Chotivanich *et al.* 2000). These results suggest that all else being equal, virulent parasites should have a selective advantage. Despite this potential for natural selection to favour increased virulence in malaria, case-fatality rates in humans are very low (0.02-0.08% Trape *et al.* 2002). Human

malaria parasites exhibit tremendous genetic diversity in nature (Walliker *et al.* 1998), and there is ample evidence that parasite genetic variation is associated with disease severity (Yoeli *et al.* 1975; Carlson *et al.* 1990; Rowe *et al.* 1997; Chen *et al.* 1998; Mackinnon and Read 1999a; Mackinnon and Read 1999b; Ariey *et al.* 2001; Ofosu-Okyere *et al.* 2001). If there is an optimal level of virulence in malaria, there is certainly sufficient genetic variation for natural selection to converge upon it. What then limits the evolution of increased virulence in malaria?

1.7.1 Vertebrate host mortality

The death of the vertebrate hosts of *Plasmodium* could place an upper limit on virulence. However, this mechanism is not sufficient to explain the virulence of the rodent malaria parasite *P. chabaudi.* A selection experiment with this parasite found that virulence in mice continued to increase even when a parasite-induced death rate of 50-75% was simulated, a substantially higher rate than occurs naturally. Death rates in Africans infected with *P. falciparum* are 60-4000 smaller (Trape *et al.* 2002) than those simulated in this *P. chabaudi* experiment (Mackinnon and Read 1999a). If these rodent and human *Plasmodium* species have similar fitness functions, it seems unlikely that host mortality could limit the virulence of *P. falciparum.* However, until the relationships between parasite virulence and transmission for this and other human malaria species are known, the strength of host death as a selective force against virulent parasites is unknown.

1.7.2 Vertebrate host morbidity

As malaria is a vector-borne disease, severe illness will not reduce the contact rates between infected and susceptible individuals. On the contrary, disease severity could increase the contact rate between infected and susceptible hosts, as individuals who are ill may exhibit less anti-vector behaviour than those who are asymptomatic (Day and Edman 1983; Ewald 1983). There may be other morbidity-associated factors that reduce selection towards higher virulence. For example, high levels of parasite-induced anaemia may reduce total gametocyte production (Mackinnon *et al.* 2002) and impede mosquito feeding success (Taylor and Hurd 2001).

1.7.3 Vertebrate host genotype

A wide variety of host factors including nutrition, genetics, age, pregnancy, immunity, housing and occupation are known to influence both the susceptibility and severity of malarial disease in humans (Miller 1988; Wernsdorfer and Wernsdorfer 1988; Riley 1996; FernandezReyes *et al.* 1997; Mackinnon *et al.* 2000; Shankar 2000; Miller *et al.* 2002). Specific host genetic traits that modulate infection and immunity include the sickle cell trait, thalassaemia, ovalcytosis, duffy blood group type, MHC locus, glucose-6-phosphate dehydrogenase deficiency, and variation in several cell surface receptors (Miller 1988; Riley 1996; Miller *et al.* 2002). The contribution of host genetic factors to disease prevalence and severity is thought to be large, but is rarely quantified. In the only known study where the relative importance of ecological and genetic factors were quantified, human genetics were shown to explain 15% of the variation in the frequency of malarial attacks, and 10% of the variation in the clinical intensity of illness (Mackinnon *et al.* 2000). Thus, in

human populations, host genetics influence infection. What is not yet known, however, is whether parasite fitness benefits accruing from virulence vary between host genotypes in such a way that no single strategy can prevail. So far, the dependency of virulence on the interaction between host and parasite genotype has been investigated only in mice (Mackinnon *et al.* 2002). In this system, no trade-offs between the success of parasite genotypes in different host genotypes were detected (Mackinnon *et al.* 2002).

1.7.4 Vector fitness

Very little is known about the evolution of virulence in any disease vector, malaria or otherwise. However, as reviewed above, parasites can impose strong fitness costs on their vectors. These fitness costs could select against malaria virulence in the vertebrate host if the following three conditions were met: 1) vector survival is reduced by *Plasmodium*, 2) the effect of *Plasmodium* on vector fitness depends on parasite genotype, and 3) parasite genotypes that cause the most damage to vertebrates induce the greatest fitness cost in vectors. All but the first of these issues have been largely ignored by malaria biologists, and due to the lack of consensus between studies, even it remains unresolved (see Chapter 2).

Although the relationship between parasite virulence in vertebrates and vectors has never been examined in malaria, there is some evidence from other vector-borne parasites that virulence in vectors and in vertebrates are linked. For example, strains of schistostomes that are the most virulent in mice are the most lethal to their snail vector (Davies *et al.* 2001), and the experimental evolution of trypanosomes towards increased virulence is significantly slower when parasites are

passaged through their triatomine vector instead of simply from mouse to mouse (Contreras *et al.* 1994).

In malaria, I envision two possible mechanisms by which selection towards increased virulence in the vertebrate host could generate a correlated reduction in vector fitness. First, the increased density of gametocytes associated with high levels of *Plasmodium* virulence in vertebrates (Mackinnon and Read 1999b) could generate high parasite burdens in mosquitoes which may be associated with decreased longevity (Klein *et al.* 1986). Second, regardless of parasite presence, blood that has endured a severe *Plasmodium* infection could be of poorer quality to mosquitoes. This could occur as a result of parasite-induced anaemia, or changes in blood chemistry that could influence mosquito blood feeding success (Hosoi 1959; Taylor and Hurd 2001).

The general aims of this thesis are to investigate the importance of *Plasmodium* genetics to mosquito fitness, and test whether vector mortality could restrict the spread and evolution of parasite strains that are virulent towards their vertebrate host.

1.8 Experimental system

All experiments described in this thesis were conducted using the rodent malaria parasite *Plasmodium chabaudi,* female inbred laboratory mice, and *Anopheles stephensi* mosquitoes.

1.8.1 The parasite

Plasmodium chabaudi was first isolated from its natural host, the thicket rat *T. rutilans,* in the Central African Republic in 1969-1970 (Killick-Kendrick and Peters 1978). Since this time, these isolates have been stored in liquid nitrogen at the University of Edinburgh. Parasite isolates were cloned to obtain genetically distinct parasite lines, each being derived from a single parasite (Beale *et al.* 1978). This was done by injecting each isolate into mice with an innoculum expected to contain only one parasite (Mackinnon and Read 1999a).

Genetic analysis of these clones revealed they were indeed distinct, with considerable diversity being detected at isoenzyme and antigen loci (within an MSP-1 protein) (Carter 1978; McLean *et al.* 1991). Since first isolation, clones have been periodically passaged through mice in order to multiply the parasite stock (Mackinnon and Read 1999a). It is possible that some adaptation towards laboratory conditions may have occurred during this process.

1.8.2 The vertebrate host

The dynamics of *P. chabaudi* in laboratory mice have been extensively studied (Killick-Kendrick and Peters 1978; Stevenson *et al.* 1982; Jarra and Brown 1985; Cox 1988; Taylor *et al.* 1997a; Taylor *et al.* 1997b; Taylor *et al.* 1998; Taylor and Read 1998; Mackinnon and Read 1999a; Mackinnon and Read 1999b; Timms *et al.* 2001; Mackinnon *et al.* 2002). Asexual parasites replicate once every 24 hours, reaching their maximum density 6-11 days after infection depending on the initial innoculum dose (Timms *et al.* 2001). Maximum gametocyte density occurs a few days after the maximum asexual parasitemia (Landau and Boulard 1978).

In laboratory mice, *P. chabaudi* infections generate very high parasite densities and sometimes cause death (Stevenson *et al.* 1982). This appears to contrast with the dynamics of *P. chabaudi* in its natural host, *T. rutilans,* where infections are chronic and characterized by relatively low parasite densities (Landau and Chabaud 1994). However, *P. chabaudi* infections in mice are also chronic, with small recrudescences occurring up to 3 months after first infection (Cox 1988). Also, experimental infection of thicket rats indicates that the primary infection cycle can be similar to those seen in laboratory mice (Carter and Diggs 1977). Thus the apparent discrepancy between infection dynamics in the natural and laboratory host may simply be due to the fact that field data are mostly drawn from periods of recrudescence.

1.8.3 The vector

The natural vector of *P. chabaudi* is unknown, but it is efficiently transmitted through *Anopheles stephensi* mosquitoes. These mosquitoes are commonly used to study the sporogony of all four rodent malaria species: *P. berghei* (Gad *et al.* 1979; Seitz *et al.* 1987; Yuda *et al.* 1999), *P. chabaudi* (Taylor *et al.* 1997b; Buckling *et al.* 1999), *P. vinckei*: (Alvarez *et al.* 1991; Gautret *et al.* 2000) and *P. yoelii* (Rowland and Boersma 1988; Hogg and Hurd 1995a; Pumpuni *et al.* 1997). *Anopheles stephensi* is found throughout south-east Asia and the Indian sub-continent where its prevalence has been linked to malaria outbreaks and epidemics (Tyagi and Chaudhary 1997). In nature, this mosquito transmits both *P. falciparum* and *P. vivax* (Bruce-Chwatt 1985), the two most important malaria parasites of humans. *Anopheles stephensi* was originally described as a zoophilic (Reisen and Boreham

1979), but more recent research suggests that it can be diverted from cattle onto humans (Hewitt *et al.* 1994). This suggests *A. stephensi* are opportunistic and will feed on a wide variety of mammalian species. The Edinburgh University colony of *A. stephensi* was obtained from stock at the London School of Tropical Medicine and Hygiene. It is maintained on rat blood.

1.8.4 Generality of model system

Ideally, the best way to study malaria evolution and its public health implications would be to work directly with a human malaria parasite. There are several reasons why this is not feasible. First of all, a major aim of this thesis is to examine the influence of infection virulence in vertebrates on vector fitness. Direct testing of this aim would require the experimental infection and/or denial of drug treatment to infected people in order to assess virulence, which would be unethical. Correlational studies relating reported level of disease severity to subsequent mosquito fitness could be conducted. However, the full range of virulence outcomes could not be tested as people with very severe disease would not be able to donate blood for feeding experiments. Also, the percentage of mosquitoes that become infected either from direct feeds on humans or membrane feeds is generally quite low (Vanderberg 1988), thus very large sample sizes would be required to obtain accurate estimates of vector fitness.

The second aim of the study, to investigate the role of parasite genetics on mosquito fitness, could not easily be conducted with a human parasite either. In the laboratory, only a small number of genotypes from human malaria parasites *(P. falciparum* or *P. vivax)* can be cultured and transmitted to mosquitoes. Thus only a

limited range of genetic diversity could be used in laboratory studies of human malaria parasites. Field studies could provide a better sample of parasite genotypes which mosquitoes could be infected with. However, levels of parasite genetic diversity in the field are so high that most studies fail to identify two infected people with the same *Plasmodium* genotype (e.g. Babiker *et al.* 1991; Conway and McBridge 1991; Paul *et al.* 1995; Robert *et al.* 1996). Given this tremendous level of genetic diversity, it would be very difficult to replicate genotype treatments in human hosts. These problems necessitate the use of an animal model of malaria.

Primates are probably the best animal model of human malaria (Butcher 1996). However due to ethical considerations and expense, their use should be limited to cases where no other malaria model system is appropriate. I do not believe this is the case for the experiments described in this thesis. The next best model of mammalian malaria is rodents. Of all rodent malarias, *P. chabaudi* is the best model of the human malaria parasite *P. falciparum,* the most deadly of human parasites (Cox 1988). Specifically, in its laboratory mouse host, *Plasmodium chabaudi* shares several life history features with *P. falciparum* including synchronous schizogony, preference for mature red blood cells, strain-specific immunity and the occurrence of maximum gametocyte density after maximum asexual density has been reached (Walliker 1983; Jarra and Brown 1985; Carter and Graves 1988; Mons and Sinden 1990). Additionally, *P. chabaudi* is the only non-human malaria species in which a range of parasite genotypes with distinct virulence phenotypes (in its vertebrate host) have been isolated, and can be repeatedly grown *in vivo* in the laboratory (Mackinnon and Read 1999b).

The development of *P. chabaudi* in its vector also shares similarities with *P. falciparum*. At their optimum temperature, the sporogonic cycle of both parasite species takes approximately 11 days (Landau and Boulard 1978; Bruce-Chwatt 1985). There are morphological similarities between the sporogonic stages of the two species: their oocysts attain approximately the same maximum size (50 μm and 55 μm in the rodent and human species respectively). This suggests a similar amount of mosquito resources are necessary to fuel the growth of each oocyst type. One dissimilarity between human and rodent malaria species is that the latter frequently give rise to large oocyst burdens in mosquitoes (e.g. > 100 for *P. berghei*, Carter and Diggs 1977). Of all the rodent species, however, *P. chabaudi* infections are associated with the lowest oocyst densities (1-50, Wery 1968; Taylor *et al.* 1997b), which are closest to the average found for *P. falciparum* in the wild (< 5; Taylor and Read 1997). Despite these persuasive arguments in favour of the *P. chabaudi* laboratory system, it is not of course human malaria. Caution is thus required when extrapolating results to disease evolution in humans.

1.9 Thesis aims and overview

The three major aims of this thesis were:

1) To estimate the general impact of *Plasmodium* infection on mosquito survival,
2) To identify whether there are genetic and environmental determinants of *P. chabaudi* virulence in their *A. stephensi* vectors,
3) To test whether there is a genetic and/or phenotypic relationship between *P. chabaudi* virulence in its vertebrate and vector host.

I defined virulence as the fitness costs accruing from a mosquito feeding on infected blood (not simply the fitness of mosquitoes that became infected). The following specific questions were addressed in each chapter:

Chapter 2: Evidence of virulence: Does a meta-analysis of all previously published studies of *Plasmodium*-vector interactions support the notion that infection generally reduces survival?

Chapter 3: Parasite genetic and environmental determinants of vector survival: Is the survival of *P. chabaudi*-infected mosquitoes dependent on parasite clone and/or genetic diversity (one clone vs. two)? Is parasite virulence towards mosquitoes affected by environmental conditions (here nutritional deprivation) ?

Chapter 4: Parasite genetic determinants of vector fecundity: Is the fecundity of *P. chabaudi*-infected mosquitoes dependent on parasite clone and/or genetic diversity (one clone vs. two)?

Chapter 5: Mechanisms of virulence: Does *P. chabaudi* virulence in vectors correspond to resource depletion in mosquitoes throughout the sporogonic cycle?

Chapter 6: Vector fitness and the evolution of virulence: Is there a genetic or phenotypic correlation between *P. chabaudi* virulence in mice and the survival and fecundity of vectors that feed on them?

In the final chapter (7), the principal findings of this thesis are reviewed, apparently contradictory data are discussed, and the evolutionary and ecological implications of this work are reviewed.

Chapter 2: What is the general impact of malaria parasites on mosquito survival?

2.1 Summary:

Despite almost a century of effort, the question of whether malaria parasites kill mosquito vectors remains open. Some direct comparisons of the longevity of infected and uninfected mosquitoes have found malaria-induced mortality, whereas others have not. Meta-analysis was used to show that, overall, malaria parasites do reduce mosquito survival. However, mortality effects are more likely to be detected in unnatural vector-parasite combinations and in studies of longer duration. Until these factors are systematically investigated, no firm generalities are possible.

2.2 Introduction:

During the two weeks that malaria parasites *(Plasmodium* spp.*)* take to complete development in the mosquito, they can cause substantial damage (Box 2.1). However, conventional wisdom postulates that natural selection will favour parasites that do not influence vector survival because a parasite that kills its vector will kill itself. Although this view of virulence evolution has been rejected on theoretical grounds (selection maximises fitness, not life expectancy (Anderson 1982; Bull 1994), it still pervades the malaria literature (Hacker 1971; Anderson and May 1991; Ewald 1994; Dye and Williams 1995). The evidence for it is contradictory. Some indirect field data support the idea (Lyimo and Koella 1992) whereas others do not (Lines *et al.* 1991). Direct laboratory comparisons of the survival of malaria-infected and uninfected mosquitoes have also produced conflicting results, with some

Box 2.1: Mechanisms by which can *Plasmodium* damage its vector

(1) Tissue damage: Mosquito midguts are perforated when ookinetes pass through them (Ramasamy *et al.* 1997). In addition to the physical damage, this perforation might increase susceptibility to bacterial infection or invasion by other parasites during subsequent feeding (Seitz *et al.* 1987; Vaughan and Turell 1996).

(2) Resource depletion: Infected mosquitoes have lower concentrations of amino acids in their haemolymph than those that are uninfected, and their midguts use eight times as much glucose (Hurd *et al.* 1995; Beier 1998).

(3) Cost of immunity: Mosquitoes can mount a diverse array of immune responses when invaded by pathogens (Barillas-Mury *et al.* 2000). *Plasmodium* infection elicits the transcriptional activation of at least 6 different immune markers in the human malaria vector *Anopheles gambiae,* particularly when parasites are invading the midgut and salivary glands (Dimopoulos *et al.* 1998; Dimopoulos *et al.* 2001). The production of these molecules could be energetically costly, and divert resources away from growth and maintenance. Some mosquitoes kill oocysts by melanotic encapuslation, a process that is known to reduce mosquito ovary size and protein content when directed against filarial worms (Ferdig *et al.* 1993). Mosquitoes selected to be refractory to *Plasmodium* have also been shown to have poorer fitness than susceptible mosquitoes in the absence of infection (Yan *et al.* 1997).

(4) Behavioural modification: Infected mosquitoes have lower amounts of salivary apyrase (a platelet inhibitor)(Simonetti 1996). Consequently these mosquitoes spend more time feeding, probe more often (Rossignol *et al.* 1984; Wekesa *et al.* 1992), are more persistent in biting (Anderson *et al.* 1999), and feed more often (Koella *et al.* 1998). These changes in behaviour are likely to increase the risk of infected mosquitoes being detected and killed while feeding.

reporting reduced mosquito survival (Buxton 1935; Thompson and Huff 1944; Maier 1973; Gad *et al.* 1979; Klein *et al.* 1982; Klein *et al.* 1986; Hogg and Hurd 1995a; Hogg and Hurd 1995b; Ferguson and Read 2002a) and others finding vector survival unaffected (Mayne 1920; Wenyon 1926; King 1929; De Buck 1936; Sinton and Shute 1938; Boyd 1940; Rossignol *et al.* 1986; Freier and Friedman 1987; Seitz *et al.* 1987; Chege and Beier 1990; Robert *et al.* 1990; Gamage-Mendis *et al.* 1993; Hogg and Hurd 1997) (Table 2.1). This inconsistency is surprising: if *Plasmodium* does impose a direct cost on mosquito survival, why is it not consistently found in controlled laboratory experiments? Understanding the reason for this variation is crucial not only to answer the general question of whether malaria parasites are detrimental to their vectors, but also to identify the conditions under which vector survival could limit the epidemiology and evolution of malaria.

2.3 Methods:

An analysis of previously published laboratory studies (n = 22, Table 2.1) was conducted to determine the overall direction and magnitude of *Plasmodium* effects on mosquito survival, and to test whether variation in outcomes could be explained by experimental design. Quantitative meta-analytic methods were used (Arnqvist and Wooster 1995; Sutton *et al.* 2000), which require that summary statistics and sample sizes are given for each study so that a standard measure of statistical effect size can be computed. This information was missing from several studies (Mayne 1920; Wenyon 1926; King 1929; De Buck 1936; Boyd 1940; Thompson and Huff 1944; Gad *et al.* 1979; Rossignol *et al.* 1986; Freier and Friedman 1987; Seitz *et al.* 1987; Gamage-Mendis *et al.* 1993). Consequently, meta-

analysis was conducted on only 11 of the original 22 studies (Table 2.2). In total, these 11 studies provided information on 24 separate experiments.

Standardised effect sizes for each of these 24 experiments were obtained by converting the one-tailed *p*-value for the test of differences in survival between infected and uninfected mosquitoes into a standard normal deviate *(z)*, and dividing by the square root of the sample size (Rosenberg *et al.* 2000). A positive value was assigned to the effect size (*r*) if the survival of uninfected mosquitoes was higher than that of infected mosquitoes. In cases where one study consisted of several different experiments, separate values of *r* were computed for each trial. The mean effect size was calculated using the program METAWIN (Rosenberg *et al.* 2000). Mixed effects analysis was used to evaluate the relationship between effect size and experimental design parameters.

Table 2.1: Studies investigating the impact of *Plasmodium* on vector survival. Column B refers to whether the species combination is known to exist in the wild: Y = yes, N = No, ? = unknown (according to Garnham 1966). When study length is unclear from the relevant paper, a missing value is given. Column C indicates whether statistics were reported: Y = yes, N = No, and N* indicates studies without statistical analysis but where raw data was given; in those cases, I did an appropriate test (see Appendix 2.6). All studies involve experimental blood feeds, except Chege & Beier (1990) and Hogg & Hurd (1997) which used wild-caught mosquitoes.

Study	Parasite	Vector	B	Study length (days)	C
Studies where *Plasmodium* reduced vector survival					
Buxton 1935	*P. praecox*	*C. fatigans*	?	5	N*
Thompson & Huff 1944	*P. rhadinurum*	*Ae. aegypti*	N	3	N
		C. fatigans	N	3	N
Maier 1973	*P. cathermerium*	*C. pipiens*	?	20	N*
Gad *et al.* 1979	*P. berghei*	*An. stephensi*	N	14	N
Klein *et al.* 1982	*P. cynomolgi*	*An. dirus*	N	65	Y
Klein *et al.* 1986	*P. cynomolgi*	*An. dirus*	N	65	Y
Hogg & Hurd 1995a	*P. yoelii*	*An. stephensi*	N	6	Y
Hogg & Hurd 1995b	*P. yoelii*	*An. stephensi*	N	6-18	Y
Ferguson & Read 2002a	*P. chabaudi*	*A.n stephensi*	N	35	Y
Studies where *Plasmodium* did not change vector survival					
Mayne 1920	*P. vivax*	*An. punctipennis*	Y	.	N
Wenyon 1926	*P. falciparum*	*Various Anopheles*	Y	.	N
King 1929	*P. vivax*	*An. punctipennis*	Y	17	N
	P. falciparum	*An. crucians*	Y		N
De Buck 1936	*P. vivax*	*An. maculipennis*	Y	.	N
Sinton & Shute 1938	*P. vivax*	*An. maculipennis*	Y	7-19	N*
Boyd 1940	P. vivax	An.. quadrimaculatus	Y	.	N
Rossignol *et al.* 1986	*P. gallinaceum*	*Ae. aegypti*	N	7	N
Freier & Friedman 1987	*P. gallinaceum*	*Ae. aegypti*	N	8	N
Seitz *et al.* 1987	*P. berghei*	*An. stephensi*	N	20	N
Chege & Beier 1990	*P. falciparum*	*An. funestus*	Y	30	Y
		An. gambiae	Y	30	Y
Robert *et al.* 1990	*P. falciparum*	*An. gambiae*	Y	5	Y
Gamage-Mendis *et al.* 1993	*P. falciparum*	*An. tessellatus*	Y	14	N
Hogg & Hurd 1997	*P. falciparum*	*An. gambiae*	Y	5	Y

Study	*r*	N	Oocyst density	Temperature (C)	Humidity (%)
Buxton 1935	0.187	99	-	23	90
Maier 1973	0.387	2692	-	25	83
Klein *et al.* 1982	0.686	720	-	26	67
Klein *et al.* 1986	0.195	280	< 10	26	67
	0.705	360	10-40		
	0.827	480	41-70		
	0.907	320	> 71		
Hogg & Hurd 1995a	0.259	207	> 75	27	80
Hogg & Hurd 1995b	0.056	101	> 75	27	80
	0.048	93	> 75		
	0.032	116	4.4		
	0.137	100	4.4		
	0.286	100	4.4		
Ferguson & Read 2002a	0.010	1527	17.2	27	70
	0.058	1814	44.6		
Sinton & Shute 1938	-0.048	60	-	24	75
	-0.001	100		24	
	0.028	40		24	
	0.060	50		24	
	0.449	60		24	
Chege & Beier 1990	0	1128	-	25.3	65.5
	0	1221			
Robert *et al.*1990	0.005	1600	-	28	80
Hogg & Hurd 1997	0.047	967	2.4	24.5	80

Table 2.2: Studies used in the meta-analysis of *Plasmodium* and vector survival. *r* is the Pearson correlation coefficient representing the standardised effect size of *Plasmodium* on mosquito survival; N, the total number of mosquitoes used in each experiment. Oocyst density is the mean number of oocysts on the midgut of an infected mosquito. Values of temperature and humidity are means.

2.4 Results:

2.4.1 Overall effect

The proportion of studies that have found a statistically significant detrimental effect of *Plasmodium* is similar to those that have found no effect (41% *vs.* 59%, Table 2.1). For all accounts of reduced survival to have arisen by chance alone (Type 1 errors), there would need to be about 360 unpublished studies with null results, in addition to about nine showing *increased survival.* Given the experimental effort involved in survival studies, and the novelty of showing that malaria is a longevity-enhancer, this degree of under reporting seems unlikely. The lack of any studies showing a significant positive effect of *Plasmodium* is interesting because if parasites can manipulate mosquito longevity, they might be expected to enhance it (at least during the time when oocysts are growing, e. g. Schwartz and Koella 2001) by, for instance, reducing fecundity and hence the longevity costs of reproduction (Hurd *et al.* 1995). The absence of this effect in any of these studies suggests such manipulation is not occurring.

Quantitative analysis shows that, in 22 out of 24 experiments, *Plasmodium*-infected mosquitoes had poorer survival than their uninfected counterparts (Table 2.2) and that overall, malaria does reduce mosquito survival (Table 2.3). The mean effect size was similar when experiments were treated as independent units, and when analysis was conducted on the average effect size of all experiments in a study. Across the 24 experiments that were examined, there was no relationship between effect size and sample size ($p = 0.26$). To include as much data as possible, all further meta-analyses were conducted at the level of experiment.

Sample	Mean *r*	95% CI of *r*	N
All Experiments	0.287	0.136 - 0.470	24
Studies (experiments pooled within a study)	0.259	0.102 - 0.447	11
Experiments of natural associations	0.061	-0.004 - 0.170	9
Experiments of non-natural associations	0.436	0.201 - 0.705	13
Experiments ending before sporozoites	0.129	0.055 - 0.218	10
Experiments ending after sporozoites	0.395	0.147 - 0.664	14

Table 2.3: Mean effect sizes of *Plasmodium* on mosquito survival. If there was no effect of malaria infection on longevity, effect size would be zero; positive effect sizes are when mortality is increased by infection. Confidence intervals (CI) were obtained by bootstrapping. Statistical analysis was conducted on Z-transformed values of *r*.

2.4.2 Choice of species

Of the 22 survival studies that were identified (Table 2.1) , ten used combinations of vector and parasite species found in nature, ten used unnatural combinations, and for two the natural vector is unknown. Of the ten studies that used an unnatural vector-parasite combination, seven found that infection decreased mosquito survival; none of the ten studies that used natural associations reported a significant effect, although all studies exhibited a tendency towards poorer survival in the infected group (Table 2.2). The magnitude of *Plasmodium* effects on mosquito survival is substantially greater when novel pairings are used (Table 2.3, $p = 0.04$, $n = 22$).

This analysis supports the notion that *Plasmodium* is harmful only in novel vector species, an idea that is often proposed to explain the lack of virulence in studies of natural infections (Chege and Beier 1990; Hogg and Hurd 1997). Assuming that the artificial combinations used in the laboratory are a random sample of novel pairings, is there an *a priori* reason to expect them to be more virulent? It is often assumed that only maladapted parasites are virulent, so that virulence is high in host-parasite associations that have not co-evolved. Yet neither theory nor empirical studies support this: both increased and decreased virulence can arise from novel host-parasite pairings (Ebert 1994; Read 1994; Woolhouse *et al.* 2001). Certainly there are many accounts of increased virulence in novel associations (Fenner and Ratcliff 1965; Taylor *et al.* 2001), but ancient virulent associations, and avirulent interactions are likely to be unnoticed (Read 1994).

Although a reduction in mosquito survival is more commonly reported in unnatural *Plasmodium*-vector combinations, there are examples of novel combinations that did not result in virulence (Seitz *et al.* 1987). Furthermore, a range of outcomes can be found for the same vector-parasite combination, with different studies reporting different effects (e.g. *P. berghei* in *A. stephensi* reduced survival (Gad *et al.* 1979) or did not (Seitz *et al.* 1987)). Comparative studies, in which the effect of malaria parasites is assessed simultaneously in natural and novel vectors in the same laboratory, are crucial to determine the relevance of co-evolutionary history to mosquito-malaria interactions.

2.4.3 Length of Study

Mosquitoes cannot transmit malaria until approximately two weeks after infection, when the parasite has transformed into a sporozoite and invaded their salivary glands. Natural selection should minimise virulence, at least until sporozoites have developed. Once sporozoites have developed, natural selection will favour parasites who can increase the biting rate of their vectors, possibly at the expense of their longevity (Koella 1999; Schwartz and Koella 2001). This prediction has received empirical support from the observation that sporozoite-infected mosquitoes are more persistent feeders and have greater feeding-associated mortality than their uninfected counterparts (Anderson *et al.* 1999; Anderson *et al.* 2000).

Even though only one of the studies in this meta-analysis allowed host biting after initial infection (Hogg and Hurd 1995b), longevity effects were more likely to be detected in studies that lasted until the sporozoite stage or longer. Effect size was positively and linearly related to study length ($p = 0.001$), with the overall effect size

for studies ending after sporozoite invasion being greater than those that ended before (Table 2.3). These time-dependent effects could arise because sporozoites cause more physiological disruption than oocyst or ookinetes, because parasites alter mosquito behaviour at the sporozoite stage (coincidentally increasing mortality) or simply because small differences in daily survival are more easy to detect over long time periods. Regardless of the mechanism, this analysis strongly suggests that virulence will be underestimated by studies that end before the completion of *Plasmodium*'s extrinsic incubation period.

2.4.4 Dose effects

Malaria biologists have long argued that *Plasmodium* is harmful to mosquitoes only when parasite load is very high (De Buck 1936; Hogg and Hurd 1995a). This idea has been used to dismiss the possibility that parasites could limit vector populations because few infected mosquitoes carry more than 1-2 oocysts in the wild (Chege and Beier 1990). However, the absence of high oocyst burdens could also be due to mortality of heavily infected mosquitoes (Koella 1999). In the laboratory, variable effects of parasite dose have been reported. In one study, mortality increased with the density of asexual parasites, but only at one of two temperatures (Gad *et al.* 1979). In other studies, mortality was unrelated to gametocytaemia (Maier 1973; Ferguson and Read 2002a, Chapter 3), and oocyst burdens have been correlated with mortality in some (Klein *et al.* 1986; Hogg and Hurd 1995b), but not all studies (Ferguson and Read 2002a, Chapter 3). We know of no studies comparing sporozoite load with mosquito survival.

It is difficult to assess the importance of parasite dose to effect size as estimates of blood stage and sporozoite densities were mostly absent from the studies reviewed here. Some analysis was possible for oocyst burden, a parameter that was reported in 5 studies (12 experiments) (Klein *et al.* 1982; Klein *et al.* 1986; Hogg and Hurd 1995a; Hogg and Hurd 1995b; Ferguson and Read 2002a, Chapter 3). In this sample, there was no relationship between effect size and oocyst burden ($p = 0.76$, $n = 12$). Clearly, the data are far from definitive, but what there is suggests that oocyst burden is not a large, universal predictor of harm.

2.4.5 Environmental conditions

Mosquito longevity varies with temperature (Buxton 1935; Gad *et al.* 1979), season (De Buck 1936), diet (Straif and Beier 1996) and larval density (Gomulski 1985; Mercer 1999). The particular rearing, climatic and nutrient regimes used in different labs could therefore influence mosquito survival. Furthermore, this variation is likely to influence the ability of mosquitoes to tolerate parasitism. Several studies have shown that *Plasmodium*-induced mortality varies with environmental conditions such as temperature (Buxton 1935; Gad *et al.* 1979), diet (Ferguson and Read 2002a, Chapter 3), adult density (Maier 1973), and bacterial infection (Seitz *et al.* 1987). The survival studies examined here were performed over a wide range of temperature (21-30 C) and humidities (57-90%). Across experiments there was no obvious relationship between temperature and effect size ($p = 0.42$, $n = 24$), but there was for humidity ($p = 0.04$), with less *Plasmodium*-induced mortality at higher humidities. Experiments are required to assess whether this association has any causal basis.

Diet is another factor that could influence mosquitoes response to *Plasmodium* but, because there was little replication of dietary conditions between studies, no quantitative analysis of this variable was conducted. Quantitative analysis was also not possible for larval density because this trait was mentioned in only 5 studies (and an effect size was computable only for three of them). It is interesting to note, however, that the mean larval density used in the three studies that found *Plasmodium* to be virulent (Hogg and Hurd 1995a; Hogg and Hurd 1995b; Ferguson and Read 2002a, Chapter 3) was almost 5 times greater than in the two that did not (Rossignol *et al.* 1986; Freier and Friedman 1987) (mean larval density in studies that showed no effect = 113 larvae/ L, mean larval density in studies that found reduced survival = 555 larvae/ L). This variation is likely to be important, as even a four-fold increase in larval density can generate a significant decrease in adult body size (Mercer 1999), which is a prime determinant of mosquito survival (Ameneshewa and Service 1996). Small mosquitoes produced from high density larval conditions may have fewer resources to combat losses imposed by parasites. Experimental tests of the relationship between larval density and susceptibility to *Plasmodium* would be valuable.

2.4.6 Choice of Control

Early research on *Plasmodium*-mosquito associations was mostly observational. Several papers published before 1950 did not use an uninfected control, but asserted their results anecdotally (Mayne 1920; De Buck 1936; Boyd 1940). Despite this, these studies are still cited as evidence for the neutrality of *Plasmodium* to their vector (e.g. Klein *et al.* 1982; Chege and Beier 1990; Robert *et*

al. 1990; Ameneshewa and Service 1996). Among the studies with controls, there still exists an important distinction in the type: (1) mosquitoes fed on uninfected blood or (2) mosquitoes without parasites regardless of the infection status of earlier blood meals. Field-based studies must use the latter design because it is not possible to ascertain the feeding history of wild-caught mosquitoes (Chege and Beier 1990; Hogg and Hurd 1997). However, using this type of control group could be misleading if pathology associated with infection arises not from parasite development but from the toxicity and/or poor nutrient quality of infected blood. If mosquitoes fed on infected blood have lower fitness even when oocysts and sporozoites do not develop, field-based studies might underestimate the effect of malaria on vector survival. This data set has too few studies that have used the 'no sporozoite' design (n =2) to draw any conclusions.

2.4.7 Genetic diversity

Field studies of *P. falciparum* and *P. vivax* have shown each species harbours an enormous amount of genetic diversity for traits that mediate infection (e. g. merozoite surface proteins, Walliker *et al.* 1998). However, most laboratory studies of vector-parasite interactions use only one species of inbred mosquito and one genetically homogeneous *Plasmodium* strain. Studies of other invertebrates and their parasites have shown that susceptibility to infection depends on parasite genotype (Webster and Woolhouse 1998; Little and Ebert 2000). These results have recently been corroborated for malaria, where the survival of mosquitoes infected with *P. chabaudi* was shown to vary with parasite genotype (Ferguson and Read 2002a, Chapter 3). One genotype was apparently benign; another reduced mosquito survival

(these effects are averaged in Table 2.2). Thus, a single study captures the contrasting conclusions of different studies. Results from field studies are not limited by genetic homogeneity, as wild-caught mosquitoes are infected with many different parasite genotypes (Babiker *et al.* 1994). However, averaging the consequences of infection over many parasite genotypes may obscure important variation in pathogenicity.

2.5 Discussion:

This analysis has identified several possible reasons why some studies have found that vectors are killed by malaria parasites whereas others have not. Studies reporting detrimental effects typically involve experiments of longer duration and unnatural vector-parasite combinations, and there might also be an effect of humidity. However, it is not easy to disentangle these various factors because they are confounded in studies to date. For example, the longest studies have been done on unnatural host-parasite combinations (35.9 *vs.* 15.1 days respectively, $F_{1,20} = 5.38$, $p = 0.03$), and the laboratory reporting the strongest effects (Klein *et al.* 1982; Klein *et al.* 1986) is also the one that maintained its mosquitoes in the driest atmosphere. Rather than over-extend the statistics of meta-analysis, we suggest that experimental manipulations of these factors in a single laboratory is required to disentangle these factors, and others such as genetic diversity.

In any case, are data on mosquito longevity in laboratory cages relevant to the natural transmission setting? Critics have suggested that the frequent use of unnatural vector-parasite combinations will generate artificially high virulence (Hogg and Hurd 1997), and that laboratory experiments exclude possible indirect

costs of infection, such as increased risk of predation and feeding-associated mortality (Anderson *et al.* 2000). This meta-analysis is certainly consistent with the first objection, further experimental tests are required to separate the effects of co-evolutionary history and experimental design.

The importance of the second issue (failure to incorporate indirect effects) is unclear. Certainly, mosquitoes face a diverse array of environmental and biotic hazards in the field, most of which are eliminated within laboratory cages. Host defensive behaviour, for example, is a potentially significant source of mortality for mosquitoes (Day and Edman 1983), yet none of the studies reviewed here allowed the possibility of post-infection anti-vector behaviour. Indirect mortality costs are probably higher for infected mosquitoes. It has been shown that, under natural field conditions, mosquitoes with sporozoites have greater feeding-associated mortality than those without (Anderson *et al.* 2000) . Laboratory results support this observation, confirming that infected mosquitoes are more persistent feeders (Anderson *et al.* 1999), and have poorer flight ability (Schiefer *et al.* 1977; Rowland and Boersma 1988), a trait which may reduce their ability to evade defensive behaviour.

Short of unethical mark-recapture experiments of experimentally infected mosquitoes, it will be difficult to estimate the relative importance of the longevity effects detected in the laboratory and those that are a result of natural host feeding. The most plausible way forward is for experiments in the laboratory to incorporate other possible sources of mortality. We know of only one study where there was simultaneous estimation of the survival of a population when kept in cages and when free-living (Chege and Beier 1990). Interestingly, this study found survival to be

higher in the free-living population, suggesting that the protective benefits of being in a cage may be outweighed by other factors. Only in the laboratory can mortality be properly evaluated over a range of conditions, such as variation in number of blood meals, and environmental stress. Setting these results into the context of the mortality experienced during a natural transmission cycle will require substantially more research, both in the laboratory and the field.

2.6 Appendix: Statistical analysis of raw data in previously published studies

(1) Buxton (1935)

Data were from 3 experiments where the number of mosquitoes dying in control and infected cages over an 11 day period was given. Data was pooled from all 3 experiments and used to compute Kaplan-Maier survival curves (SPSS 1995). The p-value from the log rank test (factor: infection status) was used to compute the effect size (r) (log-rank statistic = 3.46, p = 0.06).

(2) Sinton and Shute (1938)

Data were from 5 experiments where the number of mosquitoes dying in control and infected cages were given. Data from these experiments were not pooled as they lasted for a different numbers of days (7-19). Kaplan-Maier survival curves were computed for each experiment and differences between infected and uninfected groups were detected with the log rank test (Expt 1: log-rank statistic = 0.14, p = 0.71, Expt 2: log-rank statistic = 0.01, p = 0.99, Expt 3: log-rank statistic = 0.03, p = 0.86, Expt 4: log-rank statistic = 0.18, p = 0.67, Expt 5: log-rank statistic = 12.3, $p < 0.01$).

(3) Maier (1973)

Data were presented as the mosquito median survival of (n = 24, 18 infected, 6 uninfected). The effect size (r) was computed from the F-statistic for the ANOVA analysis of median survival as a function of infection treatment ($F_{1,22}$=4.01, p = 0.06).

Chapter 3: Genetic and environmental determinants of malaria parasite virulence in mosquitoes

3.1 Summary:

Models of malaria epidemiology and evolution are frequently based on the assumption that vector-parasite associations are benign. Implicit in this assumption is the supposition that all *Plasmodium* parasites have an equal and neutral effect on their vector, and thus that there is no parasite genetic variation for vector virulence. While some data support the assumption of avirulence, there has been no examination of the impact of parasite genetic diversity. A laboratory study with the rodent malaria parasite *Plasmodium chabaudi* and the vector *Anopheles stephensi* was conducted to determine whether mosquito mortality varied with parasite genotype (CR and ER clones), infection genetic diversity (single *vs.* mixed clone) and nutrient availability. Vector mortality varied significantly between parasite treatments, but the rank order of virulence depended on environmental conditions. In standard conditions, mixed clone infections were the most virulent but when glucose water was limited, mortality was highest in mosquitoes infected with CR. These genotype-by-environment interactions were repeatable across two experiments and could not be explained by variation in anaemia, gametocytaemia, blood meal size, mosquito body size, infection rate or oocyst burden. Variation in the genetic and environmental determinants of virulence may explain conflicting accounts of *Plasmodium* pathogenicity to mosquitoes in the malaria literature.

3.2 Introduction:

The basic reproductive rate of many infectious diseases is critically dependent on the life span of their hosts (Anderson 1982). For successful transmission to new vertebrate hosts, malaria-infected mosquitoes must survive at least as long as the extrinsic incubation period within the vector, which can be two weeks or more. Several authors have argued that this means there will be strong selection pressure on malaria parasites (*Plasmodium*) not to reduce vector survival (Ewald 1994; Dye and Williams 1995; Koella 1999). However, the evidence that malaria parasites are indeed benign in their vectors is mixed. Some indirect field data support the notion of survival costs to infection (Lyimo and Koella 1992), whereas others do not (Lines *et al.* 1991). There are substantially more data from experimental infections, but these too are contradictory. Several studies have reported reductions in vector longevity (Gad *et al.* 1979; Klein *et al.* 1982; Klein *et al.* 1986; Maier *et al.* 1987) while others have not (Sinton and Shute 1938; Freier and Friedman 1987; Chege and Beier 1990; Robert *et al.* 1990; Hogg and Hurd 1997).

One explanation for these conflicting accounts is that virulence (here defined as a reduction in survival) is not a fixed property of infection, but varies with parasite genotype. This phenomenon has been documented within vertebrate hosts of *Plasmodium*, where parasite genetic variation is associated with disease severity (Yoeli *et al.* 1975; Carlson *et al.* 1990; Rowe *et al.* 1997; Chen *et al.* 1998; Mackinnon and Read 1999a; Mackinnon and Read 1999b; Chotivanich *et al.* 2000; Ofosu-Okyere *et al.* 2001). However, the relationship between parasite genotype and mosquito survival has not been examined. Genotype-specific reductions in vector survival could arise either as a direct consequence of differential pathogenicity of

parasites to the mosquito, or indirectly as a function of parasite-induced changes that reduce the nutritional quality of host blood.

Plasmodium virulence in vectors could also be affected by the genetic diversity of an infection. Some evolutionary theory predicts that virulence should increase as the genetic diversity of parasites in an infection increases (Sasaki and Iwasa 1991; Frank 1992; van Baalen and Sabelis 1995; Frank 1996), a pattern that has been observed (reviewed in Read and Taylor 2001). Increased virulence of genetically diverse infections has been attributed to many factors, including the promotion of more virulent strains and overall parasite burdens under competition (Hargreaves *et al.* 1975), collateral damage due to the release of competition-mediated allelopathic substances (Chao *et al.* 2000), and the increased difficulty of immune control of mixed infection (Taylor *et al.* 1998). It is known that mixed genotype infections of *P. chabaudi* are more virulent in mice (Taylor *et al.* 1998), and are more infectious to mosquitoes than single genotype infections (Taylor *et al.* 1997b). The epidemiological consequences of the enhanced transmission of these mixed infections will depend critically on whether they are also more lethal to mosquitoes.

In addition to parasite genetics, environmental conditions may be an important determinant of virulence: fitness costs are often more evident in harsh conditions (Stearns 1992; Bergelson and Purrington 1996). Many laboratory studies of vector-parasite interactions, however, take place in conditions that aim to maximise vector survival. Consequently, the cost of parasitism may be frequently underestimated. This alone could generate apparently benign vector-parasite associations in some laboratory experiments even if virulence in the wild is high.

Furthermore, variation in environmental conditions between laboratories may influence the likelihood of detecting parasite-induced mortality, a hypothesis supported by meta-analysis of previously published studies of *Plasmodium* virulence in mosquitoes (Ferguson and Read 2002b, Chapter 2) .

The outcome of *Plasmodium*-vector interactions has potentially important implications for malaria epidemiology (Macdonald 1957; Dye and Williams 1995). It is also critical in understanding the conditions that shape virulence evolution (Bull 1994), both in the vector itself and in the vertebrate host. Here we conducted a laboratory study using the *P. chabaudi*-laboratory mouse model to examine whether (1) vector survival varies with parasite genotype, (2) vector mortality increases with the overall level of diversity in an infection (single versus mixed genotype infections), and (3) whether parasites cause greater mortality when vectors are maintained in sub-optimal conditions.

3.3 Methods:

Anopheles stephensi larvae were reared in standard insectary conditions of 27 ± 1°C, 70% humidity and a 12:12 light dark cycle. Eggs were placed in plastic trays (25 x 25 x 7 cm) filled with 1.5 L of distilled water. In order to reduce variation in larval growth rate and size at emergence, larvae were reared at a fixed density of 500 per tray (from first day after hatching). Larvae were fed on Liquifry™ for 5 days and then on ground Tetrafin™ Fish flakes. Larvae took 9-14 days to transform into pupae. On days 10-13 post hatching, groups of 250 pupae were placed in one of 16 emergence cages (16 x 16 x 16 cm). The adults that emerged (n=170-240) were fed

ad libitum on a 10% glucose water solution supplemented with 0.05% para-aminobenzoic acid PABA.

Two genetically distinct clones of *Plasmodium chabaudi* known as CR and ER were used (Beale *et al.* 1978) (from the World Health Organisation's Registry of Standard Malaria Parasites, University of Edinburgh). These clones were chosen because their behaviour has been extensively studied in the vertebrate host, where they are known to generate infections of similar length and parasite density (Taylor *et al.* 1997a). Clones are asexually replicated lineages derived from a single ancestral parasite which was originally isolated by serial dilution. Groups of four mice (C57BL/6J) were infected with 10^5 parasites of CR, 10^5 of ER, 10^5 of a 1:1 mix of CR and ER (5×10^4 CR + 5×10^4 ER), or were left uninfected. Previous molecular analyses of parasites in mosquitoes show that both of these genotypes will transmit from mixed infections in mice (Taylor *et al.* 1997a). From the fifth day of infection onwards, thin blood smears were taken on a daily basis. Mosquito feeds took place 14 days after mouse infection, when all infected mice had sufficiently high gametocytaemia (proportion of red blood cells infected with gametocytes > 0.1%).

To increase hunger levels, mosquitoes were deprived of glucose for 24 hours before feeding on the mice. One anaesthetised mouse was placed on each cage and mosquitoes were allowed to feed for 20 minutes (16 cages, 85-120 females per cage). After the feed, half the cages from each parasite treatment were supplied with glucose water *ad libitum*. The others were supplied with glucose water for 24 hours every second day until the experiment was terminated. In a pilot study, this feeding regime reduced the survival of uninfected mosquitoes by 50%. Cages were then

checked daily and dead mosquitoes were removed. No further blood meals were given after the first infective feed.

Fifteen to twenty females from each cage were killed with chloroform on days 8 and 9 after the blood feed. Their midguts were dissected in a drop of a 0.01 M solution of phosphate-buffered saline and examined under a microscope to assess oocyst prevalence and burden.

The entire experiment was repeated six months later (hereafter called block two). In this second block, mean blood meal size and anaemia were also estimated. Mean blood meal size was measured indirectly as the mass of hematin excreted by 4-5 mosquitoes that were taken from each cage immediately after the feed and moved individually into 30 ml tubes for hematin collection (as in Briegel 1980). Only mosquitoes that were fully engorged with blood were used for this analysis. All mosquitoes collected for this analysis excreted hematin, confirming that they had indeed taken a blood meal. Anaemia was measured as the number of red blood cells in a 2 ul sample of mouse blood taken a few hours before the feed. A final difference from the first block was that an error in glucose treatment assignment on the day of the feed meant that only one of the four CR cages was placed under glucose water deprivation, and three of the four ER cages were placed under glucose water deprivation.

In both blocks, survival monitoring continued for 35 days after the feed except in two cases where observation was curtailed due to cage damage (block 1, CR-infected cage under glucose deprivation, stopped on day 29) and problems with glucose water delivery (block 2, ER-infected cage under glucose deprivation, stopped on day 27).

3.3.1 Statistical Analysis

Statistical analysis was conducted in two stages to account for survival measurements estimated at the individual level (response variable: individual's day of death) and at the cage level (response variables: (i) median survival of all mosquitoes in a cage, and (ii) the proportion of mosquitoes in a cage surviving until day 14 post feed). Both median survival and the proportion of mosquitoes surviving until day 14 were obtained from Kaplan-Maier estimates of the survival distribution in each cage (SPSS 1995). The proportion of mosquitoes surviving until day 14 was examined as it is an index of survival at the time when mosquitoes are first able to infect new hosts. Sporozoite invasion of the salivary glands can begin as soon as 10 days after an infectious feed (Killick-Kendrick and Peters 1978), and is probably complete by day 14 (R. Carter, pers. comm.). General Linear Models were used to evaluate the relationship between each of the two cage-level survival indices and the three main treatment effects of parasite clone, glucose water treatment, and experimental block (SAS 1997). Maximal models included all higher order interactions. Non-significant terms were dropped to yield a minimum model.

To identify the mechanism for any observed parasite genetic and/or glucose water effects, the other infection covariates measured at the cage-level (host anaemia and gametocytaemia, mean blood meal size, wing length, infection rate and oocyst burden) were added individually to the minimum model for median survival. Covariates that eliminated the significance of main treatment effects when added to the minimum model, and were significant in themselves, were identified as potential causal agents of the main treatment effects. Proportion data (alive, infected) were

arcsine square-root transformed prior to analysis, and oocyst burdens were analysed as log(number of oocysts + 1).

To estimate the individual cost of infection, survival data was fitted to the Cox Proportional Hazards model (Collet 1994). A hazard ratio for each infected group was estimated as their instantaneous risk of death relative to that of the uninfected controls. A hazard ratio that is significantly greater than one indicates that the infection treatment reduced mosquito survival. Separate models were constructed for each block to examine the consistency of hazard ratio estimates between both trials.

3.4 Results:

The fate of approximately 3300 mosquitoes was observed. In both blocks, approximately 25% survived until the experiments were terminated on day 35 post-feed. The midguts of 380 mosquitoes from the infected groups were dissected. On average, 27% were infected in block one and 56% in block 2. In contrast to a pilot study, restricting provision of glucose water to alternate days had no effect on either the median survival of mosquitoes fed on uninfected mice, or the proportion of mosquitoes surviving until day 14 ($p > 0.10$ for both indices). This may have been due to improved larval rearing techniques used in the experiments reported here.

Parasite genetics and glucose water availability influenced vector mortality (Figure 3.1). Median survival was determined by the interaction between parasite clone and glucose water (Figure 3.2, $F_{3,23}=4.94$, $p<0.01$). When glucose water was continuously available, mosquitoes with mixed genotype infections had the poorest survival. However, when glucose water was available only on alternate days, it was

the CR-infected mosquitoes that had the greatest mortality. This pattern had not achieved significance by day 14 ($F_{3,23}$=2.49, p=0.086). Parasite clone*glucose water interactions were repeatable across blocks (Figure 3.2, no block interactions: p > 0.10). The minimal model of treatment effects explained 56.2% of the variation in median survival, and 68.5% of the variation for the proportion surviving until day 14 (treatment effects = block, parasite clone, glucose water and glucose water*parasite clone).

Analysis of the day of death of individual mosquitoes reinforced the conclusions from the cage-level analyses (Figure 3.3),with the parasite clone by glucose water interaction term significantly improving the fit in the Cox proportional hazard model (block 1, χ^2_7 = 57.95, p <0.01, block 2 χ^2_7= 26.80, p <0.01). There was no significant between-block variation in the hazard ratios generated for each parasite clone (Wald statistic = 2.45, df=3, p=0.48) or the nature of parasite genotype*glucose water interaction (Wald statistic = 3.17, df=3, p=0.37).

Variation in the proportion of infected mosquitoes (prevalence) and their oocyst burdens could not account for the mortality generated by the experimental treatments (Figure 3.4). Although the interaction between parasite genotype and glucose water had a strong influence on survival, it had no effect on either prevalence or mean oocyst burden (p <0.10 in both cases). Furthermore, unlike its influence on survival, the effect of parasite clone on prevalence and oocyst burden was inconsistent between blocks (significant block interactions, p < 0.05).

None of the effects of gametocytaemia, mean mosquito body size, prevalence, or oocyst burden were significant when added to the minimal model for median survival (Figure 3.5, p> 0.15 in all cases). The effect of the clone*glucose

water interaction on median survival remained significant when each of these potential explanatory variables were added to the minimal models ($p < 0.05$ in all cases). Blood meal size and anaemia were measured only in block 2. In that block, there was no evidence that survival was influenced by blood meal size ($F_{1,14}= 1.36$, $p = 0.26$) or anaemia ($F_{1,14}= 0.37$, $p = 0.55$).

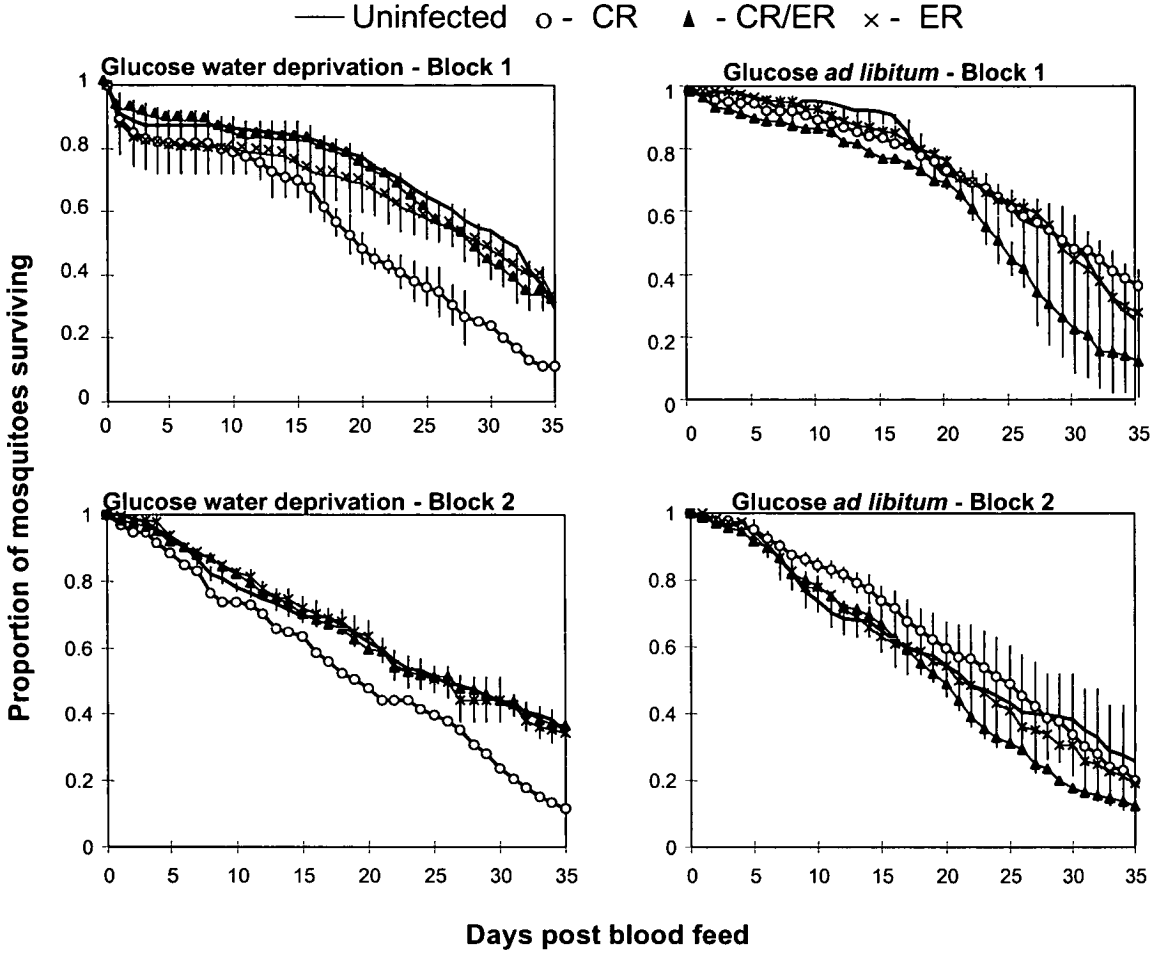

Figure 3.1: Survival curves for female *A. stephensi* mosquitoes infected with different clones of *P. chabaudi*. In graphs for experimental block 1, each line represents the mean survival across 2 cages. In block 2, there are 3 ER cages and 1 CR cage in glucose water deprivation, and 1 ER and 3CR cages in the *ad libitum* treatment. Bars represent the standard error of estimates between cages (no standard error for CR in glucose deprivation (block l) after day 29 because monitoring terminated in one of the two cages due to damage).

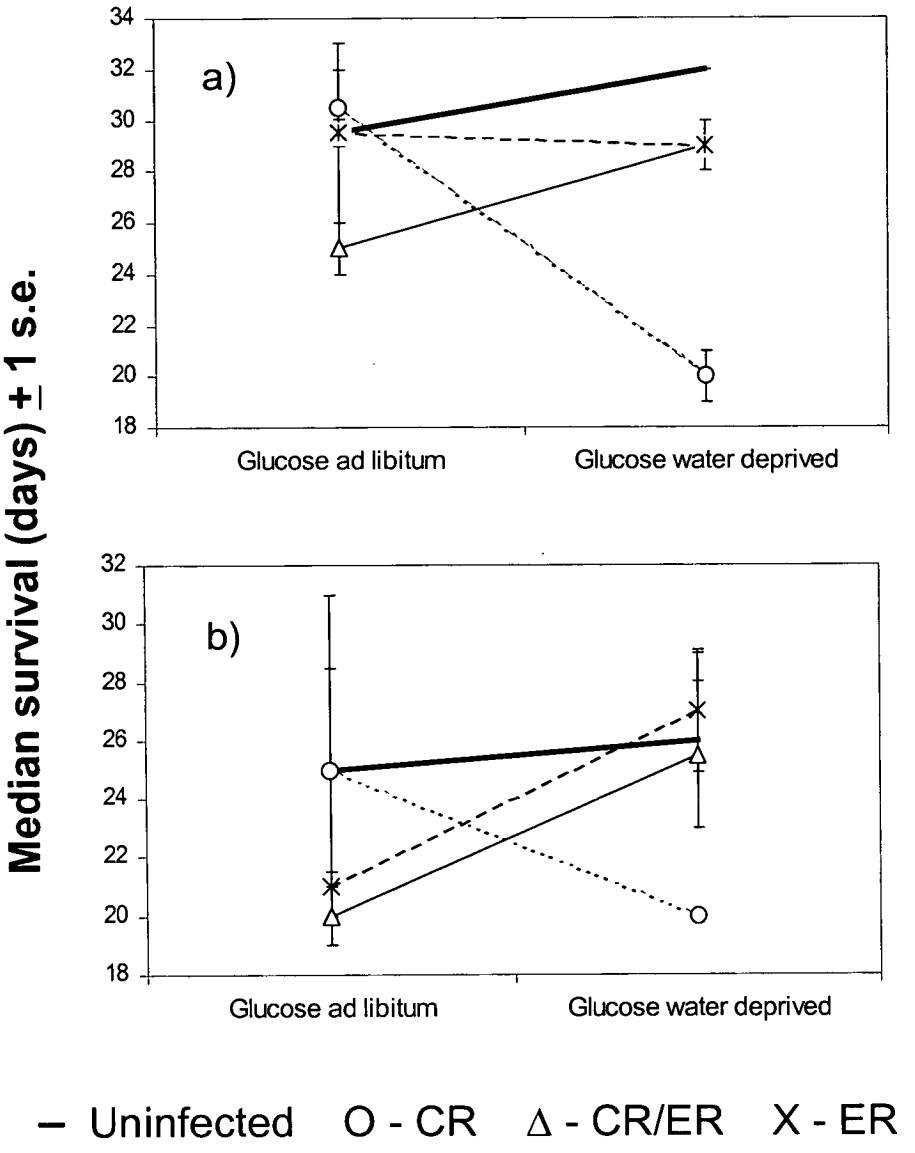

Figure 3.2: Median survival of mosquitoes infected with different clones of *P. chabaudi*, and maintained with glucose water provided *ad libitum* or every second day (a = experimental block one, b = block two). Points are treatment means (+ 1 s.e.), each point is based on two replicates, except in (b) where there was only one replicate of CR glucose water deprivation and ER glucose *ad libitum* treatment (hence no s.e. generated). Lines: small dashes = CR, large dashes = ER, thin solid = CR/ER, thick solid = uninfected.

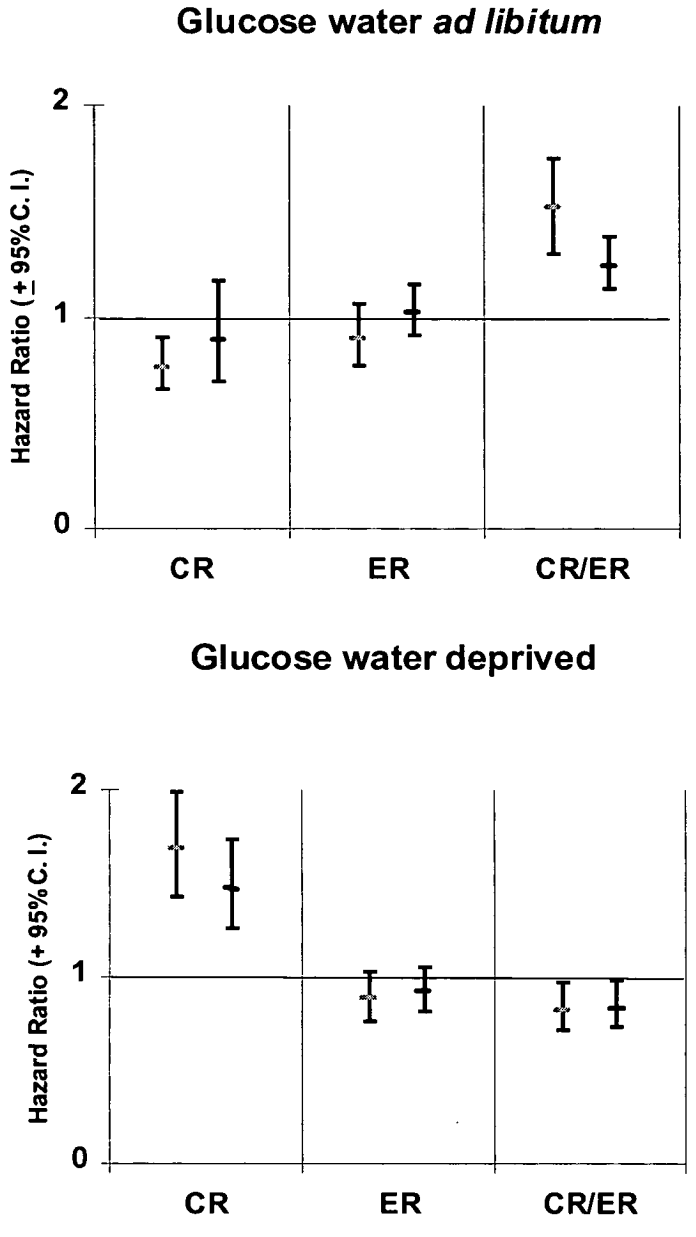

Figure 3.3: The mean and 95% confidence intervals of the hazard ratio for each infection group in standard glucose water and glucose deprived conditions. Grey central dashes are for block 1, and black central dashes for block 2.

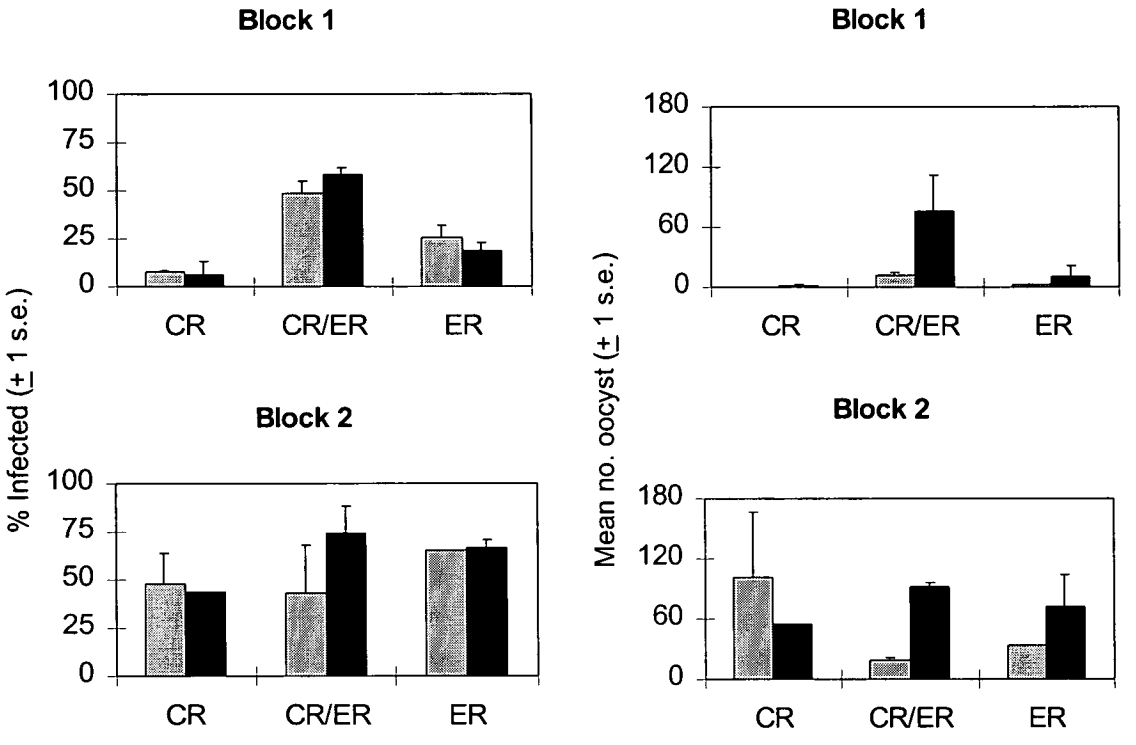

Figure 3.4: Infectivity and oocyst burdens of different infection treatments in *A. stephensi* mosquitoes. Grey bars are for mosquitoes given glucose water *ad libitum*, and black bars are glucose water deprived conditions. Each bar is based on the mean of 18-20 mosquitoes (± 1 s.e.).

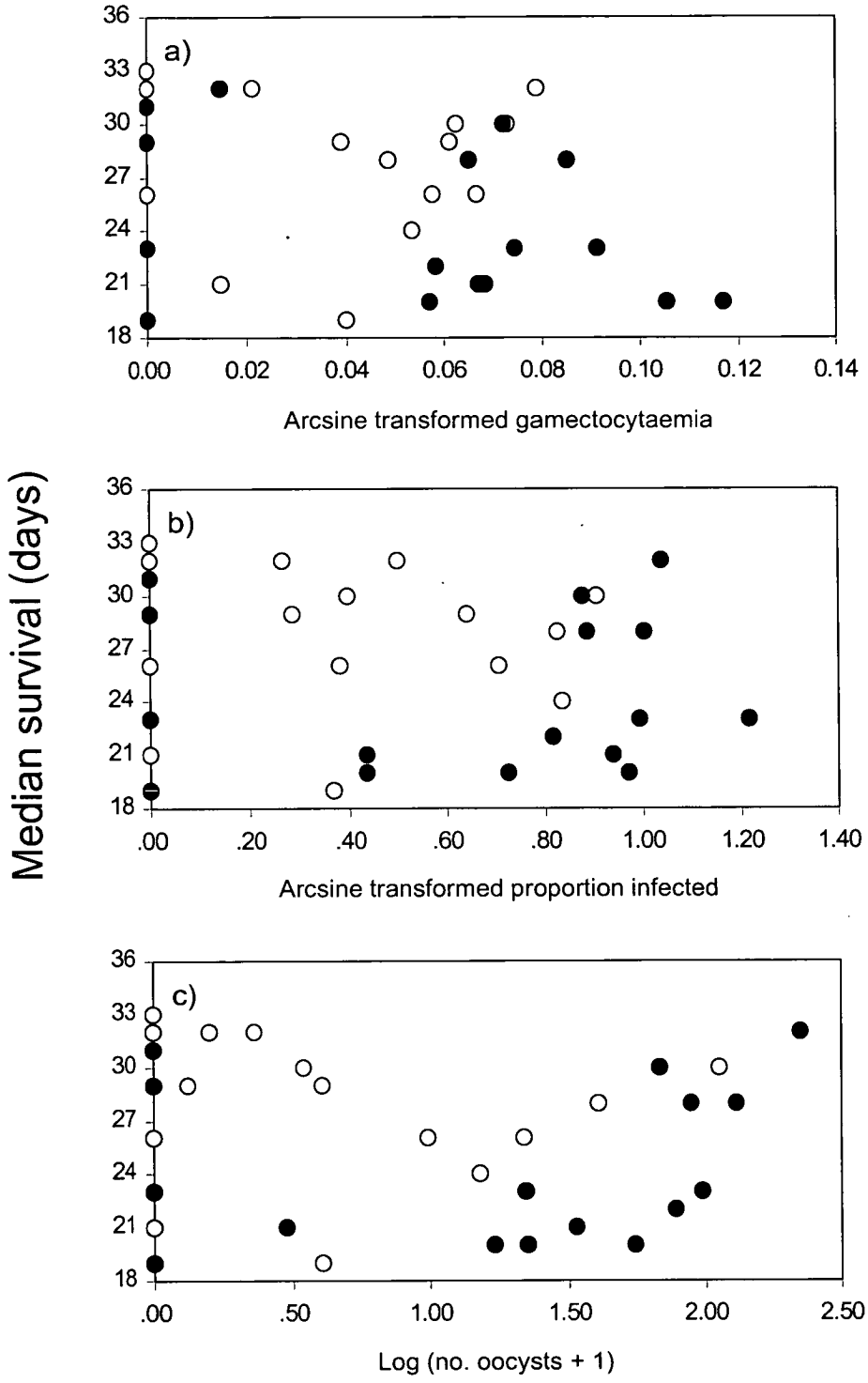

Figure 3.5: Median survival of female *A. stephensi* mosquitoes as a function of gametocytaemia (a), proportion of mosquitoes infected (b), and mean oocyst burden (c) (n=32). Open circles = block 1, closed circles = block 2.

3.5 Discussion:

The primary finding of this study was that vector mortality varies significantly with parasite genotype in an environmental condition-dependent manner. The mixed infections were more virulent when glucose water was provided *ad libitum,* but when mosquitoes were given glucose water only every second day, CR infections were more virulent. The direction of this genotype-by-environment (G*E) interaction was highly repeatable between two experiments conducted several months apart (Figures 3.1-3.3).

The interaction between parasite clone and glucose water was by far the strongest predictor of mosquito mortality, overwhelming the effect of all the other parameters that were examined, including measures of blood meal quality (size and anaemia of blood meal), and parasite load (gametocytaemia, oocyst prevalence and burden). Under glucose water deprivation in block one, CR-infections caused considerable mortality despite producing significantly lower oocyst burdens (geometric mean $\pm$ s.e. = 1.5 $\pm$ 1.4) than the other two treatments (11.9 $\pm$ 9.8 and 76.0 $\pm$ 36.0 for ER and CR/ER respectively, Figure 3.4). This result contradicts the traditional view that malaria parasites are harmful to mosquitoes only when oocyst burdens are exceedingly high (De Buck 1936; Klein *et al.* 1986). This argument has been used to refute the existence of *Plasmodium*-induced mortality in nature, as the majority of naturally infected mosquitoes carry only 1-2 oocysts (Chege and Beier 1990). These results indicate that the determinants of virulence are more complex, and that the detection of parasite-induced mortality may be hindered by focusing solely on parasite load.

It is difficult to explain why the effects of glucose water on mosquito survival varied across parasite genotypes. The result for mixed infections is particularly unexpected, as sugar-feeding usually enhances mosquito survival (Foster 1995; Straif & Beier 1996; Gary & Foster 2001). The following speculations are offered. First, in both blocks, mosquitoes with mixed infections had significantly higher oocyst burdens when they were maintained on restricted glucose water. For both the single clones, there were no such differences. Perhaps increasing glucose availability allowed the mixed clone group to mount an immune response. Activation of the invertebrate immune system can decrease survival (Moret & Schmid-Hempel 2000). Second, *Plasmodium* infection may have altered the sugar-feeding behaviour of mosquitoes in a parasite genotype-specific manner. *Plasmodium* is known to influence the blood-feeding behaviour of their vectors, specifically by increasing the frequency and persistence with which they feed (Wekesa *et al.* 1992; Koella *et al.* 1998; Anderson *et al.* 1999). It is possible that such feeding manipulation extends also to glucose-feeding, and that some parasite genotypes promoted a higher intake than others. This could be detrimental if rapid consumption leads to physiological damage, or increased exposure to bacteria that grow in glucose water (Seitz *et al.* 1987). Third, the genotype-by-environment interaction could be due to differences in sporozoite development. It is possible that sporozoite load, not oocyst burden, is the prime determinant of mosquito mortality; indeed, mortality differences became apparent only when sporozoites would be in the salivary glands (Figure 3.1). It is unknown whether or if glucose supply and parasite genotype influenced the number of sporozoites produced by an oocyst, or the ability of these sporozoites to invade the salivary glands. Certainly the efficiency with which oocyst infections develop into

salivary gland infections varies in nature (Lombardi *et al.* 1987; Beier *et al.* 1990), a phenomenon that has been attributed to the genetic diversity of parasites and their vectors (Vaughan *et al.* 1992). None of these three hypotheses (cost of immunity, feeding rate manipulation, or differential sporozoite invasion) are entirely satisfactory, as they all rely on some sort of unknown genotype specific effects. Nonetheless, all three are testable.

The survival differences reported here were not evident within the first fourteen days of mosquito infection, and were detected only when survival over the entire 35 day experimental period was considered. Thus genotype-specific mortality did not influence the proportion of mosquitoes that became infectious, only their survival through the infectious period. If survival curves obtained here (Figure 3.1) are representative of what happens in the field, differences in survival during this period could have a large effect on sporozoite rates in natural populations. The investigation of additional issues such as the importance of subsequent feeds and survival from feeds over the entire infection course will further elucidate the epidemiological consequences of these genotype-specific survival effects.

The mortality effects revealed here may be underestimates for several reasons. First, the survival estimates for mosquitoes in infected treatment groups included individuals that did not actually get infected (Figure 3.4). Second, this experiment necessarily addressed only the direct cost of infection in isolation of secondary factors such as susceptibility to predation and the ability to evade anti-vector behaviour. Sporozoite-infected mosquitoes spend more time feeding, probe more often (Wekesa *et al.* 1992), are more persistent in biting (Anderson *et al.* 1999),

and feed more often (Koella *et al.* 1998). These behavioural changes are likely to increase the mortality of infected vectors (Anderson *et al.* 2000).

The blood feeding procedure employed in these experiments may also have influenced the ability to detect virulence. Mosquitoes were allowed to take only one blood meal, after which they were maintained exclusively on glucose water. This protocol was selected to eliminate all factors that could confound our ability to detect pathogenicity arising from the first infectious feed (e.g. differential blood feeding subsequent to initial infection). Also, we wished to make our results comparable to other studies that have examined the impact of *Plasmodium*-effects on vector survival, the vast majority of which have used a similar one blood meal protocol (Buxton 1935; Thompson and Huff 1944; Maier 1973; Gad *et al.* 1979; Klein *et al.* 1982; Klein *et al.* 1986; Freier and Friedman 1987; Maier *et al.* 1987; Chege and Beier 1990; Hogg and Hurd 1995a). However, this feeding regime does not approximate the natural feeding behaviour of *Anopheles* where blood meals are taken every few days.

It is unclear how post-infection blood meals will influence *Plasmodium* virulence. The extra nutrients gained by multiple blood feeds could offset any detrimental effects of parasitism. Alternatively, extra blood meals could increase parasite virulence: they generate higher sporozoite burdens (Vanderberg and Nawrot 1968; Ponnudurai *et al.* 1990), they increase the risk of feeding associated mortality (Anderson *et al.* 2000), and they increase reproduction (Hurd *et al.* 1995). Survival costs of reproduction are well known (Stearns 1992). To our knowledge there have been no studies of *Plasmodium* virulence under different blood feeding regimes. As such, further experimentation is required to test whether the genetic variation for

virulence reported here would be maintained during repeated blood meals. Until this occurs, the epidemiological significance of these results remains unclear.

The genotype-specific mortality we report was detectable from examination of just two parasite clones. These clones represent only a fraction of genetic variation in this *Plasmodium* species (Mackinnon and Read 1999b), and are likely to be more similar than most because they have a common history of laboratory passage, and have approximately equal growth rates in their vertebrate host (Taylor *et al.* 1997a). The fact that differences are evident in a survey of only two clones indicates that the range of parasite-induced vector mortality may be much greater in the wild. In any case, this study demonstrates that there is genetic variation for virulence in the vector, a necessary prerequisite for virulence evolution. Moreoever, widespread G*E interactions of the sort discovered here could act to maintain diversity in *Plasmodium*.

This study demonstrates the ease with which conflicting accounts of virulence can be generated, even within a single vector and *Plasmodium* species in a laboratory setting. For instance, had only the clone ER been used, we would have concluded, like many others, that malaria is benign in its vector. Similarly, the increased virulence of mixed clone infections would not have been detected had all mosquitoes been maintained on reduced glucose water. This indicates that the general outcome of vector-parasite interactions can be established only by wide examination of different parasite genotypes and environmental conditions. This complexity arises even before the role of mosquito genotype has been considered. Susceptibility to *Plasmodium* can vary significantly both between (Beier 1998) and

within vector species (Yan *et al.* 1997). The elucidation of G*E*G interactions poses considerable experimental challenges.

Chapter 4: The influence of malaria parasite genetic diversity on mosquito feeding and fecundity

4.1 Summary:

Studies of invertebrate-parasite interactions frequently report that infection reduces host fecundity. The extent by which infection reduces fecundity is likely to be determined by a wide range of host and parasite factors. A laboratory experiment was conducted to evaluate the role of parasite genetics and infection genetic diversity on the fecundity of mosquitoes infected with malaria parasites. The malaria vector *Anopheles stephensi* was infected with two different genotypes of the rodent malaria parasite *Plasmodium chabaudi*, or a mixture of both. Mixed genotype infections reduced mosquito fecundity by 20%, significantly more than the either of the two single genotype infections. Mixed genotype infections were associated with high gametocyte densities and anaemia, both of which were correlated with reduced blood meal size. Blood meal size was the most important predictor of mosquito fecundity; the presence and number of parasites had no effect. Thus, mosquitoes feeding on mixed infections had reduced fecundity most probably because they took smaller blood meals. Gametocyte density also influenced the propensity of mosquitoes to feed on infected mice, with a higher percentage of mosquitoes taking a meal as gametocyte density increased. Thus mosquitoes may preferentially feed on hosts with parasites that incur the greatest fitness costs.

4.2 Introduction:

Parasite virulence is generally described as a reduction in host fitness accompanying infection. Virulence of malaria parasites towards their mosquito vectors is most commonly reported as a reduction in their fecundity (Hacker 1971; Hacker and Kilama 1974; Freier and Friedman 1976; Hogg and Hurd 1995a; Hogg and Hurd 1995b; Carwardine and Hurd 1997; Hogg and Hurd 1997; Jahan and Hurd 1997; Jahan and Hurd 1998; Ahmed *et al.* 1999). The extent by which infection reduces vector fecundity is likely the product of a wide variety of mosquito and parasite factors. The contribution of parasite genetics to malaria vector fecundity reductions has never been quantified. As parasite genetics are an important predictor of the virulence of malaria parasites in their vertebrate hosts (Rowe *et al.* 1997; Taylor *et al.* 1998; Mackinnon and Read 1999a; Mackinnon and Read 1999b; Ariey *et al.* 2001; Ofosu-Okyere *et al.* 2001; Timms *et al.* 2001; Mackinnon *et al.* 2002), and of vector longevity (Ferguson and Read 2002a, Chapter 3), it is likely they also influence vector fecundity.

It is possible that vector fecundity is influenced not only by the particular *Plasmodium* genotype with which they are infected, but also by the number of genotypes they receive. There are several reasons why infection genetic diversity should increase virulence. It may be more difficult to mount an immune response against a genetically diverse infection, and/or competition between genetically distinct parasites may involve the release of toxins that are harmful to the host as well as the parasite competitor (discussed in Ofosu-Okyere *et al.* 2001; Read and Taylor 2001). Also, evolutionary theory predicts that parasites should facultatively increase their growth rate, causing a correlated increase in virulence, when their host

is infected with another parasite strain (Sasaki and Iwasa 1991; Frank 1992; van Baalen and Sabelis 1995; Frank 1996). Such a strategy would ensure the parasite obtained some nutrients from their host before they were depleted by their competitor. In studies of rodent malaria, mixed infections generate greater weight loss and anaemia in mice (Taylor *et al.* 1998), and are more deadly to mosquitoes, at least under some environmental conditions (Ferguson and Read 2002a, Chapter 3). Whether genetically diverse infections also impose greater costs on vector reproduction is unknown.

There are at least two reasons for examining the relationship between parasite genetics and mosquito fecundity. First, the net effects of parasites on vector populations can be estimated only by considering the range of fitness costs they elicit, and in nature, many malaria-infected mosquitoes have more than one *Plasmodium* genotype (Babiker *et al.* 1994). Secondly, identification of a range of parasite genotypes with different effects on vector fecundity would permit investigation of parasite traits that elicit virulence and their potential adaptive nature (Hurd 1998; Hurd 2001). For example, it would permit tests of whether parasite strains that elicit the greatest reduction in vector fecundity have the greatest transmission success into new vertebrate hosts (adaptive for the parasite).

Parasites that reduce host fitness should select for infection avoidance and/or resistance on the part of the host. However, studies of many vector-borne diseases have shown that insect vectors preferentially bite infected hosts (Mahon and Gibbs 1982; Turell *et al.* 1984; Coleman and Edman 1988a; Coleman *et al.* 1988b; Baylis and Mbwabi 1995). The evidence that this occurs in malaria is mixed. Some studies report increased feeding on infected hosts (Day *et al.* 1983; Rossignol *et al.* 1985),

another reports avoidance of infected hosts (Freier and Friedman 1976) and another that host infection status has no influence on feeding (Burkot *et al.* 1989). The reason for this variation is uncertain, and may be due to the particular species of vector, parasite and vertebrate host used in each study, or variation in mosquito feeding between laboratory and field settings. Understanding the relationship between parasite virulence and vector biting behaviour is crucial to resolve the possible population level consequences of *Plasmodium* on mosquitoes.

Here, a simultaneous investigation of the effects of malaria parasite genetic diversity on the fecundity and blood feeding tendency of their *Anopheline* mosquito vectors was conducted. Experiments were conducted using the *Plasmodium chabaudi*-laboratory mouse model and *Anopheles stephensi* mosquitoes. The aims were to test whether (1) mosquito fecundity is generally reduced by *P. chabaudi* infection, (2) the magnitude of fecundity reduction varies with parasite genotype and/or infection genetic diversity (one parasite genotype or two), and (3) mosquito blood feeding tendency is influenced by host infection status. Such a study is useful for determining whether parasites are detrimental to their vectors, and also whether parasite effects on vector populations could be exacerbated by concentrated feeding on infected hosts.

4.3 Methods:

4.3.1 Mosquito rearing and infection

Anopheles stephensi larvae were reared in standard insectary conditions of 27 ± 1 °C, 70% humidity and a 12:12 light:dark cycle. Eggs were placed in plastic trays (25 x 25 x 7 cm) filled with 1.5 L of distilled water. In order to reduce variation in

larval growth rate and size at emergence, larvae were reared at a density of 500 per tray. Larvae, which were fed on Liquifry™ for 5 days and then on ground Tetrafin™ Fish flakes, took 9-14 days to transform into pupae. On days 10-13, groups of 250 pupae were randomly selected from the rearing trays and placed in one of 24 emergence cages (16 x 16 x 16 cm). Adults (160-240 per cage) were fed *ad libitum* on a 10% glucose solution supplemented with 0.05% PABA.

Two genetically distinct clones of *P. chabaudi* known as CR and ER were used (from the World Health Organisation's Registry of Standard Malaria Parasites, University of Edinburgh, Beale *et al.* 1978). Groups of six mice (C57BL/6J) were infected with either 10^5 CR parasites, 10^5 ER, 10^5 of a 1:1 mix of CR and ER, or were left uninfected (controls). From the fifth day of infection onwards, thin blood smears were taken on a daily basis. Mosquito feeds took place 14 days after mouse infection, when all infected mice had sufficiently high gametocytaemia (proportion of red blood cells infected with gametocytes > 0.1%). A few hours before the feed, anaemia was recorded by counting the number of red blood cells in a 2 μl sample of mouse blood. Gametocyte density was calculated as the number of red blood cells in a 2 μl sample multiplied by the gametocytaemia of the blood.

To increase hunger levels, mosquitoes were deprived of glucose for 24 hours before feeding on the mice. One anaesthetised mouse was placed on each cage (n = 24) for 20 minutes. Immediately after the feed, 4-5 fully engorged mosquitoes were removed from each cage and individually placed into 30 ml plastic tubes (9 x 2.5 cm) covered with mesh. Cotton pads soaked in a 10% glucose solution with 0.5% PABA were placed on top of each tube and replaced daily.

Blood meal size was estimated indirectly as the amount of hematin excreted over a 3 day period by the mosquitoes in tubes (as in Briegel 1980). This assay estimates blood meal size as the mass of blood protein obtained from the meal, not the volume of liquid taken in. Excretia collected in the bottom of these tubes was dissolved in 1 ml of a 1% $LiCO_3$ solution. The absorbance of the resulting solution was read at 387 nm, and compared to a standard curve made from porcine hematin (Sigma-Aldrich). Solutions with an absorbance of less than ≤ 0.01 were classified as being from mosquitoes that had blood fed, as this absorbance was indistinguishable from that of the $LiCO_3$ control.

After the 3-day hematin collection period, mosquitoes were moved to new tubes filled with 2 ml of water to allow oviposition. Fecundity was measured as the number of eggs laid over the following three days. Mosquitoes were subsequently moved into further new tubes for 1-2 days before being killed with chloroform. The midguts of mosquitoes that fed on infected mice were dissected under a microscope in a drop of 0.01 M phosphate-buffered saline solution. A cover slip was placed over each midgut and observed under a compound microscope to assess infection rate (% of mosquitoes with oocysts) and oöcyst burden (no. oocysts per gut).

The entire experiment was repeated four months later (hereafter called block two). The experimental procedure was identical in this second experimental block except that 35-40 mosquitoes were placed in tubes from each of 13 cages (three cages per infection treatment: CR, ER, CR/ER, and four uninfected controls). Increasing the number of mosquitoes sampled from each mouse allowed a more precise estimate of the proportion of feeders per treatment.

4.3.2 Statistical Analysis

General Linear Models (SAS 1997) were used to assess whether *P. chabaudi* infection and clone influenced : (1) mosquito fecundity, (2) mosquito blood meal size and (3) the propensity of mosquitoes to feed on an immobile host. Variation between the mean fecundity of the group of mosquitoes feeding on a single mouse was modelled as a function of parasite clone, experimental block, mean blood meal size, and the mean infection rate and abundance of parasites on mosquito midguts (oocysts). As block main effects are of little biological interest in their own right, they are reported only if they interacted significantly with other explanatory variables in the model. Individuals that did not feed (as defined above) were excluded from the analysis of fecundity and blood meal size.

The analysis of feeding propensity (proportion of mosquitoes that fed when exposed to an anaesthetised host for 20 minutes) was carried out only on data from block two as it had a larger sample size of mosquitoes per mouse. Feeding proportion was analysed as a function of parasite clone, host anaemia and gametocyte density.

Proportion data (feeding, infected) were arcsin square root transformed prior to analysis, and gametoctye densities and oocyst burdens and were $\log_{10}(x + 1)$ transformed. In the first block, one of the control mice died during blood feeding and mosquitoes that had fed on it were not included in our analysis.

4.4 Results:

4.4.1 Parasite clone and fecundity

Of mosquitoes that did take a blood meal (block 1: n=96, block 2: n=443), fourteen and thirty two percent did not lay any eggs in block one and two respectively. Controlling for this block difference, the proportion of mosquitoes from each mouse that did not oviposit was unrelated to infection treatment ($F_{3,31}$= 0.69, p=0.57). All subsequent analyses exclude individuals that did not lay eggs.

There was an overall effect of *Plasmodium* on mosquito fecundity: the mean fecundity of mosquitoes feeding on infected mice was lower than those feeding on the controls ($F_{1,33}$= 5.83, p = 0.03, Figure 4.1). Restricting analysis to the infected groups, parasite genetics did influence mean mosquito fecundity ($F_{2,23}$= 3.41, p = 0.05). The mean fecundity of mosquitoes that fed on mixed infections was lower than those fed on CR-infected or uninfected blood (Figure 4.1, pairwise t-tests: Bonferroni adjusted $p < 0.025$ in both cases). The fecundity of mosquitoes with single clone infections (CR and ER) did not differ from each other, or from the uninfected controls (pairwise t-tests, Bonferroni adjusted $p>0.20$ in all 3 cases). Although mosquitoes in block two laid a greater number of eggs than in block 1 ($F_{1,31}$= 73.9, $p < 0.01$), the effects of parasite clone on mosquito fecundity did not differ significantly between the two blocks (Block*genotype interaction: $F_{2,21}$= 0.76, p = 0.48), .

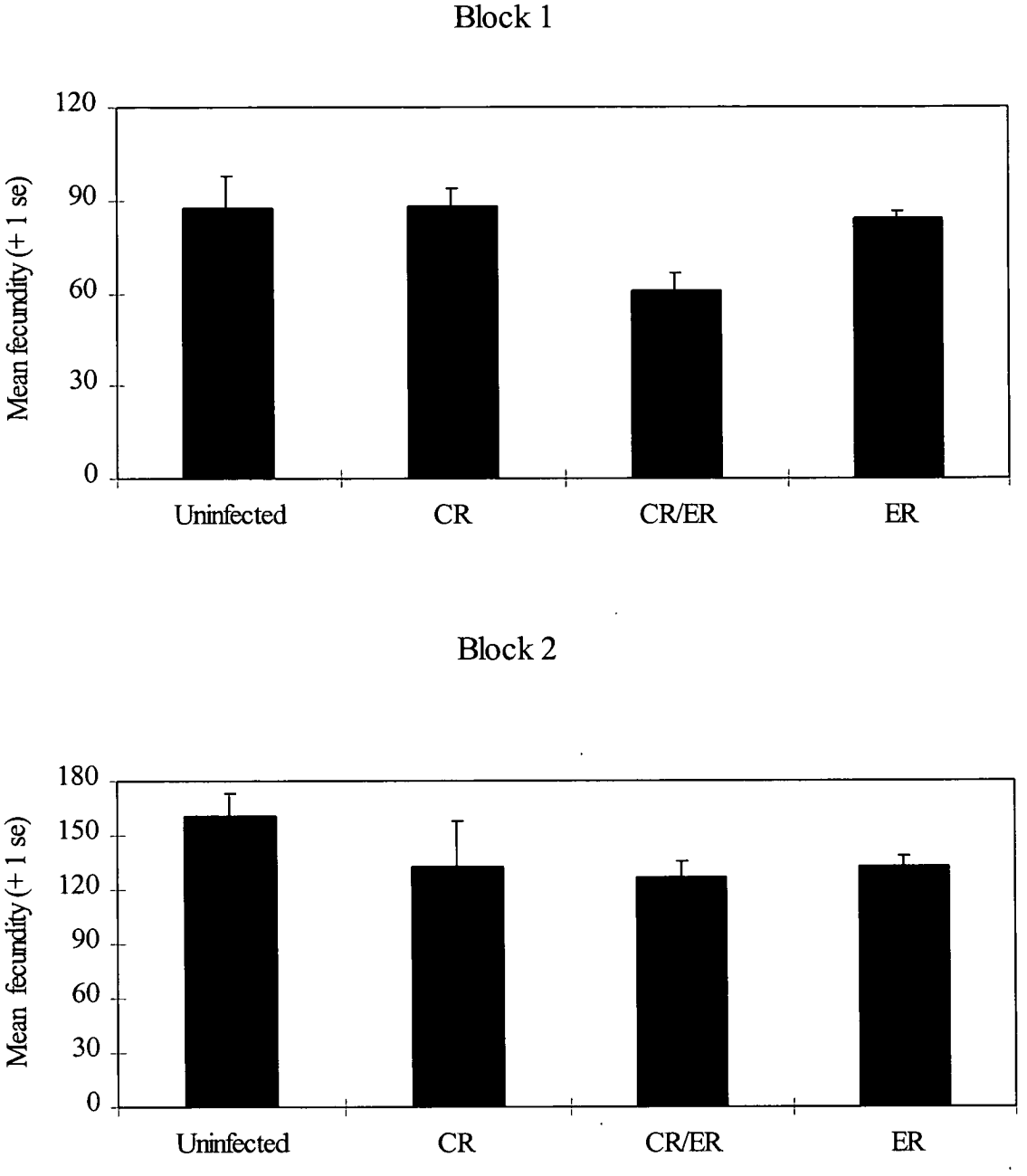

Figure 4.1: The mean fecundity of mosquitoes (no. eggs $\pm$1 standard error) after feeding on mice infected with single and mixed clone *P. chabaudi* infections in the two experimental blocks. Each bar represents the grand mean of the mean mosquito fecundity per mouse.

4.4.2 Parasite genetics and transmission

Several infection properties differed between the treatments (Figure 4.2). Gametocyte density of mice with mixed infections was higher than those with either of the two single clone infections ($F_{2,23} = 4.77$, $p = 0.02$), although the mean infectivity and number of oocysts was not (infection rate: $F_{2,23} = 0.37$, $p=0.69$, mean oocyst burden: $F_{2,23} = 0.69$, $p=0.51$). Pooling all parasite treatments, blood meals taken from infected mice were approximately 25% smaller than those taken from uninfected mice ($F_{1,33} = 16.13$, $p < 0.01$). Blood meal size varied between infected groups also, with only the mixed infections and the ER genotype causing a reduction in blood meal size relative to the controls (Bonferroni adjusted $p < 0.01$ in both cases). Finally, the red blood cell density of mice that had experienced *Plasmodium* infection was approximately 20% lower than those that had not ($F_{1,33} = 13.78$, $p < 0.01$), but did not differ significantly between the three infected groups ($F_{2,23} = 0.44$, $p = 0.65$, Figure 4.2).

4.4.3 Explaining the parasite genetics – fecundity relationship

Can the effects of parasite clone on mosquito fecundity be explained by the dynamics of each type of infection? Restricting analysis to the infected groups, neither mean mosquito infection rate ($F_{1,22} = 0.17$, $p=0.69$), mean oocyst burden ($F_{1,22} = 1.52$, $p=0.23$), red blood cell density ($F_{1,22} = 0.10$, $p=0.91$) nor gametocyte density ($F_{1,22} = 0.002$, $p=0.96$) could explain variation in mean fecundity when added to a statistical model including parasite clone and experimental block. Furthermore, none of these variables had any association with fecundity when considered

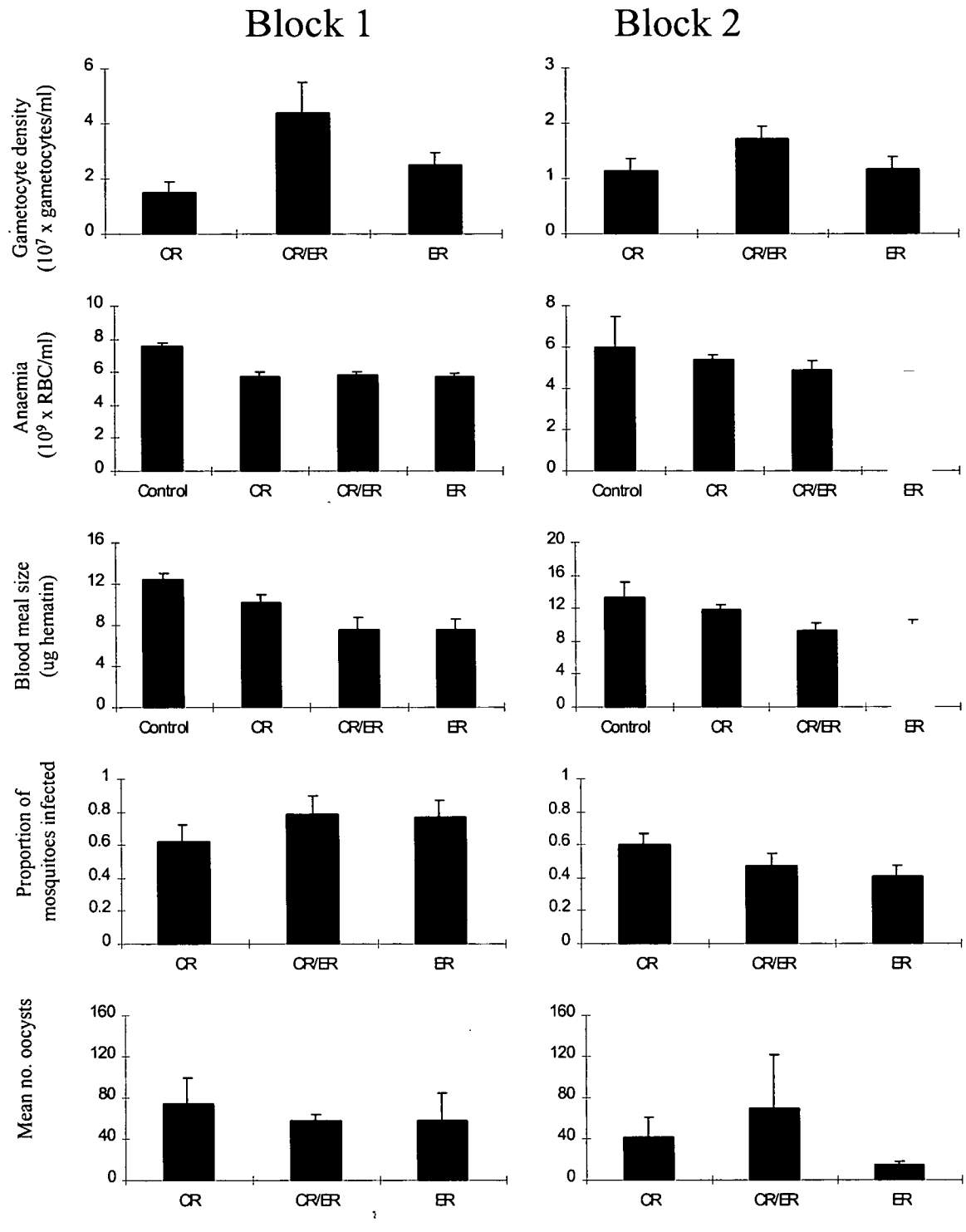

Figure 4.2: Properties of blood from uninfected mice and those infected with three genetically different *P. chabaudi* infections (mean ± 1 s.e.). Data are grand means from separate mice (no. mosquitoes per mouse = 4-5 in block 1, 35-40 in block 2).

independently ($p > 0.1$ in all cases). Mean blood meal size, however, did explain additional variation in fecundity when added to a statistical model that included parasite clone ($F_{1,22}=4.15$, $p = 0.05$), and was also significant when considered on its own ($F_{1,24}=5.95$, $p = 0.02$, Figure 4.3). Furthermore, the inclusion of blood meal size reduced the explanatory power of parasite clone to the point where it was no longer significant ($F_{2,22}=2.59$, $p = 0.10$). This suggests that the effects of parasite genetic variation on mosquito fecundity is driven by differences in the blood meal size taken from infected mice.

4.4.4 Blood meal size

Parasite clone, red blood cell density, gametocyte density and their experimental block interactions were combined in a statistical model to test their effects on mean blood meal size. Across infected groups, both red blood cell density ($F_{1,23} = 11.87$, $p < 0.01$, Figure 4.4a) and gametocyte density ($F_{1,23} = 10.51$, $p < 0.01$, Figure 4.4b) were related to blood meal size. Vector fecundity increased with host red blood cell density, but fell with increasing gametocyte density. Both red blood cell density and gametocyte density remained significant in the absence of one another ($p<0.01$ in both cases), and regression analysis indicated that these two variables were not correlated ($F_{1,25} = 1.41$, $p =0.25$, $r^2=0.02$). Parasite clone was no longer a significant predictor of blood meal size when gametocyte density and red blood cell density were included as explanatory variables (when both factors included in model, parasite clone: $F_{2,21} = 1.81$, $p = 0.19$).

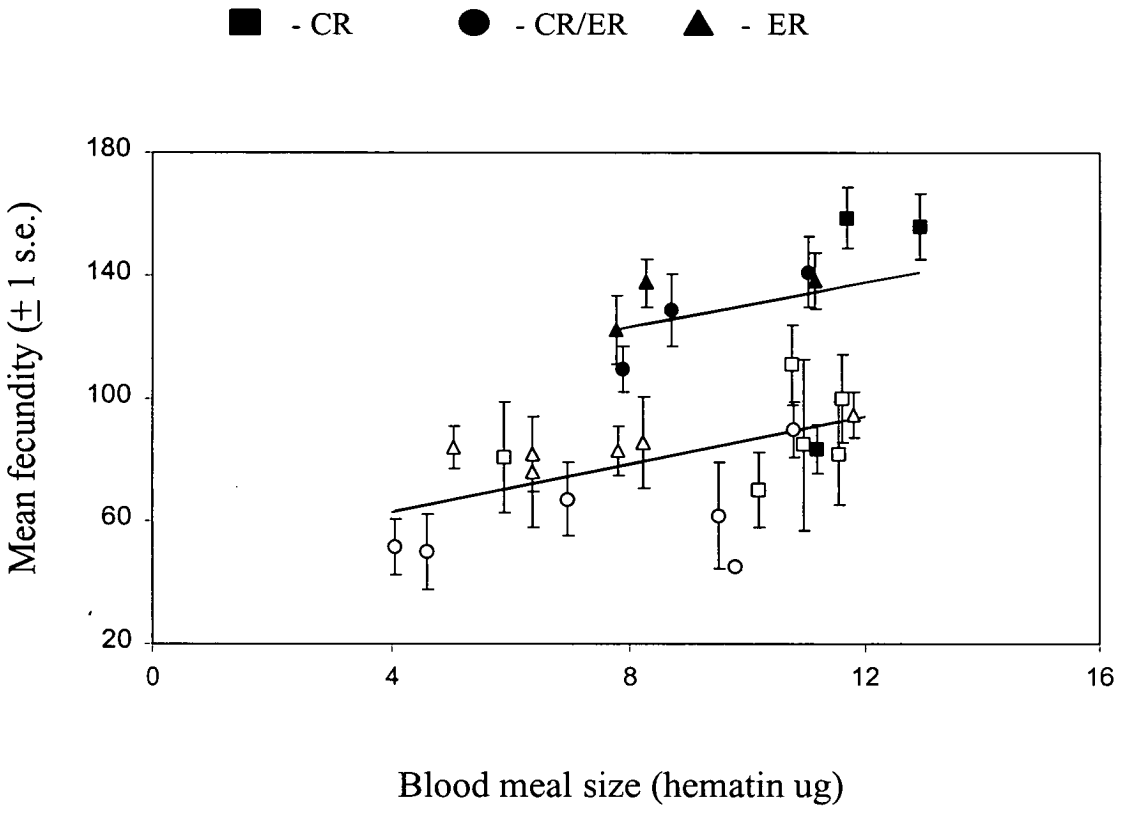

Figure 4.3: Relationships between the mean blood meal size taken from infected mice and the mean fecundity of *A. stephensi* mosquitoes. Symbols indicate the parasite clone of each infected mouse. Block 1 data are open symbols, block 2 data are black symbols. The slope of the regression lines do not differ between blocks (see text).

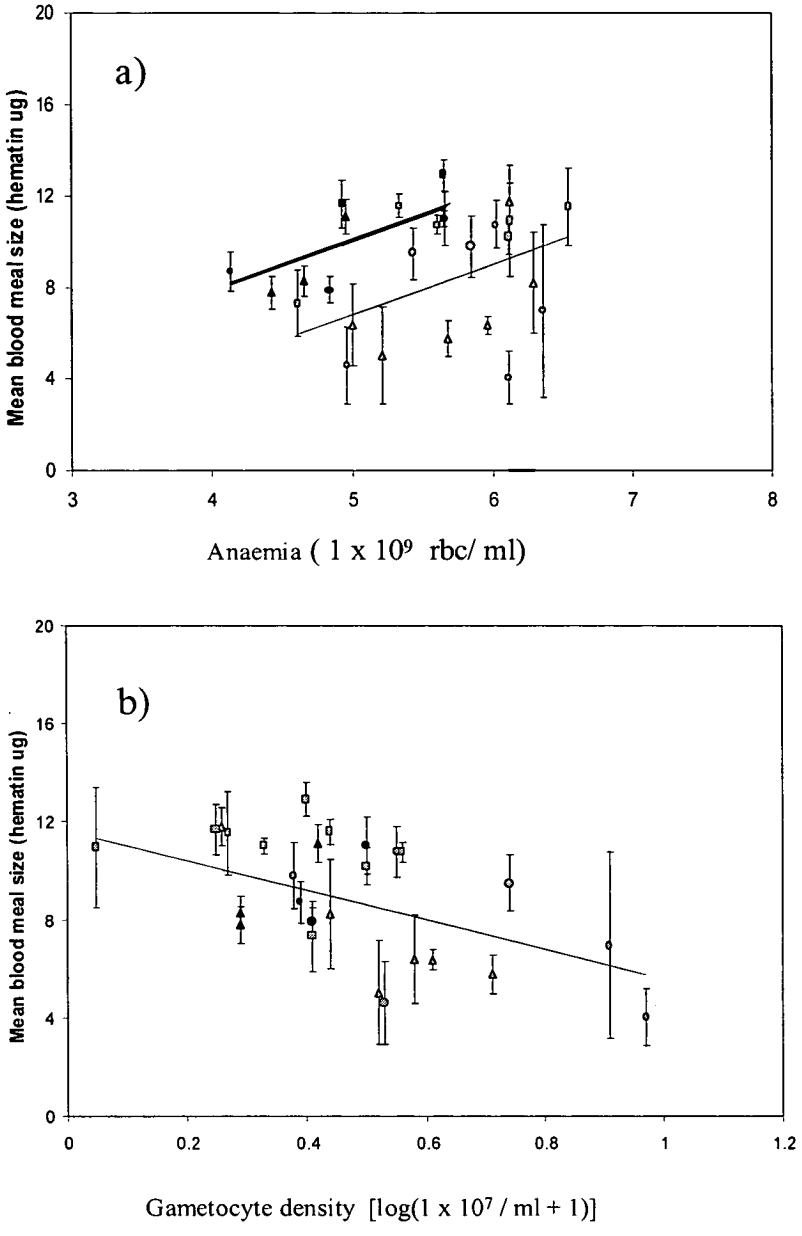

Figure 4.4: Relationships across infected mice between mean mosquito blood meal size and mouse red blood cell density (a) or mouse gametocyte density (b). Error bars are 1 s. e. Symbols indicate the parasite clone. Block 1 data are grey, block 2 data are black. Two regression lines are shown only when experimental block influenced the relationship (a: black line = block 1, grey line = block 2)..

4.4.5 Proportion feeding

Across both blocks, 71-97% of the mosquitoes that were collected for hematin analysis had taken a blood meal within the 20 minute mouse exposure period. A greater proportion of mosquitoes fed when exposed to infected hosts (92% vs. 81%, $F_{1,11}=10.07$, $p < 0.01$). The proportion that fed did not significantly vary between hosts with different *Plasmodium* clones ($F_{2,6}=0.95$, $p = 0.44$), but was significantly related to the gametocyte density of the host to which they were exposed ($F_{1,11}=16.77$, $p < 0.01$), with the proportion of vector feeding events increasing with mouse parasite load (Figure 4.5). This correlation was maintained when uninfected mosquitoes were excluded ($F_{1,7}= 5.50$, $p=0.05$). Mouse anaemia was not strongly related to feeding proportion ($F_{1,11}= 4.31$, $p = 0.06$).

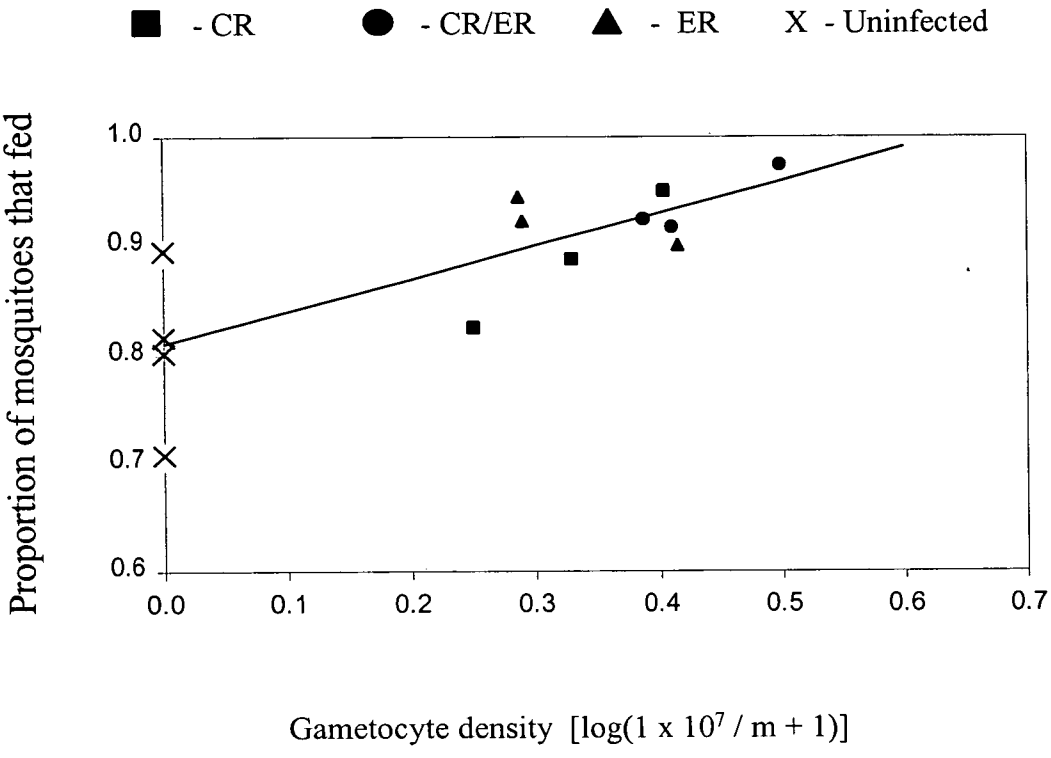

Figure 4.5: Gametocyte density of mice and the proportion of mosquitoes that took a blood meal from them in a twenty minute period (data from block 2 only).

4.5 Discussion

4.5.1 Parasite genetics and mosquito fecundity

So far as we are aware, this study provides the first evidence that genetic diversity within *Plasmodium* infections determines the magnitude by which mosquito fecundity is reduced. It is also the first demonstration that *P. chabaudi* reduces vector fecundity, strengthening the case that this is a general outcome of *Plasmodium* infections in mosquitoes (Hacker 1971; Hacker and Kilama 1974; Freier and Friedman 1976; Hogg and Hurd 1995a; Hogg and Hurd 1995b; Hogg and Hurd 1997; Jahan and Hurd 1997; Ahmed *et al.* 1999). Mosquitoes with mixed infections laid approximately 20% less eggs than those fed uninfected blood. The detrimental effects of *P. chabaudi* were due largely to the mixed clone infection. Neither of the two single-clone treatments generated significantly lower fecundity than the uninfected controls.

The increased pathogenicity of mixed infections apparently arises indirectly from the interaction between gametocyte abundance, red blood cell density and blood meal size. Mixed infections produced higher gametocyte densities and made mice more anaemic in this experiment and others (Taylor *et al.* 1997b; Taylor *et al.* 1998; Read and Taylor 2001). As hematin intake was positively related to red blood cell density (Figure 4.4a), and negatively to gametocyte density (Figure 4.4b), mosquitoes feeding on mixed genotype infections tended to take smaller blood meals than those feeding on hosts with single-clone infections. This result matches previous studies of other malaria species where mosquitoes took smaller blood meals from mice with high parasitemia (Hogg and Hurd 1995a). As blood meal size is a prime correlate of mosquito fecundity both in this study and others (e.g. Reisen and

Emory 1976; Briegel 1990a; Briegel 1990b), the small blood meals taken from hosts with mixed infections likely rendered mosquitoes less fecund.

Despite having higher gametocyte densities, mixed infections did not produce higher infection rates or oocyst burdens in mosquitoes. This result contrasts with an earlier study where the increased gametocyte densities of mixtures did translate into higher oocyst prevalence (Taylor *et al.* 1997b). Variation between that study and mine may be due to the fact that different parasite doses were used, and that the CR and ER genotypes were combined in a different ratio.

It is not possible to pinpoint which factor, anaemia or gametocyte density, was responsible for the decrease in mosquito blood meal size. The fact that these two variables were not correlated with each other on the day of blood feeding, and that they were both were significant when combined and in isolation, suggests they have independent effects. The association between blood meal size and gametocyte density, thus, is not obviously a by-product of mice with high gametocyte burdens also being very anaemic. Assuming both effects are independent, how can they be explained? It is not surprising that a positive association between red blood cell density and blood meal size was found. Hematin content, the measure of blood meal size, is related to the number of red cells that a mosquito digests, not the volume of liquid it imbibes. Mosquitoes feeding on an anaemic host will intake a lower hematin mass than those feeding on a healthy host, even if both ingest a similar volume of blood. The association between blood meal size and anaemia reported here is thus likely a product of variation in the quality of parasitised blood (as indexed by the amount of red cells per meal), rather than differences in the quantity of blood consumed.

It is less clear how gametocyte abundance could influence blood meal size. One possibility is that mosquitoes choose to reduce their intake when they encounter highly gametocytaemic blood, and another that gametocytaemic blood is more difficult to imbibe. There has been little investigation of the first, intriguing possibility, but the second is strengthened by evidence that several properties of host blood, including molecular composition (Hosoi 1959) and the presence of antibodies (Srikrishnaraj *et al.* 1993) are known to influence different aspects of female mosquito feeding and fecundity. Properties of gametocyte-infected blood (e.g. antibodies, or pH - Dearsly *et al.* 1990), or the gametocytes themselves, could have similarly interfered with feeding efficiency and reduced blood meal size.

If the correlation between gametocyte density and blood meal size is a general phenomenon, variation in vector fecundity between single parasite genotypes may occur in nature even though it did not in this study. The two single clone infections that were used (CR and ER) produce similar levels of gametocytes. However, more extensive studies of genetic variation both within this *Plasmodium* species (Mackinnon and Read 1999b, Chapter 6) and the human malaria parasite *P. falciparum* (James *et al.* 1932; Graves *et al.* 1984) have shown that gametocyte density can vary significantly between parasite genotypes. Use of parasite clones which differed in gametocyte production may have revealed effects on mosquito fecundity due to single genotypes, in addition to the effects of infection diversity reported here.

No association between oocyst presence or number and mosquito fecundity was found, suggesting that reductions in egg output do not arise from direct nutrient competition between host reproductive tissue and growing parasites, a hypothesis

strengthened by similar findings in other studies (Hacker and Kilama 1974; Hogg and Hurd 1995b; Ahmed *et al.* 1999). In this parasite system, it is most likely that fecundity reductions are due to the reduced size of infected blood meals, and not the action of the parasites within the mosquito. However, the possibility that parasites had direct effects on mosquito resources immediately after ingestion cannot be ruled out. Mosquitoes may have expended a large amount of resources mounting immune responses to invading parasites, stopping them from developing into oocysts. Immune responses are costly in insects, and can drain resources that would have otherwise have been directed to survival and reproduction (Ferdig *et al.* 1993; Moret and Schmid-Hempel 2000). Additionally, parasites may have directly reduced mosquito fecundity by interfering with the rate of egg production (Carwardine and Hurd 1997; Jahan and Hurd 1998).

4.5.2 Parasite genetics and mosquito feeding

Parasites that reduce host fitness should select for infection avoidance on the part of the host. However, in this study mosquitoes had a greater propensity to feed on mice with high gametocyte densities than on those that were uninfected (Figure 4.5). This observation matches results from studies of other vector-borne diseases have also shown that insect vectors preferentially bite infected hosts (Mahon and Gibbs 1982; Turell *et al.* 1984; Coleman and Edman 1988a; Coleman *et al.* 1988b; Baylis and Mbwabi 1995), and some (Day *et al.* 1983; Rossignol *et al.* 1985) but not all studies of malaria (Freier and Friedman 1976; Burkot *et al.* 1989).

It is difficult to envision why mosquitoes should have an increased tendency to feed on hosts of poor quality (those which generate low fecundity) unless parasites

are somehow manipulating mosquito behaviour by making infected hosts more attractive. In any case, this study challenges the notion that the increased feeding success of mosquitoes on gametocytaemic mice is due to the reduced anti-vector behaviour of infected individuals (Day and Edman 1983). In this experiment, all mice were under anaesthetic. Thus host odour or physiology must also be responsible for preferential feeding, in addition to any effect of host behaviour.

4.5.3 Conclusions

This study demonstrates that the genetic composition of malaria infections does influence mosquito fecundity. The fecundity reductions reported here could be a pathological by-product of infection, or they could be the outcome of an adaptation of the parasite and/or vector to increase their own fitness (Hurd 2001). For example, mosquitoes could benefit from reducing their fecundity when parasitised, if by doing so they could increase their longevity and lifetime reproductive success. Similarly, parasites could benefit by reducing their vector's fecundity if doing so increases mosquito longevity (and thus parasite transmission opportunities). As mosquito survival was not monitored in this experiment, the relationship between vector fecundity reduction and longevity could not be directly tested. However, evidence from a separate study of mosquito survival suggests that, under similar conditions, the infection generating the greatest fecundity reduction (mixture of CR and ER) is also the one that causes the greatest vector mortality (Ferguson and Read 2002a, Chapter 3). Thus the fecundity reductions observed in this system may help offset the mortality costs of infection, but they do not eliminate them.

Could the effects of *Plasmodium* on mosquito fecundity have any influence on vector population dynamics? This possibility is usually dismissed (Dye and Williams 1995) as only a small percentage of mosquitoes (1-6%) harbour parasites in the wild (Beier 1998). Although only a small proportion of mosquitoes become infected, a much larger proportion will feed on infected blood. Up to 80% of humans are infected with *Plasmodium* in some endemic regions (Molineux and Gramiccia 1980). If mosquitoes have a greater tendency to feed on infected individuals, the encounter rate of mosquitoes with infected blood could potentially be very high. As the fecundity costs demonstrated here require only that mosquitoes feed on infected hosts, not that they develop oocysts, a high percentage of vectors could experience *Plasmodium*-associated fitness reductions. Detailed demographic studies are required to assess whether these effects could influence vector dynamics.

Chapter 5: The energetic budget of *Anopheles stephensi* infected with *Plasmodium chabaudi*

Note: Work presented in this chapter was conducted jointly with Dr. Ana Rivero, I.C.A.P.B. Both H. F. and A.R. planned and jointly conducted all parts of the experiments described below and their statistical analysis. AR and HF made substantial contribution to the writing.

5.1 Summary:

Evidence continues to accumulate indicating that malaria parasites (*Plasmodium sp.*) reduce the survival and fecundity of their mosquito vectors (*Anopheles sp.*). Interpretation of the possible epidemiological and evolutionary consequences of these parasite-induced fitness reductions would be improved by understanding the physiological basis of these shifts. This study tested whether the reduction in vector fecundity and longevity associated with three genetically different *Plasmodium chabaudi* infections was correlated with energy reserve depletion and/or reallocation in *A. stephensi* mosquitoes. Infected mosquitoes were expected to have fewer energetic resources than uninfected mosquitoes, with energy levels being the lowest in mosquitoes infected with the most virulent parasite genotype. However, there was no evidence that *P. chabaudi* influenced the overall energetic budget of mosquitoes: infected mosquitoes had the same amount of three key physiological resources (lipids, glycogen, proteins) as those that were uninfected. Furthermore, mosquitoes infected with the most virulent parasite genotypes had an increased abundance of glucose relative to the controls. This is consistent with

Plasmodium manipulating mosquito sugar-feeding behaviour in order to increase its own transmission.

5.2 Introduction:

Models of malaria epidemiology and evolution are frequently based on the assumption that vector-parasite associations are benign (Anderson and May 1991; Gandon *et al.* 2002). However, evidence that malaria parasites (*Plasmodium sp.*) reduce the survival (Ferguson and Read 2002b, Chapter 2) and fecundity (Hacker 1971; Hacker and Kilama 1974; Freier and Friedman 1976; Hogg and Hurd 1995a; Hogg and Hurd 1995b; Hogg and Hurd 1997, Chapter 4) of their mosquito vectors (*Anopheles sp.*) continues to accumulate. Our ability to identify the possible epidemiological and evolutionary consequences of these parasite-induced fitness reductions has been hampered by a poor understanding of the origin and physiological basis of these shifts. Some explanations for *Plasmodium* virulence in its vectors are that parasites damage vital organs such as the midgut or salivary glands (Sinden and Billingsley 2001), reduce feeding efficiency (e.g. change the level of salivary gland proteins, Shandilya *et al.* 1999), and/or increase the risk of secondary infection by other pathogens (Seitz *et al.* 1987). An additional explanation and one of particular interest for its potential adaptive implications, is that the reduction in fecundity and longevity could be the result of a depletion or reallocation of the mosquito energetic resources (Maier *et al.* 1987; Hurd 2001).

Energetic allocation is central to many theories concerning life history shifts (Chippindale *et al.* 1993). Energetic constraints imposed by parasite development have been implicated in the observed change in life history parameters in several host-parasite systems (Toft 1991; Kearns *et al.* 1994; Sorensen and Minchella 1998). In malaria vectors, energy depletion could arise either as a result of direct resource competition between *Plasmodium* and the mosquito (Maier *et al.* 1987), because

infected mosquitoes require extra nutrients to compensate for parasite damage (e.g. tissue repair) or to fuel the cost of mounting an immune response (Ferdig *et al.* 1993; Ahmed *et al.* 2002).

In this study, we examined whether energy loss could account for the virulence of the rodent malaria *P. chabaudi* in its *A. stephensi* mosquito vector. Recent laboratory studies of this parasite have shown that infection reduces the longevity (Ferguson and Read 2002a, Chapter 3) and fecundity (Chapter 4) of these mosquitoes. The magnitude of fitness reductions varies with the genetic composition of the infection (Ferguson and Read 2002a, Chapters 3 and 4). The physiological basis of these patterns of virulence is unknown. It is possible that different parasite genotypes have different physiological requirements, generate blood meals of variable nutritional quality, and/or induce changes in mosquito resource use and nutrient uptake – any of which could generate variation in virulence.

We infected mosquitoes with one of two genetically different *P. chabaudi* clones or their mixture. These treatments are known to generate variable levels of virulence (reduction in survival and fecundity) in *A. stephensi* mosquitoes (Chapters 3 and 4). After infection, we measured the abundance of three key insect energetic and nutritional resources :glucose, glycogen and lipids (Gillot 1980; Clements 1992; Nijhout 1994; Rivero and Casas 1999). Levels of protein, an important structural component that is only rarely metabolised for energy (Clements 1992), were also measured. These resources were analysed at two different time points during parasite development: 1) when parasites are growing on the mosquito midgut as oocysts (7-8 days after infection) and 2) when the transmissible sporozoite stages have invaded the mosquito salivary gland (14 days post-infection).

We made two predictions about the effect of *P. chabaudi* on mosquito energy reserves: 1) that mosquitoes infected with *Plasmodium* would have fewer energetic resources than control (uninfected) mosquitoes, and 2) that the energetic budget of infected mosquitoes would be negatively correlated with parasite genotype-specific virulence. In addition, we tentatively expected shifts in the energetic budget of mosquitoes between the oocyst stage and the sporozoite stage of the parasite's development, although the direction of change is difficult to predict. Oocysts are metabolically demanding (Maier *et al.* 1987) and thus may impose a larger drain on mosquito resources than sporozoites. However, evolutionary theory predicts that natural selection should minimise parasite virulence during the period when the parasite is growing but cannot yet be transmitted (i. e. at the oocyst stage, Schwartz and Koella 2001). Thus from an adaptive standpoint (and if energy depletion and virulence are correlated), energetic depletion may be expected to be greater at the sporozoite rather than oocyst stage of development.

5.3 Methods:

5.3.1 Mosquito rearing and infection

Anopheles stephensi larvae were reared in standard insectary conditions at 27 ± 1° C, 70% humidity and a 12:12 light:dark photocycle. Eggs were placed in plastic trays (25 x 25 x 7 cm) filled with 1.5 L of distilled water. In order to reduce variation in adult size at emergence, larvae were reared at a fixed density of 500 per tray. Larvae were fed on Liquifry™ for 5 days and then on ground Tetrafin™ fish flakes. On days 10-13, groups of 250 pupae were randomly taken from the re

emerged (ca. 160-240 per cage) were fed *ad libitum* on a 10% glucose solution supplemented with 0.05% PABA.

Two clones of *Plasmodium chabaudi* known as CR and ER were used (from the World Health Organisation's Registry of Standard Malaria Parasites, University of Edinburgh, Beale *et al.* 1978). Previous studies have shown that while ER is relatively benign in mosquitoes, CR reduces their longevity when access to sugar is restricted, and a combination of CR and ER (henceforth CR/ER) reduces both their longevity and fecundity when glucose is provided *ad libitum* (Ferguson and Read 2002a, Chapters 3 and 4). Mice (C57BL/6J) were infected with either 10^5 parasites of CR (n =3), 10^5 of ER (n = 3), or 10^5 of a 1:1 mix of CR and ER (henceforth CR/ER, n = 3). Four uninfected mice were maintained as controls (total = 13 mice).

From the fifth day of infection onwards, thin blood smears were taken on a daily basis. Mosquito feeds took place 14 days after mouse infection, when all infected mice had sufficiently high gametocytaemia (proportion of red blood cells infected with gametocytes, > 0.1%). On the day of the feed, anaemia (number of red blood cells in a 2 μl blood sample) and gametocyte density (anaemia x gametocytaemia) were also recorded.

Mosquitoes were deprived of glucose for 24 hours before the feed. Blood feeds were carried out by placing one anaesthetised mouse on top of a randomly chosen cage for 20 minutes. Immediately after the feed, 35-40 fully engorged females were taken from each of the cages and placed individually into 30 ml plastic tubes covered with mesh. Food was henceforth provided in the form of a cotton pad soaked in a 10% glucose solution (with 0.5% PABA) that was placed on top of each tube.

Mosquitoes were kept in the tubes for three days to allow all hematin (a by-product of the decomposition of haemoglobin) to be excreted. Blood meal size was indirectly estimated from the amount of hematin excreted (as in Briegel 1980). For this purpose, on the day of collection (day 3 post-infection), all excreted hematin was dissolved in 1 ml of a 1% $LiCO_3$ solution. The absorbance of the resulting mixture was read at 387 nm, using the $LiCO_3$ solution as a blank, and compared to a standard curve made with porcine serum hematin (Sigma-Aldrich). Solutions which were within the error range of the $LiCO_3$ blanks (absorbance ≤ 0.01) were eliminated from the analysis and mosquitoes that had been held in these tubes were classified as non-feeders.

After the 3-day hematin collection period, mosquitoes were moved to new 30 ml tubes containing approximately 3ml of water to allow oviposition. After three days in oviposition tubes, mosquitoes were again transferred, and all eggs laid in the previous tubes were counted. On day 7 after infection, half of the mosquitoes that fed on each mouse were randomly selected and killed with chloroform. One of their wings was removed and measured along its longest axis as an index of body size. Their midguts were dissected to determine oocyst prevalence and load (see below). The remaining mosquitoes were left in the tubes for a further seven days to allow the parasite to reach the sporozoite stage. At this time (day 14), all remaining mosquitoes were killed, measured as above, and their salivary glands dissected to determine if sporozoites were present (see below).

5.3.2 Mosquito dissections

Mosquitoes were dissected under a binocular microscope in 100 µl of 0.01 M phosphate-buffered saline (PBS). After dissection, midguts were transferred to a new slide with a pin, placed under a cover slip and observed under a compound microscope to assess parasite presence and burden (no. oocysts per gut). Mosquitoes collected at the sporozoite stage were also dissected in 100 µl of PBS. Salivary glands were transferred to a new slide, crushed, and observed under a compound microscope to determine if there were sporozoites (sporozoite density was not estimated). For the CR, ER and CR/ER treatments, mosquitoes where at least one oocyst or sporozoite was found were classified as 'infected', the rest as 'uninfected'. At both stages, the bodies of mosquitoes fed on uninfected mice were dissected and treated in the same way as the rest. The dissected bodies of all the mosquitoes that fed on each mouse at each parasite stage were divided into two groups. Half of these individuals were randomly allocated to the quantification of lipids, glycogen and sugars (which can be carried out on the same specimen) and half to the quantification of proteins (see below).

5.3.3 Quantification of lipids, sugars and glycogen

The dissected bodies of individuals allocated to the quantification of lipids, sugars (glucose and trehalose) and glycogen were transferred individually into Pyrex glass tubes (7.3 cm length, 1 cm diameter) and crushed with a glass rod. The PBS solution in which each female was dissected was recovered with a pipette and added to the tube. One hundred µl of sodium sulphate (which adsorbs glycogen) and 750µl of a 1:2 chloroform-methanol solution (which dissolve lipids and sugars

respectively) were added to these tubes which were then covered and left to react at room temperature for 24h. Subsequent chemical analysis was carried out in blocks of 24-25 randomly chosen tubes. For each chemical analysis block, a blank tube was prepared following the same exact procedure without the mosquito body.

Quantification of lipids, glycogen and sugars on the same specimen was carried out using a colorimetric technique developed for mosquito analysis (vanHandel 1985a; vanHandel 1985b; vanHandel 1988). Briefly, sample tubes were centrifuged and the chloroform-methanol supernatant was separated into two fractions: one for lipid analysis and the other one for sugar (glucose and glycogen) analysis. For lipid determination, the solvent was evaporated completely in a heating block and sulphuric acid was added to the tubes which were then reheated to convert the unsaturated lipids to water-soluble sulphonic acid derivatives (vanHandel 1985b). These develop a deep pink colour after addition of a vanillin-phosphoric acid reagent which is read in spectrophotometer at OD_{525}. Lipid concentrations were obtained from a standard curve computed with vegetable oil.

For sugar determination , the solvent was evaporated in a heating block and then heated with anthrone-sulphuric acid reagent (vanHandel 1985a). Heat breaks down the sugars in mosquito bodies to their glucose units and anthrone binds to them, turning the mixture green. Tubes are then read at OD_{625} and sugar concentrations are obtained from a standard curve computed with glucose. Finally, the precipitate in the original tube containing the glycogen was washed with methanol to eliminate residual sugars, and then heated with anthrone and read at OD_{625}. Glycogen concentrations were obtained from a standard glucose curve.

5.3.4 Quantification of proteins

Bodies of mosquitoes allocated to the quantification of proteins were placed in an 1.5 ml Eppendorf tube, crushed with a glass rod and 100 µl of a solution of physiologic water (0.15 molar NaCl) and 0.001% Triton X-100 (Sigma-Aldrich) was added. Blanks consisted of physiologic water and Triton solution but no crushed mosquito. Tubes were left for 5 days at 4 °C to allow the Triton to dissolve the proteins in the body.

Protein analysis was carried out using the Bradford dye-binding micro-assay procedure (Bradford 1976). For this purpose, 80 µl of the sample was extracted into a plastic tube (7.5 cm length, 1 cm diameter), to which 720 µl physiologic water and 200 µl Bradford reagent (Bio-Rad Laboratories, Munich, Germany) were added. After 15 min, samples were read in a spectrophotometer (Jenway-6300) at OD_{595}. Protein concentrations were obtained from a standard curve based on porcine serum albumin (Sigma-Aldrich).

5.3.5 Statistical Analysis

Data were analysed with GLM models (SAS 1997). The energetic value of glucose and glycogen is 16.74 J per mg, and that of lipids is 37.65 J per mg (Clements 1992). Analyses were done both on total energetic reserves (summed caloric value of lipids, glycogen and glucose) and on each resource individually (lipids, glucose, glycogen and protein). The abundance of each resource (and their sum) was modelled as a function of parasite clone (control, ER, CR, or CR/ER), mouse (nested within clone), body size, chemical analysis block and blood meal size. The maximal model was simplified by sequentially eliminating non-significant terms

and interactions. After the minimal model (the model including only significant terms and interactions) was obtained, its appropriateness was tested by inspecting a plot of the residuals against the fitted values. Significant values given in the text are for the minimal model while non-significant values are those obtained prior to the deletion of the variable from the model.

5.4 Results

The average energetic resources available to mosquitoes were 7.00 J (± 0.19 s.e.) at the oocyst stage and 7.14 J (± 0.31 s.e.) at the sporozoite stage. Contrary to expectations, the energetic content of mosquitoes fed on uninfected control mice was no different from those fed on infected mice (pooling all parasite groups), either at the oocyst ($F_{1,76} = 0.01$, ns) or sporozoite stage of parasite development ($F_{1,66} = 0.70$, ns). Similarly, there were no differences in the total energy content of mosquitoes across parasite treatments (control, ER, CR, CR/ER: oocyst: $F_{3,86} = 1.21$, ns, sporozoite: $F_{3,56} = 1.31$, ns). In no case was there significant variation in energy levels between mosquitoes feeding on different mice within the same parasite treatment ($p > 0.05$).

The abundance of particular energy reserves, however, did vary between parasite clones. At the oocyst stage, mosquitoes that fed on CR and CR/ER-infected mice contained approximately 50% more sugar than those in either the control or the ER treatments (Figure 5.1). This difference, which was very significant ($F_{3,105} = 4.06$, $p < 0.01$), could not be explained by differences in blood meal size ($F_{1,79} = 0.07$, ns). This difference in sugar abundance was lost by the sporozoite stage, however, where mosquitoes from different parasite treatments had similar amounts

($F_{3,76} = 0.31$, ns, Figure 5.1). Sugar levels in mosquitoes at the sporozoite stage were generally lower than at the oocyst stage ($F_{1,184} = 4.32$, $p<0.05$).

Unlike sugars, levels of glycogen, lipids and proteins were unaffected by parasite treatment either at the oocyst (lipids: $F_{3,91} = 0.90$, ns; glycogen: $F_{3,82} = 0.21$ ns; proteins: $F_{3,79} = 0.08$, ns) or sporozoite stage (lipids: $F_{3,62} = 0.80$, ns; glycogen: $F_{3,71} = 0.28$ ns; proteins: $F_{3,61} = 0.17$, ns). Similarly, blood meal size was not related to the abundance of glucose, glycogen or protein at either stage ($p > 0.05$ in all cases). However, blood meal size was negatively correlated with the level of lipids in mosquitoes at the sporozoite stage ($F_{1,76} = 8.31$, $p<0.01$).

In order to further explore the nature of the increase in sugars in CR and CR/ER infected mosquitoes (oocyst stage), an additional analysis was conducted where mosquitoes within each parasite group were distinguished as either being infected or uninfected (those with patent oocyst infections and those without). Mosquitoes allocated to the control treatment (fed uninfected blood) were excluded. This allowed us to determine whether the increase in glucose was due to the presence of the parasite itself, or prevalent in all mosquitoes that fed on infected blood. The presence or absence of oocysts in the gut was defined as a binary explanatory factor. Oocyst presence, irrespective of parasite clone, was a strong predictor of sugar abundance ($F_{1,64} = 8.33$, $p <0.01$). The increased sugar levels in CR and CR/ER mosquitoes was only found in mosquitoes that had at least one oocyst on their midgut ($F_{3,61} = 3.14$, $p = 0.033$ for infected mosquitoes and $F_{3,61} = 1.55$, ns for uninfected ones) (Figure 5.2). Amongst mosquitoes with oocysts, their number was not related to sugar abundance ($F_{1,35} = 0.97$, $p = 0.33$).

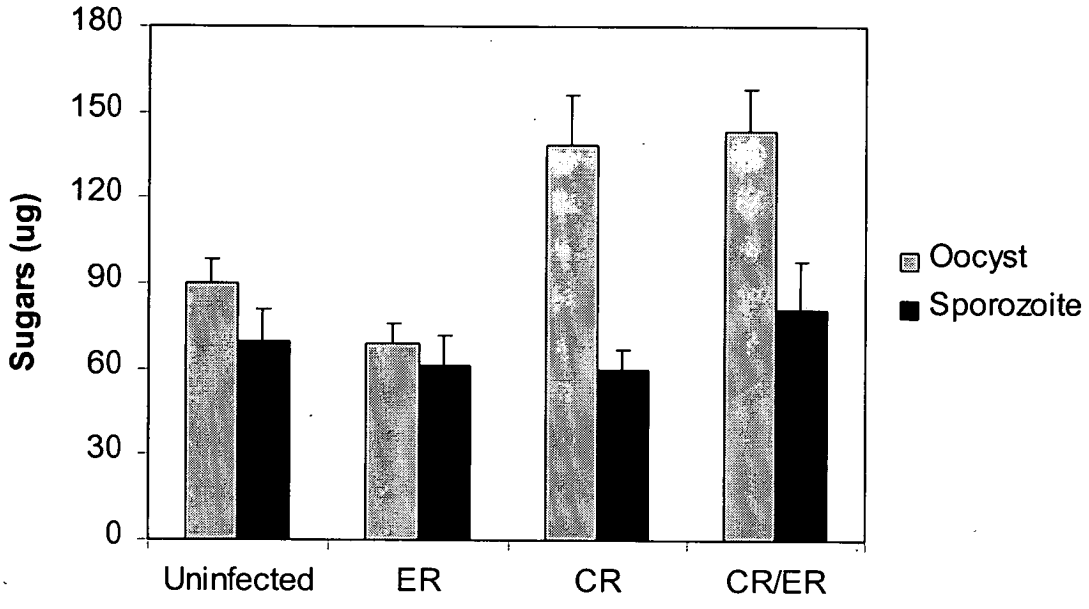

Figure 5.1: Amount of sugars (ug) in mosquitoes fed on mice with one of four *P. chabaudi* treatments at the oocyst (day 7 post infection, grey bars) and sporozoite stage (day 14 post infection, black bars) of parasite development. Error bars are standard errors.

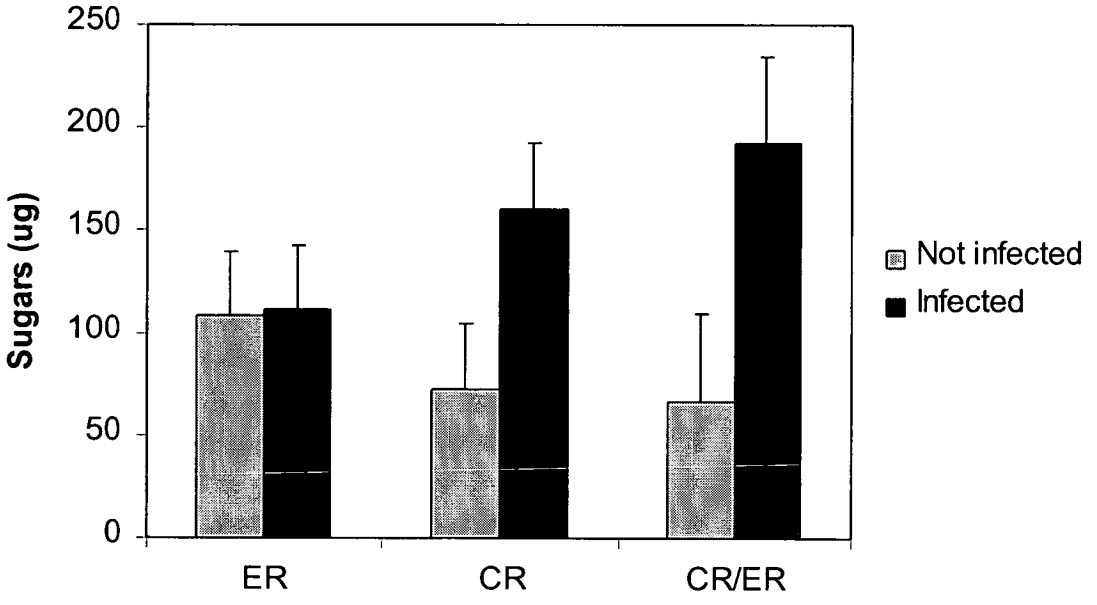

Figure 5.2: Amount of sugars (ug) in mosquitoes infected fed on mice with three *P chabaudi* treatments at the oocyst stage of parasite development (day 7 after infection). Grey bars are means for mosquitoes without patent oocyst infections, and black bars are for those with at least one oocyst. Error bars are standard errors.

5.5 Discussion

Plasmodium chabaudi infection had no impact on the overall energy levels of *A. stephensi* mosquitoes: mosquitoes fed on infected blood of any sort had the same summed resource level as those fed on uninfected controls. Nor did the observed level of parasite virulence (high = CR/ER mixture and CR, low = ER) correlate with the summed abundance of all three key mosquito energy reserves. Energy depletion cannot therefore account for the variation in fecundity and longevity observed in mosquitoes infected with different *P. chabaudi* genotypes (Ferguson and Read 2002a, Chapter 3 and 4). Also, there was no temporal shift in the net abundance of resources as mosquitoes passed from the oocyst to sporozoite stage of *Plasmodium* infection.

Across each of the four individual resource types, the only *Plasmodium* associated-change was an increased level of sugars (sugars measured were soluble carbohydrates: glucose and trehalose), key insect nutritional and energetic resources. Specifically, at the oocyst stage of parasite development, mosquitoes with the more virulent infections (CR and CR/ER clone treatments) contained up to 50% more sugars than control mosquitoes or mosquitoes infected with the less virulent parasite clone (ER). Although statistically significant, this increase in glucose was not sufficient to generate a detectable increase in the overall energy budget of mosquitoes in these parasite treatments (sugars contain only half as many calories as lipids per unit weight).

Sugars are a key ready-to-use source of metabolic energy in insects, and are the sole energy source for mosquito flight (Clements 1992). Free sugars such as

glucose are either the product of the breakdown of stored molecules (Nijhout 1994; Clements 1999) or are ingested *de novo* from the blood meal (Foster 1995) or flower nectar and honeydew (Yee and Foster 1992; Foster 1995; Holliday-Hanson *et al.* 1997; Burket *et al.* 1999; Clements 1999; Takken and Knols 1999). There are thus three different possibilities for the origin of the excess sugars in mosquitoes infected with the virulent clones of *P. chabaudi*: 1) a reallocation of resources within the mosquito as a response to the infection, 2) qualitative or quantitative differences in the glucose content of ingested blood or 3) differences in the amount of sugars ingested post-infection.

Shifts in resource allocation in response to parasite infection are well documented, although they are often inferred from shifts in life history traits, such as fecundity and longevity (Sorensen and Minchella 1998; Hurd 2001; Hurd and Warr 2001), and are only rarely measured directly (e.g. Kearns *et al.* 1994; Brown *et al.* 2000). In this experiment, we tested for nutrient reallocation by measuring the levels of other resources in the body that are known to produce glucose as a result of their catabolism: glycogen and protein (Nijhout 1994; Clements 1999). We did not find a significant reduction in the levels glycogen or protein concomitant with the increased levels of sugars in some parasite groups, and thus the possibility of resource reallocation was dismissed. The only alternative explanation for these results is that the increased levels of sugars were acquired *de novo*.

It is unlikely that the extra sugars came from host blood. The sugar content of blood is low (Foster 1995), particularly in *Plasmodium*-infected hosts (Elased and Playfair 1994; Elased and Playfair 1996; Elased *et al.* 1996). Also, blood is consumed to acquire the proteins necessary for egg production rather than for its

caloric value (Clements 1992). Even if the concentration of glucose in blood does not vary with infection, mosquitoes in infected groups could acquire a greater amount of it if they take larger blood meals than those feeding on uninfected hosts. However, variation in glucose levels between parasite treatments could not be linked to variation in blood meal size. Nor could variation in glucose levels be attributed to blood meal quality. The increase in glucose was observed only in mosquitoes that fed on blood infected with CR or CR/ER and subsequently developed oocysts. The glucose levels of mosquitoes that fed on the same infected blood but did not develop oocysts were no different from those of the controls. The observed increase in glucose is therefore not a by-product of a general qualitative change in the blood of mice infected with the parasite.

The high levels of glucose found in mosquitoes from the CR and CR/ER treatments, must therefore have arisen from increased glucose intake subsequent to infection (mosquitoes allowed to feed on glucose *ad libitum* after infection). Furthermore, since this increase in glucose was observed only in oocyst-infected mosquitoes, increased glucose feeding was, directly or indirectly, the result of infection by the parasite. It is well known that malaria parasites can modify mosquito blood-feeding behaviour (Wekesa *et al.* 1992; Koella *et al.* 1998; Anderson *et al.* 1999), and that this modification can be advantageous for the parasite (e.g. Anderson *et al.* 1999). It is unknown whether parasites can also influence mosquito sugar-feeding behaviour, and if so, whether these changes are parasite-driven or a host response.

There are at least three different ways in which manipulation of mosquito sugar feeding behaviour at the oocyst stage could benefit the parasite. First, there is

evidence that sugar-availability reduces the frequency of host biting in the laboratory, possibly because sugar loaded mosquitoes are constrained in their ability to obtain blood meals due to space limitations in the abdomen (Foster and Eischen 1987; Straif and Beier 1996). Host biting incurs mortality risks for mosquitoes due to the defensive behaviour of the vertebrate hosts (Day and Edman 1983), and this risk may be disproportionately high in infected mosquitoes (Anderson *et al.* 2000). Increased sugar feeding could thus benefit the parasite by reducing mortality associated with blood feeding while the parasite is growing. Second, parasites may benefit from increasing mosquito sugar feeding as this could provide more resources for growing oocysts. One study reported that oocyst-infected midguts used up to 8 times the amount of glucose as non-infected midguts (discussed by Schiefer *et al.* 1977). Unfortunately, this often cited study was not published, so the question of whether oocyst infected midguts have high glucose requirements remains unconfirmed. Finally, a tantalising possibility is that *Plasmodium* induces enhanced glucose uptake in order to neutralise the mosquito immune system. In many organisms, a glucose overload impairs the production of nitric oxide (e.g. Prabhakar 2000; Golderer *et al.* 2001; Kimura *et al.* 2001), a molecule that *A. stephensi* uses to combat *Plasmodium* development (Luckhart *et al.* 1998; Luckhart and Rosenberg 1999; Han *et al.* 2000). The role of glucose in NO production in mosquitoes, and the possibility that parasites could manipulate sugar intake in order to fight off the vector immune system, are intriguing and should be explored further.

It is also possible that enhanced sugar feeding could be a mosquito response to parasitism. By increasing the consumption of sugars, which are important precursors of all carbon-based chemical compounds and an essential resource for

mosquito maintenance (Clements 1999), infected mosquitoes could minimise or compensate for the harm caused by the parasite invasion of midgut epithelial cells, or competition for host metabolic products (Maier *et al.* 1987). In another study, mosquitoes infected with the CR/ER mixture had greater mortality (Ferguson and Read 2002a, Chapter 3) when sugar was provided *ad libitum* than when it was restricted. Thus increasing the availability of glucose did not improve vector longevity, suggesting that an increased intake of glucose does not directly benefit the vector.

In conclusion, although we found no evidence that virulence in *P. chabaudi-A. stephensi* system is the product of energy depletion, these results indicate that parasitism, specifically by virulent genotypes, impacts the rate of mosquito resource acquisition (glucose intake). Further investigation of the physiological role of glucose in *Plasmodium*- infected mosquitoes, and its interactions with parasite genetics, is required to evaluate adaptive hypotheses for the phenomenon. Ultimately, whether increased sugar feeding is adaptive for the parasite, for the mosquito, or simply a by-product of the infection, will be determined by which party's fitness is enhanced by this behaviour. Resolution of this issue will provide insight into the adaptive nature of vector-parasite interactions and their epidemiological consequences.

Chapter 6: Malaria parasite virulence in its vertebrate and vector hosts

6.1 Summary:

Several laboratory studies of malaria parasites (*Plasmodium* sp.) and some field observations suggest that parasite virulence, defined as the harm a parasite causes to its vertebrate host, is positively correlated with transmission. Given this advantage, what limits the continual evolution of higher parasite virulence? One possibility is that while more virulent strains are more infectious, they are also more lethal to mosquitoes. In this study, we tested whether the virulence of the rodent malaria parasite *Plasmodium chabaudi* in the laboratory mouse was correlated with the fitness of mosquitoes it subsequently infected. Mice were infected with one of seven genetically distinct clones of *P. chabaudi* that differ in virulence. Weight loss and anaemia in infected mice were monitored for a 16-17 day period before *Anopheles stephensi* mosquitoes were allowed to take a blood meal from them. Virulence in mice was positively correlated with mosquito infection rate but not with mosquito survival. Reductions in vector survival depended on parasite clone and oocyst burden, but were unrelated to virulence in the mouse. Vector fecundity was also influenced by parasite clone but, contrary to expectations, increased with parasite virulence in mice. These results suggest that vector fitness should not limit the emergence of higher malaria virulence in vertebrates. The strong parasite genetic effects associated with both fecundity and survival, however, suggests that vector fitness could be an important selective agent for malaria parasites.

6.2 Introduction:

Theoretical studies have shown that natural selection will favour the evolution of increased pathogen virulence when there is a positive genetic correlation between disease severity and transmission success (Levin and Pimentel 1981; Anderson 1982; Bremermann and Pickering 1983; Anderson and May 1991; Sasaki and Iwasa 1991; Frank 1992; Bull 1994; Read 1994; Ebert and Herre 1996; Frank 1996; Gandon *et al.* 2002). By virulence, we refer to the harm inflicted upon a host by a pathogen, a trait that can be measured in terms of a reduction in host fitness (survival or fecundity), or the severity of sub-lethal symptoms. Empirical studies suggest that positive relationships between pathogen virulence and transmission are common, and found in many pathogen taxa (e. g. Lipsitch and Moxon 1997; Ebert 1998; Mackinnon and Read 1999b).

Given these virulence-associated transmission benefits, the question arises as to what limits the continual evolution towards higher virulence. This issue is particularly perplexing for parasites that cause malaria (*Plasmodium* sp.), which along with HIV are agents of the most significant infectious disease of modern humans. Studies of rodent malaria suggest that *Plasmodium* virulence is positively correlated with transmission success (Mackinnon and Read 1999a; Mackinnon and Read 1999b) and thus virulent parasites should have a selective advantage Comparable studies of human malaria parasites are necessarily lacking, but there is evidence that the multiplication rate of *P. falciparum* in culture, a possible correlate of transmission potential, is greater in patients with severe malaria than in those who are asymptomatic (Chotivanich *et al.* 2000).

The source of selection against virulent strains of human malaria might be malaria-induced host mortality. This is the conventional explanation for why pathogens are not more virulent (Ewald 1994), and it might be correct in the malaria context. A selection experiment with the rodent malaria *P. chabaudi,* a species that shares many life history traits with the human parasite *P. falciparum* (Cox 1988), showed that higher virulence continued to evolve even when a parasite-induced death rate of 50-75% was simulated (Mackinnon and Read 1999a). The case fatality rate of *P. falciparum* in humans in Africa is substantially less than this: possibly as high as 10% but most probably 1% or less (Snow *et al.* 1999; Trape *et al.* 2002). Such low death rates are certainly insufficient to limit *P. chabaudi* virulence in laboratory mice (Mackinnon and Read 1999a), but whether they are sufficient to limit *P. falciparum* virulence in humans is unknown. Resolving this issue requires quantitative data on the relationship between the risk of host death and transmission in *P. falciparum* (i.e. would a strain inducing a 2% case fatality rate have lower fitness than one killing 1% of hosts?). For ethical reasons, such data cannot be obtained for any of the human malaria species, at least given current technologies.

However, there are a number of other candidate sources of selection against virulence (e. g. Ebert 1998; Day 2001). Here I consider the as yet untested possibility that the evolution of increased virulence is constrained by the mortality of the mosquito vectors of *Plasmodium* (*Anopheles* sp.). The role of vectors in virulence evolution has been largely ignored, probably because they are generally assumed to be unaffected by the pathogens that use them for dispersal (Ewald 1983; Ewald 1994). However, *Plasmodium* can reduce vector longevity (Koella *et al.* 1998; Anderson *et al.* 1999; Koella 1999; Ferguson and Read 2002a; Ferguson and

Read 2002b, Chapters 2 and 3) and fecundity (Hacker 1971; Hacker and Kilama 1974; Freier and Friedman 1976; Hogg and Hurd 1995a; Hogg and Hurd 1995b; Hogg and Hurd 1997, Chapter 4), and these virulence effects differ between parasite genotypes (Ferguson and Read 2002a, Chapters 3 and 4). Variation in the survival of mosquitoes infected with different *Plasmodium* genotypes could thus constrain the evolution towards higher virulence in their vertebrate host.

There are at least two possible mechanisms by which selection towards increased virulence in the vertebrate host could generate a correlated reduction in vector fitness. First, the increased transmission associated with high levels of *Plasmodium* virulence in vertebrates (Mackinnon and Read 1999b) could generate high parasite burdens in mosquitoes that may increase their mortality (Klein *et al.* 1986). The second possibility is that regardless of parasite presence, blood that has endured a severe *Plasmodium* infection could be of poorer quality to mosquitoes. This could occur as a result of parasite-induced anaemia, or changes in blood chemistry that could influence blood feeding success (Hosoi 1959; Taylor and Hurd 2001).

Here we tested whether the survival and fecundity of female *Anopheles stephensi* mosquitoes infected with the rodent malaria parasite *P. chabaudi* was correlated with the virulence of parasites in their mouse host. We know of only one other comparison of the virulence of different life stages of any pathogen or parasite that has multi-host life cycles: across five strains of schistosome worms, virulence in laboratory mice was positively but not significantly related to the mortality of the snail vectors they infected (Davies 2000; Davies *et al.* 2001). Thus there is at least

some evidence that vector survival could select against virulence in a parasite's vertebrate host.

6.3 Methods:

6.3.1 Mouse infections

Seven different clones of *Plasmodium chabaudi* known as AJ, AQ, AS, BC, CW, CR and ER were used (Mackinnon and Read 1999b). Clones are asexually replicated lineages derived from a single ancestral parasite which was isolated by serial dilution (Mackinnon and Read 1999b). These clones were chosen because their behaviour has been extensively studied in laboratory mice where they are known to generate infections of varying severity (Mackinnon and Read 1999b).

Groups of seven female mice (C57BL/6J, Harlan England) were injected with 10^5 parasites of one of the 7 clones, or were left uninfected to act as controls (n=56). The control group were given sham injections that contained only the inoculation medium of calf serum and ringers solution. From the first day after injection (day 1), daily measurements of weight and anaemia (red blood cell density) were taken from all mice. Anaemia was calculated by diluting a 2 ul sample of tail blood in 40 ml of isoton, and processing the resulting solution in a Coulter Counter (Coulter Electronics, Luton, England). Additionally, thin blood smears were taken on a daily basis from all mice in the infected treatments to determine the proportion of red blood cells that were infected with asexual parasites (parasitemia) and the proportion infected with gametocytes (gametocytaemia). Gametocytes are sexual forms that, unlike asexual parasites, can be transmitted to mosquitoes. Daily estimates of asexual parasite density and gametocyte density were calculated as the proportion of red blood cells infected with each parasite type multiplied by red blood cell density.

Total asexual parasite production and total gametocyte production were estimated for each infected mouse as the area under the curve of the density of each parasite stage from day 5 – 17. One mouse in the AJ treatment did not develop any parasites after being injected and was eliminated from analysis.

6.3.2 Mosquito rearing and infection

Anopheles stephensi larvae were reared in standard insectary conditions of 27 ± 1 °C, 70% humidity and a 12:12 light:dark cycle. Eggs were placed in plastic trays (25 x 25 x 7 cm) filled with 1.5 L of distilled water. In order to reduce variation in larval growth rate and size at emergence, larvae were reared at a density of 500 per tray. Larvae, which were fed on Liquifry™ for 5 days and then on ground Tetrafin™ Fish flakes, took 9-14 days to transform into pupae. On day 10, 11 and 12, all pupae collected were pooled in a large container, mixed, and randomly placed in one of 48 emergence cages (16 x 16 x 16 cm). Over this 3-day period, each cage received approximately 280 pupae. After emergence, adults were fed *ad libitum* on a 10% glucose solution supplemented with 0.05% PABA.

Mosquito feeds took place on days 16 and 17 after mouse infection, the point at which gametocytes were detectable in most blood smears from infected mice. Due to deaths during the infection period, only 44 out of the original 56 mice survived long enough to be fed to mosquitoes ($n_{day16} = 23$, $n_{day17} = 20$). Within a parasite clone group, approximately half of the surviving mice were fed on day 16 and half on day 17. To increase hunger levels, mosquitoes were deprived of glucose water for 24 hours before being allowed to blood feed on mice. One anaesthetised mouse was placed on each cage and mosquitoes were allowed to feed for 20 minutes (mean = 75 females per cage). Immediately after the feed, 10 engorged mosquitoes were

randomly taken from each cage and individually placed into 30 ml plastic tubes (9 x 2.5 cm) covered with mesh. These mosquitoes were used in the fecundity experiments (see below). Mosquitoes remaining in cages were used for survival experiments.

Prior to blood-feeding, mosquitoes in one of the cages had a very poor hatching rate and exceedingly high mortality. Atypical mortality continued in this cage after infection of mosquitoes (using the clone AQ): mortality by day 9 in this cage = 100%, all other infected cages = 11.2%, s.e. = 1.9. Mosquitoes in this cage were pre-disposed to high mortality before and after the application of the infection treatment, possibly as a result of bacterial contamination in the larval bowl, and so were discarded from analysis.

6.3.3 Mosquito survival

On each feed day, approximately half of the cages in each parasite clone treatment were allocated to a glucose water *ad libitum* treatment, and the other half to glucose water deprivation. This treatment was applied because previous experiments have shown that glucose availability influences parasite clone virulence in *A. stephensi* (Ferguson and Read 2002a, Chapter 3). With the exception of the ER-clone, there were at least two replicates of each parasite clone and glucose water treatment combination. As only three mice in the ER group survived until blood feeding, mosquitoes from all three feeds were maintained in the same glucose water conditions (deprivation). Under the deprivation treatment, mosquitoes were given access to glucose water only one day out of every two, whereas availability was constant in the *ad libitum* treatment.

After the blood feed, cages were checked daily and dead mosquitoes were removed. No further blood meals were given. One petri dish containing water was placed in each cage two days after the blood feed to allow mosquitoes to oviposit. These dishes were removed the following day and the eggs discarded. Fifteen females from each cage were removed on days 8 and 9 after the blood feed. These mosquitoes were killed with chloroform, and their midguts were dissected in a drop of a 0.01 M solution of phosphate-buffered saline and examined under a microscope to assess oocyst prevalence and burden. One wing was removed from all mosquitoes that died and measured as an index of body size. Wing length was measured under a dissecting microscope as the longest axis from the base of the wing to the tip (not including the hairs). Survival monitoring was terminated 45 days after the blood feed, when all remaining survivors were killed, counted, and measured.

6.3.4 Mosquito fecundity

Mosquitoes transferred into tubes were fed by cotton pads soaked in a 10% glucose solution with 0.5% PABA. These pads were placed on top of each tube and replaced daily. Blood meal size was estimated indirectly as the amount of hematin excreted over a 3-day period (as in Briegel 1980). Hematin was dissolved in 1 ml of a 1% $LiCO_3$ solution. The absorbance of the resulting solution was read at 387 nm, and compared to a standard curve made from porcine hematin (Sigma-Aldrich). Solutions with an absorbance of less than ≤ 0.05 nm were classified as being from individuals that had not fed, as this absorbance was indistinguishable from that of the $LiCO_3$ control. As the mosquitoes selected for the fecundity experiment were a random sampling of the mosquitoes used in the survival experiments, estimates of

mean blood meal size were used as explanatory variables in the analysis of both experiments.

After the 3-day hematin collection period, mosquitoes were moved to new tubes filled with 2 ml of water to allow oviposition (following Hogg and Hurd 1995a). Fecundity was measured as the number of eggs laid over the following three days. Mosquitoes were subsequently moved into new tubes for 1-2 days (7-8 days post blood feeding) before being killed with chloroform. The midguts of mosquitoes that fed on infected mice were dissected as described above and examined for oocysts. One wing was removed from all mosquitoes at the end of the experiment and measured as above.

6.3.5 Statistical Analysis

Four measures of virulence were measured in each mouse: maximum % weight loss from weight on day 0, maximum % red blood cell density loss from value on day 0, cumulative total weight loss (sum of differences between starting weight and weight during each day of the infection: 1-16) and cumulative total red blood cell loss (sums of differences between starting red cell density and red cell density during each day of the infection: 1-16). The latter two measures were selected to reflect infection chronicity, as they their value increases with the time taken for an animal to regain normal weight and red cell density. These four variables were combined in a Principal Components analysis to produce one representative measure of mouse infection virulence. Data collected from mice that died before day 16 post infection were not included in this analysis.

The two aims of the statistical analysis were to: 1) investigate the relationship between the virulence of *P. chabaudi* infection in mice and their transmission to mosquitoes and 2) test whether the virulence of *P. chabaudi* infections in mice is correlated with their virulence in mosquitoes. The first aim was accomplished by using General Linear Models (SPSS 1995) to test the relationship between mouse infection virulence score and 3 measures of parasite transmission: total gametocyte production, oocyst infection rate and mean oocyst burden (mean no. of oocysts per infected mosquito). In addition to virulence score, parasite clone and feed day (for oocyst burden and infection rate only) were included as explanatory variables.

The virulence of *P. chabaudi* infections in mosquitoes was assessed by their survival and fecundity. Two measures of survival were computed: 1) median survival time per blood feed and 2) the proportion of mosquitoes in each cage surviving until day 14 after each blood feed. Mosquitoes are vertebrate-infective after 14 days, when *Plasmodium* sporozoites are fully developed and present in the mosquito mouthparts. Both survival measures were obtained from Kaplan-Maier estimates of the survival distribution in each cage (SPSS 1995).

Genetic correlations between mosquito fitness traits and parasite virulence in mice were assessed by evaluating the relationship across clone means. As the genetic variants we used were clonal lineages, non-additive as well as additive sources of variation will be included in the genetic correlations we obtain. As such, these correlations may overestimate those occuring in parasites allowed to recombine. Phenotypic correlations were calculated across all infections and, where there were significant effects of clone, across infections within clones by fitting clone

as a factor (analogous to analysis of covariance). I tested the relationship between each of the two cage-level survival indices and the five main treatment effects: infection status (control or *P. chabaudi* –infected blood), mouse virulence score, parasite clone, blood feeding day (16 or 17), and glucose water treatment (SPSS 1995). Maximal models included all main effects and their interactions, non-significant terms were dropped to yield a minimum model. A similar analysis was conducted for mean fecundity, where the main treatment effects were the same as in the survival analyses except that there was no glucose water treatment. After the significance of the main treatment effects had been assessed, a second round of GLM models were used to identify whether any of the 6 additional infection explanatory variables that were collected (gametocyte density on feed day, oocyst infection rate, mean oocyst burden, anaemia on feed day, mean blood meal size and mosquito body size) could explain further variation in vector survival or fecundity, either when considered independently or added to the minimum statistically significant model of main treatment effects.

Before analysis, all proportion data (weight loss, red cell density loss, gametocytaemia, parasitemia, mosquitoes that laid eggs, mosquitoes surviving until day 14, mosquitoes infected) were arcsine square root transformed, and values of total gametocyte density, gametocyte density on the day of blood feeding, oocyst burden and maximum asexual parasite density were $\log(x + 1)$ transformed. Throughout, means are reported ± one standard error.

6.4 Results:

6.4.1 Virulence in vertebrate host

Plasmodium chabaudi infection induced substantial morbidity and mortality in mice (Figure 6.1). Over the 16 day monitoring period, infected mice lost up to 3-30% of their initial body weight, and 27-87% of their red blood cells. Twelve out of the initial group of 48 infected mice died within two weeks of infection (Table 6.1). The severity of infection in surviving mice varied substantially between parasite clones (Table 6.1). The most virulent clone was AQ, which induced the greatest total weight loss in survivors and killed just under half its hosts. The AS and CW clones were the least virulent. All parasite clones induced reductions in weight and red blood cell density in contrast to the uninfected controls.

When all four mouse virulence traits were combined in a principal components analysis, the first principal component (PC1) explained 68.9% of the variation in traits. This virulence score (PC1) was positively correlated with each of the four virulence traits (maximum % weight loss $r = 0.92$, maximum % of red blood cell loss $r = 0.88$, cumulative total weight loss $r = 0.85$, cumulative total red cell loss $r = 0.64$). Clone-specific death rates tended to increase with the virulence score of each clone based on surviving mice (r=0.65, p=0.11, Figure 6.2), suggesting that this virulence measure (PC1) is representative of both morbidity and mortality risk.

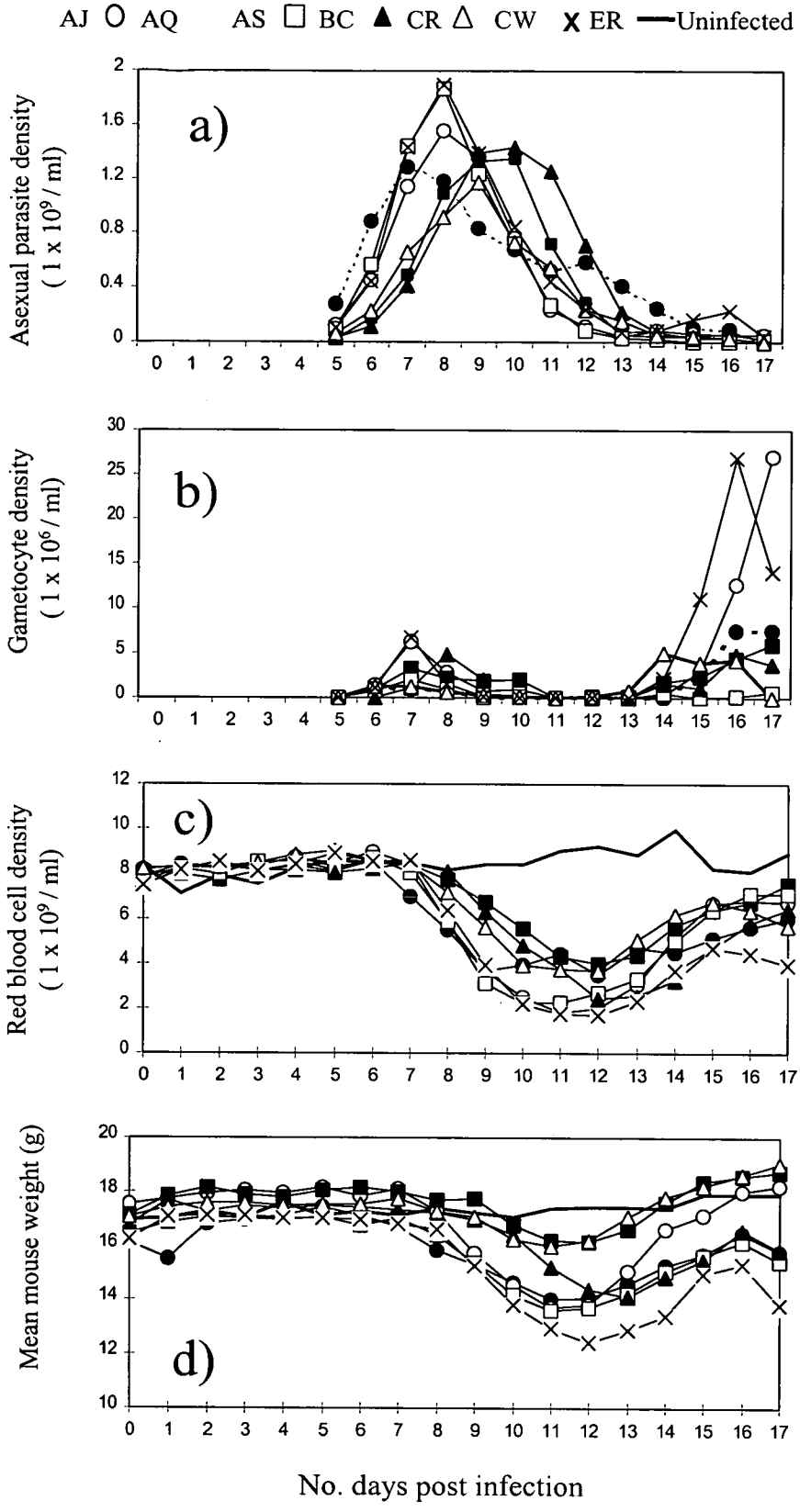

Figure 6.1: Kinetics of mean asexual parasite density (a), gametocyte density (b), red blood cell density (c), and weight (d) of laboratory mice infected with different clones of *Plasmodium chabaudi.*

Trait	Parasite clone								S. E
	AJ	AQ	AS	BC	CR	CW	ER	UN	
% Mortality	28	43	14	28	0	0	57	0	
Maximum PA^{A}	34.2	36.3	28.5	41.5	43.1	24.0	36.8	-	0.02
Day of maximum PA^{C}	10.0	9.3	9.8	9.0	10.9	9.9	8.7	-	0.23
Maximum PD (1×10^9 / ml)^{B}	1.96	1.67	1.77	2.04	1.72	1.26	1.95	-	0.07
Day of PD^{C}	9.0	7.8	9.3	8.4	9.3	9.1	8.0	-	0.19
Total PD (1×10^9 / ml) * day^{A}	6.74	5.69	5.19	6.03	6.66	4.77	7.30	-	0.20
Maximum WL (g)^{A}	3.21	3.90	1.66	3.14	2.97	1.40	3.33	0.17	0.22
Minimum RBCD (1×10^9 / ml)^{A}	2.10	2.01	3.60	1.97	2.2	3.25	1.74	6.32	0.26
Cumulative total WL loss (g * day)^{A}	9.52	15.22	-6.14	6.62	8.03	-3.69	5.15	-6.12	1.62
Cumulative total RBCD loss (1×10^9 / ml * day)^{A}	23.70	27.35	20.82	31.03	34.97	23.84	36.63	-1.95	2.83
Overall virulence score (PC1)^{A}	0.43	0.89	-1.05	0.48	0.39	-0.87	0.66	-	0.17

Table 6.1: Mean value of virulence and parasite abundance traits for mice infected with different clones of *Plasmodium chabaudi*. 'UN' in the parasite clone row gives values for uninfected mice. Values in the 'S.E.' column are standard errors for each trait, pooled over all parasite treatments. In the trait column, PA = asexual parasitemia, PD = asexual parasite density, WL = weight loss, and RBCD = red blood cell density. Subscripts on traits give the *p* value for the statistical test of differences between parasite treatments where A = $p<0.01$, B = $p<0.05$, and C = $p>0.05$ (not significantly different across groups). Values were computed only for mice that survived.

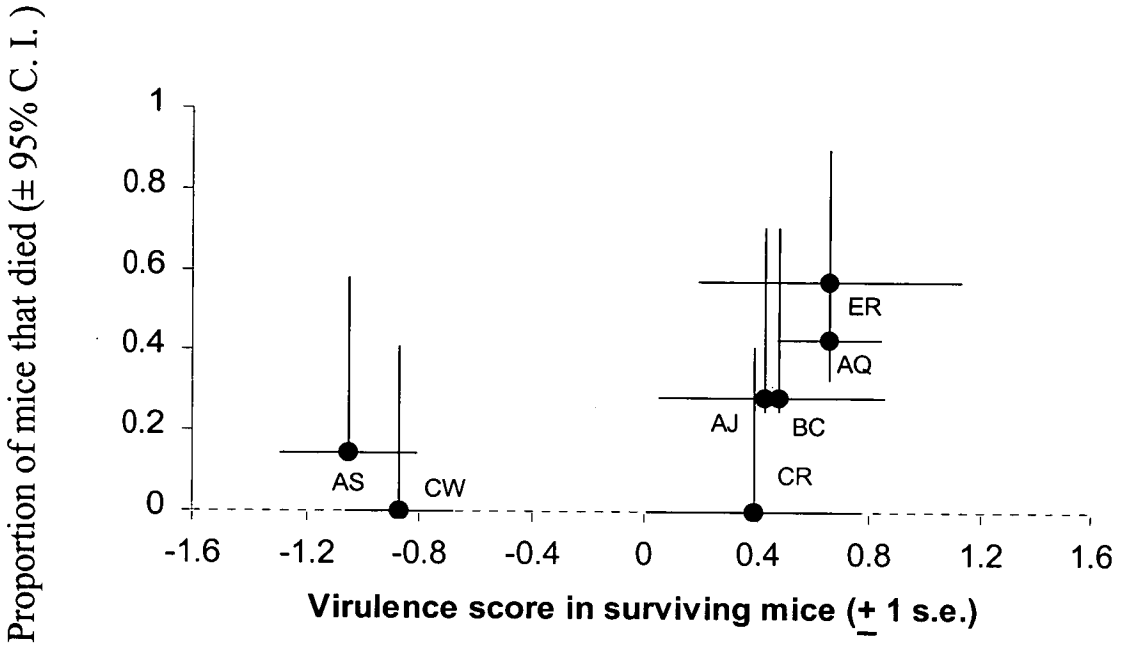

Figure 6.2: Relationship between the virulence score of different *P. chabaudi* clones computed from the morbidity of surviving mice, and the proportion of mice (out of 7) that each clone killed. The 95% confidence intervals for the proportion of mice killed are derived from the binomial distribution.

6.4.2 Virulence in vertebrate hosts and transmission to vectors

Specific details of all the minimal statistical models described in all proceeding sections are presented in Appendix 1. Ranking parasite clones by their virulence, there was a general tendency for measures of parasite transmission (gametocyte abundance, oocyst infection rates and burdens) to rise with virulence rank (Figure 6.3a-d). One consistent exception to this trend was the BC clone, which was highly virulent but which produced few gametocytes. This clone may be an outlier, as in contrast to all other clones, its transmission phenotype has changed significantly since first isolated. Specifically, its formerly high gametocyte production (similar to AQ and ER clone, Mackinnon and Read 1999b) has been lost, a phenomenon occasionally observed in the laboratory when *Plasmodium* is serially passaged (Mons 1986; Kemp *et al.* 1992; Day *et al.* 1993). BC is the only clone in which this reduction has occurred, in all others, serial passage has been associated with increased gametocyte production. All analyses of virulence and transmission were conducted both with and without the BC clone to assess if its inclusion significantly altered general relationships. Unless otherwise stated, the exclusion of BC had no qualitative influence on results, and statistics are for the analysis of all clones.

Total gametocyte production varied across parasite clones ($F_{6,28}$=6.27, p,0.01, Figure 6.3a). Including data from mice that died prior to blood feeding did not qualitatively change the relationships between parasite clone ($F_{6,41}$=2.98, p=0.02, Figure 6.3a), thus clones producing the most gametocytes in survivors retained an overall advantage even when losses due to death were incorporated. Across all infections, there was no significant relationship between total gametocyte production

and virulence ($F_{1,34}$=0.99, p=0.33, without BC: $F_{1,29}$=2.94, p=0.10), however there was across infections within clones (phenotypic correlation, $F_{1,28}$=4.54, p=0.04, Figure 6.4a). There was no relationship across clones (genetic correlation n.s., Table 6.2).

A higher proportion of mosquitoes became infected from blood feeds on day 16 and day 17 (43.4% vs. 27.2%, $F_{1,32}$=6.52, p=0.02), likely due to the increased gametocyte densities in infected mice that were fed to mosquitoes on day 16 ($F_{1,34}$=4.05, p=0.05). Accounting for this variation between days, there was a significant phenotypic relationship between mouse virulence score and mosquito infection rate ($F_{1,32}$=5.37, p=0.03, Figure 6.4b). Although a similar positive trend was evident, this association was not statistically significant at the genetic level (Table 6.2). Mean parasite load in mosquitoes (oocyst burden) varied between clones ($F_{6,28}$=5.16, p<0.01, Fig 6.3d), but was not strongly influenced by virulence either at the genetic (Table 6.2) or phenotypic level ($F_{1,33}$=2.68, p=0.11, Figure 6.4c).

Clones that induced the greatest virulence were associated with small blood meal sizes (Figure 6.3e, Table 6.2). Phenotypic relationships across infections were weaker: across all infections, mouse virulence score was unrelated to blood meal size ($F_{1,34}$=0.23, p=0.63) and there was no consistent relationship across mice within a clone ($F_{1,28}$=2.63 p=012). Interestingly, although not significant, there was a tendency towards increased blood meal size from virulent infection within each clone group, which contrasts with the negative relationship found across clone means (Table 6.2).

Figure 6.3: Parasite clone differences in total gametocyte production (day 5-17 post-infection) (a), maximum gametocyte density (b), gametocyte density on the day of blood feeding (c), mean oocyst infection rate (d) and mean mean oocyst burden (e). Parasite clones are ordered from least virulent to most virulent (left to right). In (a), black bars are based on means of mice that survived till the blood feeds, grey bars include data from mice that died earlier in the infection. Bars are standard errors.

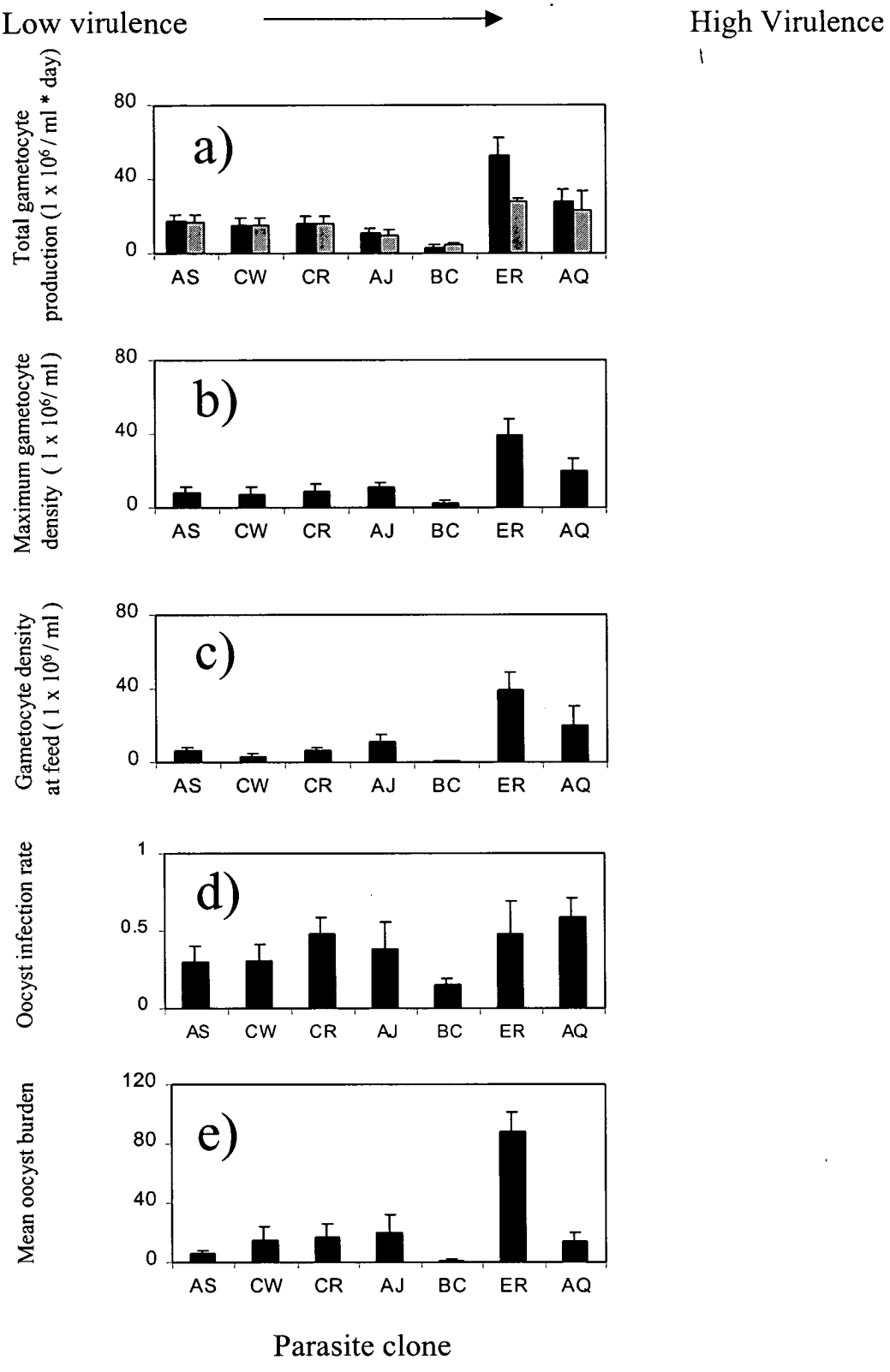
Low virulence
High Virulence
Total gametocyte production (1 x 10⁶ / ml * day)
Maximum gametocyte density (1 x 10⁶/ ml)
Gametocyte density at feed (1 x 10⁶ / ml)
Oocyst infection rate
Mean oocyst burden
a)
b)
c)
d)
e)
AS CW CR AJ BC ER AQ
Parasite clone

Figure 6.3

Figure 6.4: Relationship between *P. chabaudi* infection virulence in mice (PC1) and a) total gametocyte production, b) proportion of mosquitoes infected and c) mean oocyst burden. Regression lines indicate that the effect of virulence was statistically significant. Graphs where points are represented by symbols corresponding to clones (all but b) indicate that the effect of parasite clone was also statistically significant. Where only grey and black circles are used (b), the effect of parasite clone was not statistically significant, but 'feed day' was; black dots = day 16 feeds, grey dots =day 17 feeds. There were no significant interactions between any of the three transmission parameters and parasite clone.

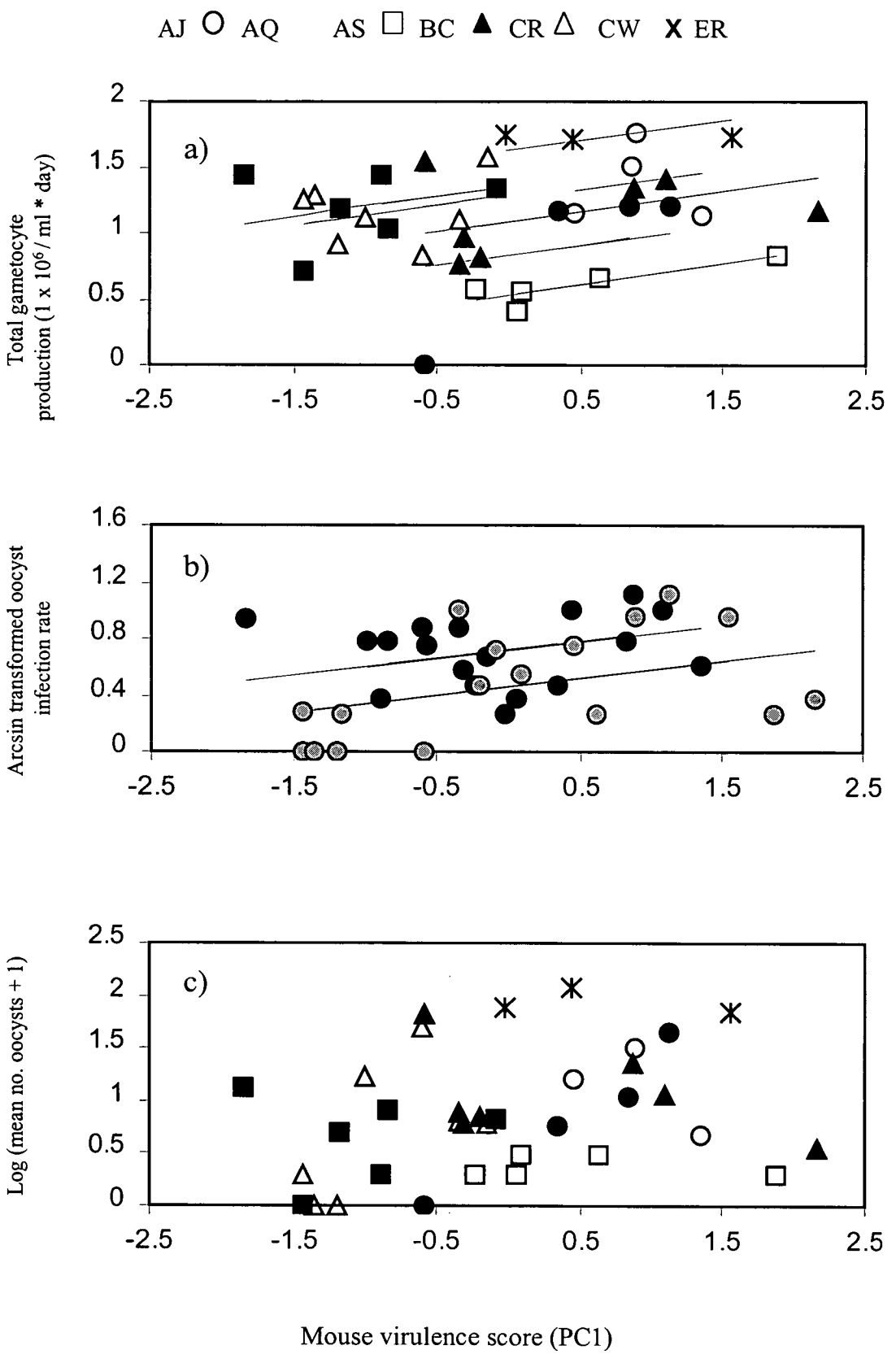
AJ ○ AQ AS □ BC ▲ CR △ CW X ER
a)
Total gametocyte production (1 x 10⁶ / ml * day)
2
1.5
1
0.5
0
-2.5
-1.5
-0.5
0.5
1.5
2.5
b)
Arcsin transformed oocyst infection rate
1.6
1.2
0.8
0.4
0
c)
Log (mean no. oocysts + 1)
2.5
Mouse virulence score (PC1)

Figure 6. 4

Trait	B	C	D	E	F	G	H	I
Virulence in vertebrate (A)	0.12	0.45	-0.89**	0.41	0.47	0.46	0.32	0.02
Total gametocyte production (B)	-	0.87*	0.18	0.77*	0.88**	-0.42	-0.08	0.29
Gametocyte density at feed (C)		-	-0.12	0.84*	0.94**	-0.20	0.22	-0.06
Blood meal size (D)			-	-0.03	-0.13	-0.73	-0.46	-0.05
Infectivity to mosquitoes (E)				-	0.82*	-0.16	0.12	0.16
Oocyst burden (F)					-	-0.30	0.01	-0.11
Mosquito median survival (G)						-	0.76*	0.15
Proportion of mosquitoes surviving until day 14 (H)							-	-0.25
Mean mosquito fecundity (I)								-

Table 6.2: Genetic correlations between *P. chabaudi* virulence in mice and their transmission and fitness costs to their mosquito vectors. Numbers are Pearson correlation coefficients (r) between the clone means (n=7) for each trait pair. Subscripts indicate the significance level of the correlation: no subscript: $p>0.05$, ' *' = $p<0.05$, ' **' = $p<0.01$.

6.4.3 *Plasmodium* virulence in vertebrate hosts and vector survival

The survival of 3175 mosquitoes was tracked for 45 days. Only 5% of these mosquitoes survived until the end of the experiment, with the average median survival (across cages) being 24.2 days (range: 13-33 days). Five hundred and twenty individuals from the initial cohort were dissected to assess oocyst prevalence and intensity, of which 35.8% were infected.

There was no evidence of a genetic or phenotypic correlation between parasite virulence in mice and mosquito survival. The mean virulence score of each clone was not correlated with its mean effect on mosquito median survival or the proportion surviving until day 14 (Table 6.2). Similarly, across infections, virulence score (PC1) could not explain significant variation in mosquito survival (Figure 6.5; Median survival: $F_{1,33}=1.18$, $p=0.29$; proportion surviving until day 14 $F_{1,33}=0.02$, $p=0.89$).

6.4.4 Other predictors of vector survival

Pooling over all parasite clones and glucose water treatments, the mean survival of infected mosquitoes was no different from those in the control groups (Median survival: $F_{1,40}=0.57$, $p=0.45$, Proportion alive at day 14: $F_{1,40}=0.89$, $p=0.35$). However, infection did influence the impact of glucose water treatment on survival. The median survival of mosquitoes blood fed on day 16 was approximately 3.5 days lower than those fed on day 17 ($F_{1,40}=5.63$, $p=0.02$). Accounting for this feed day variation, the median survival of infected mosquitoes was highest when glucose water was provided *ad libitum*, whereas uninfected mosquitoes survived best when it was limited (infection status * glucose interaction: $F_{1,37}=3.78$, $p=0.06$, Figure 6.6).

Across clones, there was a consistent tendency for mosquito median survival to be negatively associated with measures of parasite abundance (gametocytes, infection rate, and oocyst burden) and blood meal size, but these trends were not statistically significant (Table 6.2). Clearer results were obtained from analysis of phenotypic relationships. Combining all infection-related explanatory variables and their feed day interactions into a GLM model, the only significant predictors of median survival were parasite clone ($F_{6,27}$=2.65, p=0.04, Figure 6.7) and mean oocyst burden ($F_{1,27}$=6.43, p=0.02, Figure 6.8a). Similar results were obtained from the analysis of the proportion of mosquitoes surviving until day 14, where again parasite clone ($F_{6,25}$=3.03, p=0.02) and mean oocyst burden ($F_{1,25}$=3.03, p=0.02) were important. Unlike the analysis of median survival, the influence of oocyst burden on the proportion of mosquitoes surviving until day 14 varied between feed days ($F_{1,25}$=6.46, p=0.02), appearing to be more crucial for mosquitoes infected on day 16 than day 17 (Figure 6.8b & 6.8c). This discrepancy between days may be due to the fact that oocyst burdens were generally higher from feeds on day 16 than 17 ($mean_{16}$= 23.0, ± 7.8 vs. $mean_{17}$=11.21, ± 4.7 , $F_{1,33}$=3.73, p=0.06).

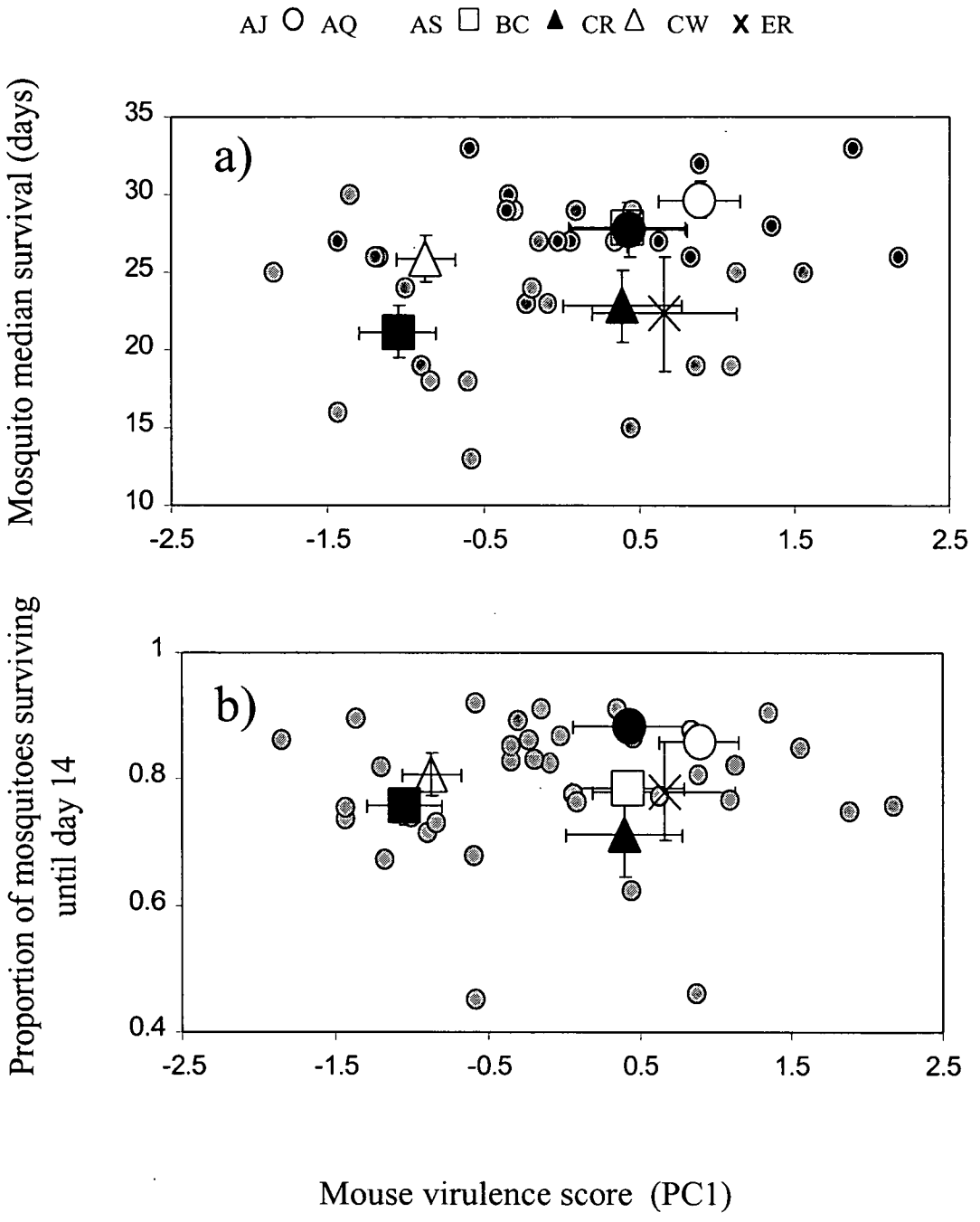

Figure 6. 5: Genetic and phenotypic relationships between *P. chabaudi* virulence in mice and vector survival: a) mean values of mosquito median survival, b) mean values of the proportion of mosquitoes surviving through the first 14 days of infection. Grey dots represent the survival of a group of 60-80 mosquitoes after feeding on an infected mouse. The large symbols are the mean values from all mouse feeds on the 7 different *P. chabaudi* clones that were used to infect mice (bars are standard errors).

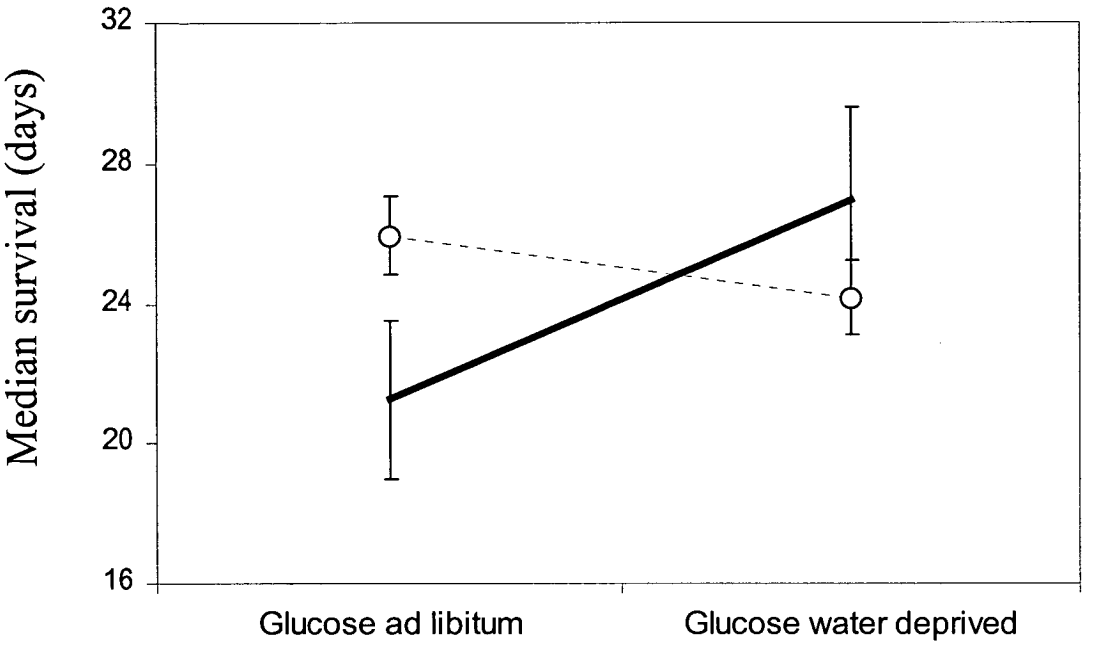

Figure 6.6: The mean value of the median survival of *P. chabaudi* infected and uninfected mosquitoes under two different glucose water regimes. Plotted points are least squares means (± s.e.) controlling for difference in day of feed (glucose*infection*day interaction $F_{1,34}$=0.25, p=0.62). Solid lines are for uninfected mosquitoes, dotted lines are for *P. chabaudi* infected mosquitoes (pooled across parasite clones).

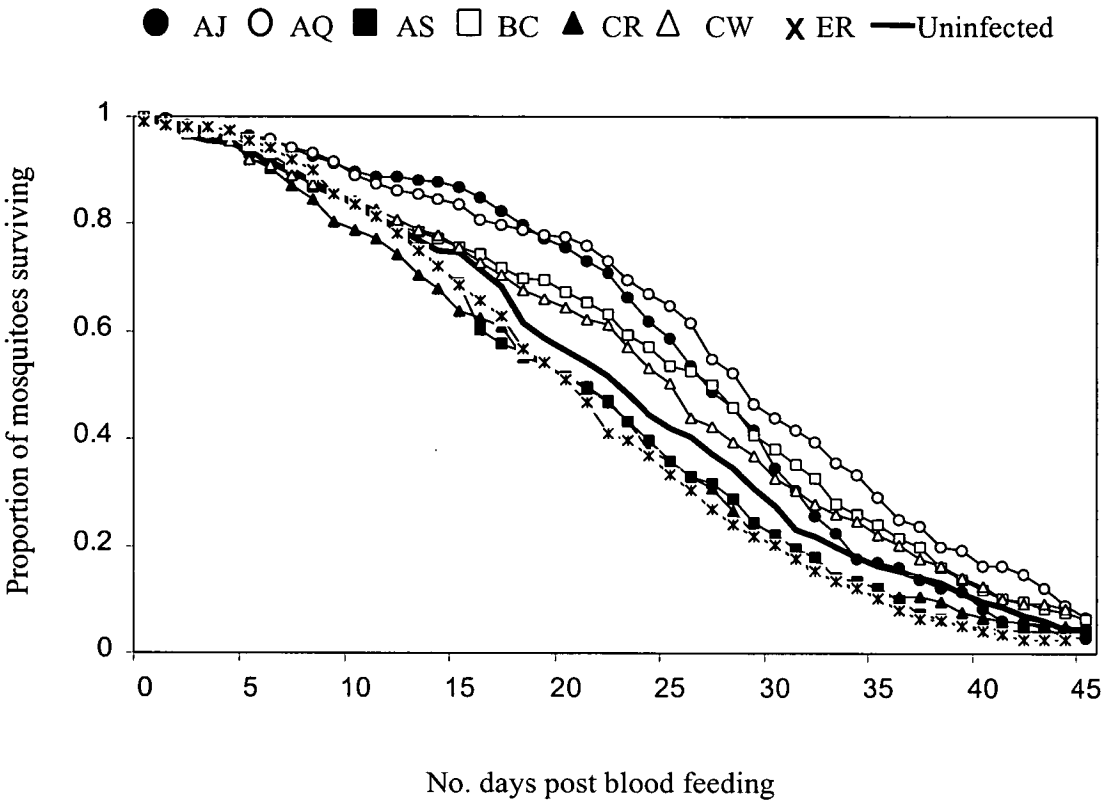

Figure 6.7: Survival curves of mosquitoes infected with different clones of *P. chabaudi.* Each curve represents the mean survival across all cages infected with the same parasite clone.

Figure 6.8: Relationships between *P. chabaudi* clone, oocyst burden, and mosquito survival: a) median survival, b) the proportion of mosquitoes surviving until day 14 from day 16 mouse infection blood feeds, c) the proportion of mosquitoes surviving until day 14 from day 17 mouse infection blood feeds. Regression lines indicate a significant effect of oocyst burden on survival. Small symbols represent the survival of a group of 60-80 mosquitoes after feeding on an infected mouse, large symbols are the mean values from all mouse feeds on the 7 different *P. chabaudi* clones that were used to infect mice (bars are standard errors).

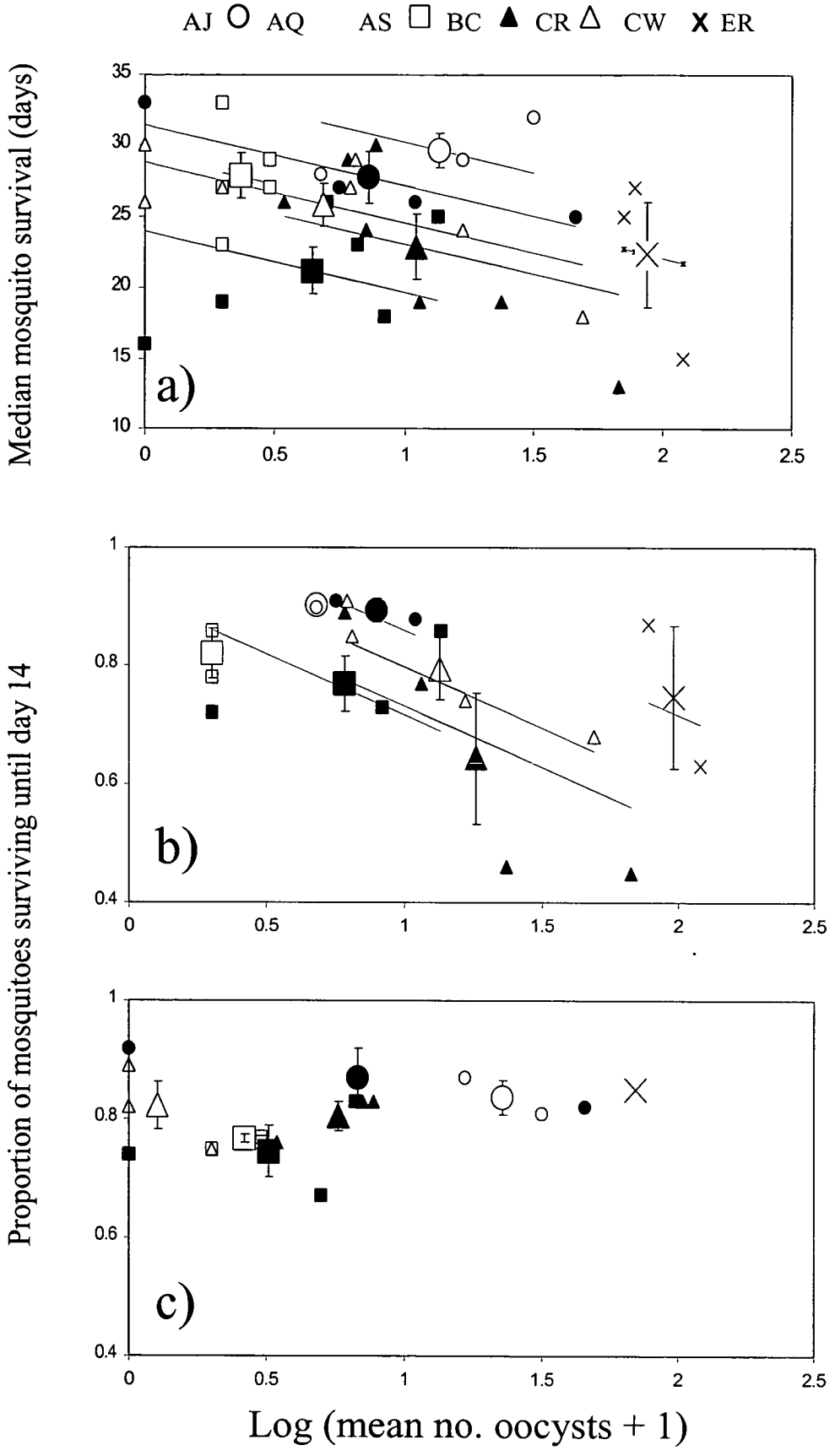
AJ AQ AS BC CR CW ER
Median mosquito survival (days)
a)
b)
c)
Proportion of mosquitoes surviving until day 14
Log (mean no. oocysts + 1)

Figure 6.8

6.4.5 *Plasmodium* virulence in vertebrate hosts and vector fecundity

Of mosquitoes that took a blood meal, approximately 13% did not lay any eggs. The proportion of mosquitoes that did not oviposit was unrelated to mouse infection status (*P. chabaudi* infected or not: $F_{1,40}=1.50$, $p=0.28$), *P. chabaudi* clone ($F_{6,28}=1.22$, $p=0.33$) or mouse virulence score ($F_{1,33}=1.49$, $p=0.23$). All subsequent analyses exclude individuals that did not lay eggs.

Three hundred and twenty mosquitoes laid eggs, with an average clutch size of 108 (± 1.7). Unlike the analyses of transmission and mosquito survival, interpretation of fecundity results was strongly influenced by whether the BC clone was considered an outlier or not. When BC was included in the analysis, there was no phenotypic relationships between the mean fecundity of infected mosquitoes and mouse virulence score (across infections: $F_{1,33}=0.27$, $p=0.61$; across infections controlling for clone differences: $F_{1,27}=0.89$, $p=0.35$). Moreover, none of the variation in mean fecundity was significantly associated with parasite clone ($F_{6,28}=1.27$, $p=0.30$). However, in the absence of BC, there were significant relationships between virulence and fecundity within clone groups (Effect of virulence within clone groups: $F_{1,23}=3.87$, $p=0.06$, effect of clone controlling for virulence: $F_{6,23}=3.58$, $p=0.05$). Contrary to expectations, these relationships within clone groups were positive, with mosquitoes feeding on more virulent infections laying more eggs (Figure 6.9). In this analysis, parasite clone and virulence effects were present only when these variables were fitted together, neither were significant when tested on their own ($p<0.10$ in both cases). There was no genetic correlation between clone virulence and mosquito fecundity (Table 6.2).

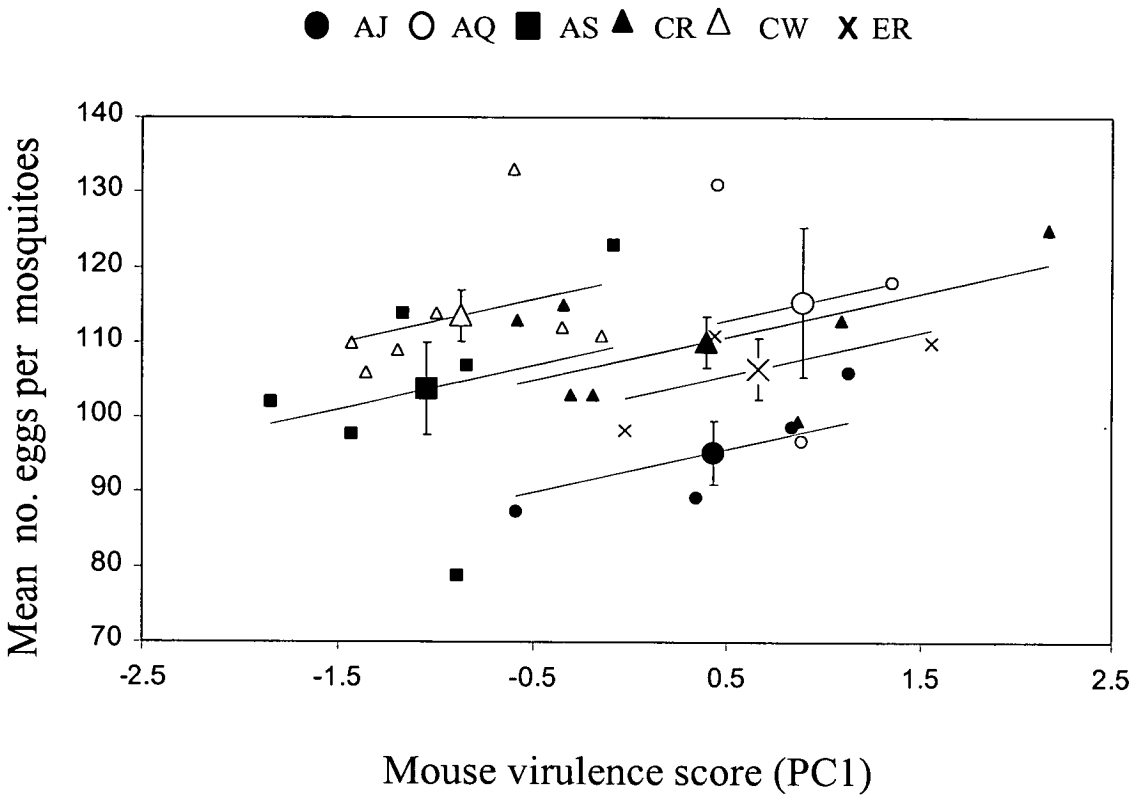

Figure 6.9: Genetic and phenotypic relationships between *P. chabaudi* virulence in mice and vector fecundity. Small symbols represent the mean fecundity of a group of 8-10 mosquitoes after feeding on an infected mouse. The large symbols are the mean values from all mouse feeds on the 7 different *P. chabaudi* clones that were used to infect mice (bars are standard errors).

6.4.6 Other predictors of vector fecundity

The mean fecundity of mosquitoes feeding on infected blood was generally similar to that of the controls (108.0 $\pm$ 2.1 vs. 109.5 $\pm$ 7.2), but the role of blood meal size on fecundity differed between the two groups (Figure 6.10). Combining data from both infected and uninfected mosquito feeds, the only significant predictors of mean fecundity were mouse infection status ($F_{1,38}$=5.27, p=0.03), blood meal size ($F_{1,38}$=5.03, p=0.03), and the interaction between infection status and blood meal size ($F_{1,38}$=5.53, p=0.02). Whereas the fecundity of control mosquitoes increased with blood meal size, variation in blood meal size had no effect on the fecundity of infected mosquitoes.

Restricting analysis to infected mosquitoes, none of the nine explanatory variables that were measured, either when evaluated independently (p>0.25 in all cases) or when combined in a GLM model, were related to fecundity when the BC clone was included. When the BC clone was excluded, then the minimal model for fecundity included both clone and virulence (Figure 6.9). The effect of each clone on mosquito fecundity was not correlated with any other measure of parasite load or infectivity, either with or without the BC clone (Table 6.2).

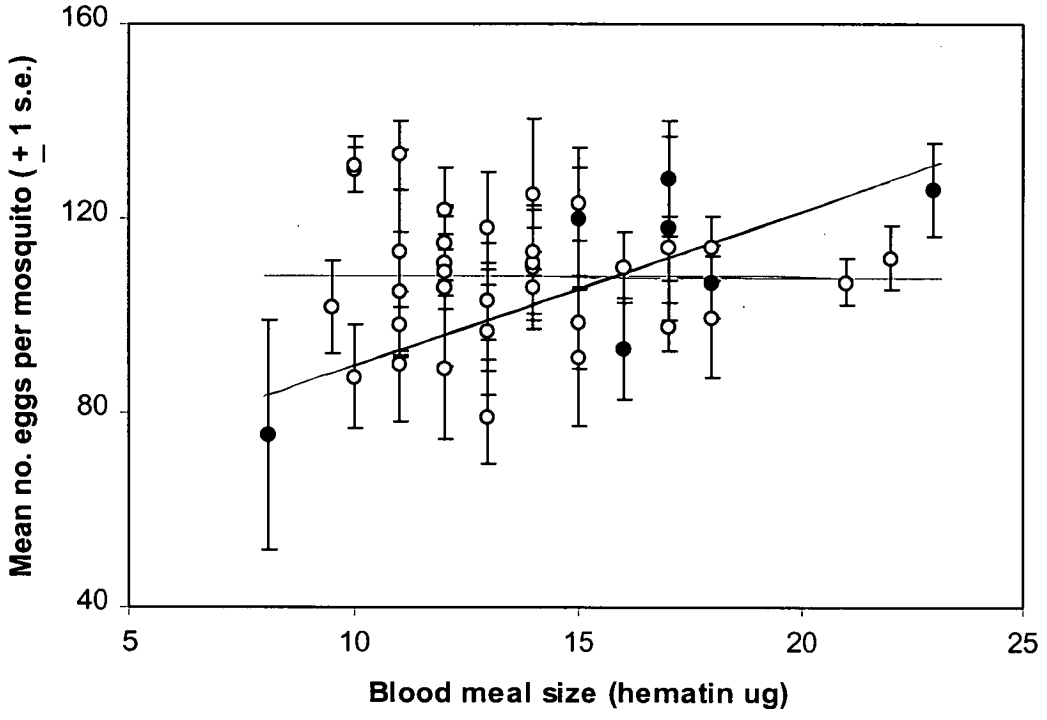

Figure 6.10: The relationship between blood meal size and fecundity in mosquitoes fed uninfected mouse blood (closed circles, black regression line) and mosquitoes fed *P. chabaudi* infected blood (open circles, grey regression line). Bars are one standard error. The slope of the relationship between blood meal size and fecundity was significantly greater than zero for uninfected mosquitoes ($p = 0.02$), but not for those feeding on infected blood ($p = 0.92$).

6.5 Discussion:

6.5.1 Vector survival

We found no evidence of a relationship between the virulence of malaria parasites in their vertebrate host and vector survival. Neither the virulence of individual infections, nor the average disease severity induced by different parasite clones, could explain variation in mosquito survival. Nor did we detect evidence of an indirect relationship between parasite virulence in mice and mosquito survival: infection properties correlated with virulence (e.g. blood meal size, mosquito infection rate) did not influence mortality. These results suggest that the evolution of increased virulence in malaria is not restricted by vector mortality.

Although there was no relationship between virulence in the vertebrate host and vector survival, *Plasmodium*-infection nonetheless affected vector survival. Parasite infection, parasite clone and parasite (oocyst) burden were all predictors of mosquito survival. However, these effects were complex. Infected mosquitoes did have poorer survival than uninfected ones, but only when glucose water was limited. When glucose water was provided freely, uninfected mosquitoes had the greatest mortality. This environmental dependency was detected in a previous study of *P. chabaudi*, where the survival of the uninfecteds was also poorer when glucose water was provided freely (Ferguson and Read 2002a, Chapter 3). Thus it appears that environmental conditions mediate the fitness effects of *Plasmodium,* an observation that may help explain why studies of the effect of *Plasmodium* on vector survival have yielded conflicting results (Ferguson and Read 2002b, Chapter 2).

The survival consequences of parasite clone were more direct than those associated with simple infection status. Of the 7 clones we used, three induced

greater mortality than the controls (AS, CR, ER), and four did not (AJ, AQ, BC, CW) (Figure 6.7). This extends our previous finding (with just two clones) that *P. chabaudi* infection can reduce mosquito survival, and that parasite genotype affects the magnitude of virulence (Ferguson and Read 2002a, Chapter 3). At least in the environmental conditions studied to date, there is no general effect of *P. chabaudi* on survival; rather a range of outcomes may occur under different parasite clones.

Pooling across feed days, the difference in median survival between mosquitoes with the most and least lethal clone (AS and AQ respectively) was 8.5 days. This constitutes an almost 30% reduction in longevity and, assuming mosquitoes blood feed a maximum of once every 2-3 days (Gillies 1953), the loss of 3-4 transmission opportunities. The clone effects report here were evident not only during the period when mosquitoes were capable of infecting new hosts, but also before sporozoites had developed. Thus the parasite genetic effects observed here could influence not only the frequency of transmission events, but the probability of transmission ocurring at all. If duplicated in a natural transmission setting, mortality effects of this nature and magnitude could have sizeable effects on malaria epidemiology and evolution.

Also important to mosquito survival was oocyst burden. Oocyst burden was negatively associated both with mosquito median survival and survival through the 14-day parasite incubation period. Oocyst burden has been linked to longevity in some *Plasmodium*-vector combinations (Klein *et al.* 1986; Hogg and Hurd 1995b), but not in previous experiments with the *P. chabaudi* - *An. stephensi* system (Ferguson and Read 2002a, Chapter 3). This discrepancy between experiments may be due to differences in mean oocyst burdens (this study = 17.3 $\pm$ 4.6, study where

oocyst burden not important = 43.2, ± 11.2). If oocyst burden is non-linearly related to mosquito survival, effects may only be detectable across a small range of oocyst densities, best approximated in this experiment than in the previous one (Ferguson and Read 2002b). This would match the observations of Klein (1986), who found that oocyst burden was related to mosquito survival only over some ranges.

The effect of oocyst burden on longevity could have facilitated an indirect link between infection virulence in mice and mosquito survival. In this study and another (Mackinnon and Read 1999b), the proportion of mosquitoes infected increases with infection virulence in mice. As mosquito infection rate is generally positively correlated with oocyst burden (Medley *et al.* 1993; Billingsley *et al.* 1994; Taylor *et al.* 1997a), one would predict that virulent infections should generate the highest oocyst burdens in mosquitoes, which in turn would induce the greatest mortality. This did not occur in our experiment, because, whilst mosquito infection rate was positively correlated with virulence to mice, oocyst burden was not (although there was a non-significant positive trend, p=0.11). The lack of association between infection virulence in mice and oocyst burden in this experiment may be a consequence of the smaller blood meals taken from mice infected with the most virulent clones (Fig 6.3e).

6.5.2 Vector fecundity

Plasmodium virulence in vertebrates did influence one key component of vector fitness: fecundity. Within each group of mosquitoes infected with the same parasite clone, the number of eggs they laid *increased* with the virulence of the mouse infection (Figure 6.9). Across the natural virulence spectrum of most clones,

mosquito egg production increased by about 5-15%. Why should mosquito fecundity increase with infection virulence in the vertebrate host? The relationship is unlikely to be driven by direct parasite effects, as no measure of parasite load (gametocytes, infection probability or oocyst burden) had an effect on egg production. It is also unlikely to be driven by blood meal size: clones that were the most virulent were associated with small blood meals. This leaves blood meal quality as the most likely explanation. Variation in several components of host blood have been associated with malaria disease severity (e.g. abundance and composition of amino acids, immune molecules and toxins, Kurtzhals *et al.* 1998; Chen *et al.* 2000; Enwonwu *et al.* 2000). Mosquitoes require 10 different amino acids to complete oogenesis (Hurd *et al.* 1995), and the specific mixture they ingest has a large effect on egg development (Uchida 1993). It is possible that virulence induces specific amino acid changes in host blood that actually increase vector fecundity. Further examination of the importance host blood components to vector fecundity, and their variation under mild and severe malaria infections, would be of great interest.

Virulence effects on fecundity were evident only when variation due to parasite clone was taken into account. Mean fecundity dropped by approximately 18% between clones with smallest and greatest effect on egg production (AQ and AJ respectively). *Plasmodium* genetic diversity has previously been linked to variation in mosquito fecundity (Chapter 4), where mosquitoes feeding on mice infected with a mixture of two parasite clones laid significantly fewer eggs than those with single clone infections or those that were uninfected. In this study as in others, neither gametocyte or oocyst burden was related to mosquito fecundity (Hacker and Kilama

1974; Hogg and Hurd 1995b, Chapter 4). Thus as with virulence, the effect of parasite clone on fecundity is presumably mediated by changes in blood meal quality.

Similar to vector survival, *P. chabaudi* infection had no general effect on mosquito fecundity, but it did influence the efficacy of other fitness-enhancing factors. Specifically, the presence of parasites disrupted the positive correlation that is usually observed between blood meal size and fecundity (Figure 6.10). This has also been documented in *P. yoelii nigeriensis* infections of *A. stephensi* (Hogg and Hurd 1995b). As discussed by Hogg and Hurd (1995b), it seems unlikely that this disruption is due to competition for blood meal resources as there is no evidence that parasite load (the force of competition) influences fecundity. Instead, the efficacy of the blood meal conversion into eggs could be derailed by parasite manipulation of another key component of oogenesis subsequent to blood feeding, such as the rate of egg resorption (Carwardine and Hurd 1997), formation and uptake of vitellogenin (Jahan and Hurd 1998) and occurrence of apoptosis in follicle cells (Hopwood *et al.* 2001).

6.5.3 The evolution of *Plasmodium* virulence

This study found no evidence of a negative relationship between the virulence of *Plasmodium* infections in their vertebrate and vector hosts. In fact, the only association detected between infection virulence in vertebrates and vector fitness was a positive one: within each parasite clone treatment, mosquito fecundity increased with infection virulence in mice. Vector survival, a more important determinant of parasite transmission success than fecundity, had no relationship whatsoever to

infection virulence in mice (Figure 6.5). On the basis of this evidence, it is unlikely that vector fitness limits the evolution of higher virulence in the vertebrate host of *Plasmodium.*

We caution however, that this conclusion is based on examination of only the direct fitness costs imposed by parasites, and then in an animal model in controlled laboratory conditions. *Plasmodium* is known to alter mosquito feeding behaviour (Rossignol *et al.* 1984; Wekesa *et al.* 1992; Koella *et al.* 1998; Anderson *et al.* 1999) and flight ability (Schiefer *et al.* 1977; Rowland and Boersma 1988), both of which may influence the ability of mosquitoes to avoid predators and/or evade anti-vector behaviour. In the case of feeding behaviour, these alterations have been associated with increased mortality of infected mosquitoes (Anderson *et al.* 2000). Neither of these two sources of mortality were taken into account in this experiment, and thus their relationship to infection virulence in vertebrates is unknown. Until the magnitude of these indirect costs is more thoroughly investigated, the possibility that vector fitness selects against virulence in vertebrates cannot be entirely dismissed.

Another proviso is that these results concern only single clone infections. In nature, most infected people (Conway *et al.* 1991; Ofosu-Okyere *et al.* 2001) and many infected mosquitoes (Babiker *et al.* 1994; Paul *et al.* 1995) carry more than one *Plasmodium* genotype. In a rodent malaria model system, mixed infections have been associated with increased disease severity in mice (Taylor *et al.* 1998) and poorer vector survival in one of two environmental settings (Ferguson and Read 2002a, Chapter 3). An association between virulence in the vertebrate and vector could thus arise via the affects of mixed infection. But in the absence of direct

evidence of such a link, it seems premature to speculate on the consequences for the evolution of virulence.

Assuming these results do fairly reflect the natural situation, where does this leave us with respect to understanding the limits to malaria virulence in nature? As no association between parasite virulence in vertebrates and invertebrate hosts was detected, it seems more likely that other factors such as host genotype (Gupta and Galvani 1999; Regoes *et al.* 2000) or the availability of resources within hosts such as red blood cells (Mackinnon *et al.* 2002) are capping virulence evolution. Nonetheless, vectors may be an important determinant of virulence in vertebrates, even if not via their survival. For example, it is unknown whether virulent and avirulent parasites clones produce sporozoites with the same efficiency, nor whether these sporozoites are equally infective to new hosts. Bottlenecking of malaria parasites through mosquitoes may also impact on virulence evolution (Bergstrom *et al.* 1999). Studies of trypanosomes, for instance, have shown that the rate at which parasites evolve higher virulence is reduced when parasites are passed through the insect vector as well the mouse host (Contreras *et al.* 1994). Finally, even if mosquitoes play no role in the evolution of malaria virulence in the vertebrate host, there is absolutely no understanding of the nature of selection acting on *Plasmodium* virulence determinants expressed in the vector. Conventional wisdom that malaria is avirulent to its vector is clearly wrong (Figs 6.5, 6.7, 6.10).

Chapter 7: General Discussion:

In this chapter, I will briefly summarize how the sum of results in this thesis addressed the initial three research aims I set out. These findings have generated several issues that require further discussion, particularly with respect to their epidemiological and evolutionary implications.

7.1 Principal findings:

7.1.1. Is *Plasmodium* virulent to its vectors?

Defining parasite virulence as reduction in host fitness, there is ample evidence to suggest that *Plasmodium* can be virulent to its vector, but the magnitude of the effect is highly variable. In this thesis, virulence was quantified as a reduction in both survival and fecundity. Evidence of *Plasmodium*-induced fecundity reduction in vectors is widespread. Indeed, including research in this thesis, I am unaware of any study that has failed to detect some detrimental effect of *Plasmodium* on mosquito egg production (Hacker 1971; Hacker and Kilama 1974; Freier and Friedman 1976; Hogg and Hurd 1995a; Hogg and Hurd 1995b; Hogg and Hurd 1997; Ahmed *et al.* 1999). These fecundity reductions appear to be a pervasive feature in both unnatural and natural vector-parasite systems (e. g. Hogg and Hurd 1997), and can be deemed the most consistent pathology of *Plasmodium* infection in mosquitoes.

Plasmodium effects on survival are less compelling, but meta-analysis revealed evidence of an overall negative effect of parasitism. Certainly there are qualifiers to this conclusion: survival effects are significantly mediated by parasite genotype (Chapter 3, Chapter 6) and possibly the co-evolutionary history of the vector-parasite association (Chapter 2). Indirect costs of *Plasmodium* on vector

mortality (e.g. increased susceptibility to predation and anti-vector behaviour) were not addressed in this thesis, and are still largely unexplored. Where they have been studied, the indirect effects of *Plasmodium* are sizeable (e. g. Schiefer *et al.* 1977; Anderson *et al.* 2000). This suggests that the consistent tendency towards reduced survival I report may be an underestimate of the cumulative cost of infection in the wild. In conclusion, virulence in terms of fecundity and survival reduction is a likely outcome of vector-parasite interactions in malaria.

7.1.2 Virulence determinants in malaria vectors

The most consistent predictor of malaria parasite virulence in mosquitoes, whether it be measured in terms of survival or fecundity, was the genetic composition of the infections on which mosquitoes blood fed. Parasite clone was a more consistent determinant of mosquito fitness than any measure of parasite load, or even the presence of parasites (oocysts) themselves. The primacy of clone effects could not be eliminated by introducing environmental variation (glucose deprivation). In the most extensive study of clone virulence (Chapter 6), glucose provision had no effect on clone virulence (Chapter 6), and in another more limited investigation, it altered the rankings of virulence (Chapter 3) but not the existence of clone-specific effects.

The dominance of clone effects on mosquito fitness mirrors what is observed in studies of *Plasmodium* infection in vertebrates. In *P. chabaudi* and *P. falciparum*, parasite genotype is a determinant of numerous measures of parasite growth, disease severity, and transmission success (James *et al.* 1936; Carlson *et al.* 1990; Rowe *et al.* 1997; Taylor *et al.* 1997a; Mackinnon and Read 1999b; Chotivanich *et al.* 2000; Ariey *et al.* 2001; Ofosu-Okyere *et al.* 2001; Timms *et al.* 2001). Together, these

results from vertebrates and vectors suggest that parasite genetics have a role in determining both disease outcome in malaria and the efficiency of vector transmission. How these effects compare to those imposed by host genotype and/or environmental conditions is an important issue for future study.

7.1.3 Vector fitness and the evolution of malaria virulence in vertebrates

In the *P. cbabaudi* laboratory system, I found no evidence of an association between parasite virulence in their vertebrate and vector host. Neither the level of virulence in the mouse host, nor any of the infection properties that are correlated with it (e. g. gametocyte density and mosquito infection rate Taylor *et al.* 1997b; Mackinnon and Read 1999a; Mackinnon and Read 1999b) were negatively related to mosquito survival or fecundity (the only association with vertebrate virulence was a positive one with vector fecundity). Thus mosquito mortality is unlikely to select against virulence in the vertebrate host, and these data give no reason to hypothesize that virulence cannot evolve independently in each of the two hosts. Virulence in vectors cannot be explained as a by-product of virulence evolution in the vertebrate. Thus why it occurs, whether it is adaptive, and more generally, the nature of selection on it, are still open questions.

7.2 Questions arising

7.2.1 Mechanisms of virulence in the vector

Why should parasite clone have an effect on vector fitness? These effects could not be consistently explained by clone-specific variation in parasite load, proximate measures of resource quality such as blood meal size or anaemia, or

variation in resource acquisition (glucose intake, lipid, protein and glycogen usage). In only one of the four studies could the significant effects of parasite clone be explained by the infection properties I measured: parasite clone effects on mosquito fecundity were linked to variation in blood meal size in one study (Chapter 4, but not in another: Chapter 6; discussed below).

One explanation for these genetic effects is that they are indicative of life-history variation between different parasite clones. It could be that clones associated with virulence develop more quickly in their vectors, produce more sporozoites, are more damaging to mosquito tissue, elicit a stronger and more costly immune response, or induce behavioural changes that are correlated with decreased longevity. A closer examination of the efficiency of sporogony and mosquito behaviour is required to test these hypotheses.

7.2.2 *P. chabaudi* virulence in vectors: maladaptation?

All of the experimental results in this thesis were obtained using an artificial vector-parasite combination that does not occur in nature. As illustrated in Chapter 2, there is reason to believe that virulence (at least in terms of vector survival) is commonly seen in artificial associations such as this one, and less often found in co-evolved associations. Does this mean that my results are simply an artefact of laboratory conditions and not useful towards a general understanding of vector-parasite interactions? Hopefully not. The most common criticism of experiments using unnatural vector-parasite associations is that they are predisposed towards virulence because they generate unnaturally high oocyst burdens. While it is true that mean oocyst burdens in my studies were substantially higher than in nature, they varied broadly (range of average oocyst burdens: 0-223). Across this wide range,

there was no consistent effect of oocyst burden on virulence. Research in Chapter 3 indicates that under one environmental setting, the CR clone induced significant vector mortality even though it was associated with a mean oocyst burden of only 1.4, well within the 1-2 range seen in *P. falciparum*-infected mosquitoes in the wild (Beier 1998). Furthermore, parasite-related fecundity reductions were not related to any measure of parasite load. If these largely dose-independent fitness reductions in *A. stephensi* are simply a result of a maladapted vector and parasite association, it suggests that parasites are able to make themselves less virulent in co-evolved systems either by altering the per parasite damage they inflict (perhaps by differential cell invasion pathways, Sinden and Billingsley 2001), altering their immunogenicity, or by altering the blood composition in their natural vertebrate host in a way to minimise damage to vectors. None of phenomena have been demonstrated, but study of them would be very interesting.

Finally, the virulence I describe in the *P. chabaudi* system cannot simply be dismissed as a maladaption. If this were the case, virulence should have been associated with all parasite clones as all are equally 'maladapted' to the *A. stephensi* vector. Certainly, there is a great need to test whether parasite genetics have similar importance in natural vector-parasite associations, and if they can be detected in the face of more intense environmental variation. However there is currently no reason to believe these laboratory-based results are without relevance.

7.2.3 *P. chabaudi* virulence in vectors: Parasite manipulation ?

As summarized above, *P. chabaudi* virulence cannot reliably be attributed to resource competition between parasite and vector (neither parasite presence nor

density was consistently related to vector fitness, nor were there signs of resource depletion in infected mosquitoes). One possibility is that the observed fitness reductions are a consequence of parasite manipulation to increase their own transmission success (Hurd 1998; Hurd and Lane 1998; Hurd 2001). For example, by curtailing vector fecundity, parasites could reduce their vector's mortality costs of reproduction (Stearns 1992), increasing the probability that the vector survives long enough for the parasite to be transmitted. There is growing evidence that *Plasmodium* can down-regulate the mosquito egg-production pathway, irrespective of their density (Carwardine and Hurd 1997; Jahan and Hurd 1998; Hopwood *et al.* 2001).

The adaptive consequences of vector survival reduction are less clear. Reduced vector survival might benefit *Plasmodium,* but only if it appeared later in the mosquito's life (after sporozoites had developed), and if these costs were correlated with increased transmission back to the vertebrate host (faster sporozoite development and/or higher biting rates, e.g. Koella *et al.* 1998).

The adaptive nature of vector fitness declines were not examined in this work. However, the *P. chabaudi* system could be ideal for testing evolutionary hypotheses. Unlike other vector-parasite systems where there appears to be a uniform effect of *Plasmodium,* there is substantial genetic variation within *P. chabaudi* for virulence towards vectors. This provides a unique opportunity to evaluate whether clones that have the greatest effect on vector fecundity and survival also have the greatest transmission success to the next vertebrate host, a prerequisite of the manipulation hypothesis that has been notoriously difficult to test.

7.3 Apparently conflicting data

7.3.1 Glucose provision and virulence

In two studies (Chapters 3 and 6), mosquito survival was monitored under two different levels of glucose water provision: *ad libitum* and deprivation. My expectation was that glucose water deprivation would reduce the survival of all mosquitoes, and amplify any differences between the infected and control groups. This did not occur. A curious yet repeatable feature of both studies was that the survival of uninfected mosquitoes was slightly greater under glucose deprivation. In contrast, the effect of glucose deprivation on infected mosquitoes was variable. In the study that used two clones and their mixture (Chapter 3), the effect of glucose treatment on survival varied between parasite treatments (clone x glucose water treatment interaction). This effect was repeated in both experimental blocks (Figures 3.2 and 3.3). In the second study in which seven single clone infections were examined, no such clone-interaction was evident (Chapter 6), instead there was a more general interaction between infection status (*P. chabaudi* or not) and glucose water treatment (Figure 6.6).

It is unclear whether the lack of a consistent parasite clone-by-glucose water interaction reflects a genuine discrepancy between experiments, or is simply due to differential sampling. Many more clones were sampled in the second study and each had a higher degree of replication. Thus survival estimates under both conditions were likely more precise, and the weighting of any one clone smaller. The only clone treatment that was duplicated in both experiments was CR (in Chapter 6, ER clone was only kept in one glucose condition). This clone had a consistent, across-study association with glucose water: in all experiments, it was most deadly when the

mosquitoes it infected were glucose-deprived. Thus there may be no qualitative difference in the effect of glucose water between studies.

7.3.2 Variable effect of oocyst burden on survival

In no case was oocyst burden related to mosquito fecundity. The effect of oocyst burden on survival was more variable. Out of 3 separate experiments (Chapter 3– two experimental blocks, Chapter 6 – one block), oocyst burden was unrelated to survival in two and negatively related to it in one. How can this discrepancy be explained? There are several possibilities. The first is discussed in Chapter 6: mean oocyst burden varied between experiments. If oocyst burden is non-linearly related to mosquito survival, effects may only be detectable across a small range of oocyst densities, best approximated in the second study experiment than in the first. This would match the observations of (Klein *et al.* 1986), who found that oocyst burden was related to mosquito survival only over some ranges.

An alternative hypothesis is that variation in the role of oocyst burden between studies may be due to temporal variation in infection dynamics. In the study that found an association between oocyst burden and survival (Chapter 6), mosquitoes were blood fed on mice with 16 and 17 day old infections. Across these two days, the role of oocyst burden was stronger on the first day than on the second (possibly because higher oocyst densities resulted from feeds on the first day). In the two experiments that found no effect of oocyst burden on infection (Chapter 3), mosquitoes were blood fed on mice with 14 day old infections. It is possible that there is a host blood factor that varies across the days when gametocytes are being produced (e. g. pH, Dearsly *et al.* 1990) which influences the formation of oocysts

and their effect on vector survival. Finally, it could simply be that there is a fixed relationship between oocyst burden and survival, but there is sufficient noise around survival estimates to make it difficult to detect unless samples sizes are high. The 'per block' sample size in the study that detected an effect of oocyst burden was almost 3 times higher than those in which no effect was found (35 vs. 12 infected cages respectively).

7.3.3 Main effect of parasites

The overall effect of *P. chabaudi* on vectors, regardless of parasite clone, is not clear cut. With respect to survival, uninfected mosquitoes had higher survival than infected ones in one study (Chapter 3), but not another (Chapter 6). The major difference between these two studies was that a wider range of single clone infections was used in the study that found no general effect of parasites on survival (7 vs. 2). Comparison of these studies indicates that the two clones used in the first study are amongst the most virulent to mosquitoes (in comparison to the other five studied in Chapter 6). Had two apparently less virulent clones been used in the first study (e.g. AQ and BC), perhaps no overall effect of infection status would have been detected. Also, the study in which a general effect of parasites was found included mosquitoes with mixed-clone infections (CR + ER). As detailed in Chapter 3, there are many reasons to predict that genetically diverse infections will be more virulent than their homogenous counterparts. This was true for *A. stephensi* mosquitoes under one of two environmental conditions. I believe variation in clone choice and infection diversity between studies is the reason for the differing

conclusions about the main effect of parasites on survival within my studies and perhaps in other peoples (Chapter 2).

Similar to vector survival, *P. chabaudi* infection *per se* had a variable effect on mosquito fecundity. In Chapter 4, I reported an overall detrimental effect of *P. chabaudi* on vector fecundity. However, this difference was driven primarily by mosquitoes who had mixed-clone infections, as the fecundity of individuals infected with single clones (CR and ER) was generally no different from the controls (Figure 4. 1). In Chapter 6, mosquitoes were infected with seven single-clone infections, but no clone mixtures. In this experiment, the effect of each clone on fecundity was variable, and no general effect could be detected when they were pooled. Thus as with survival, choice of clone may be responsible for the differences in the main effect of parasitism between studies.

Thus overall, it appears that there is no general effect of *P. chabaudi* on mosquito fitness. Rather, a range of outcomes may occur under different parasite clones. If this also occurs in natural associations, it would suggest that a general immune response to *Plasmodium*, especially if costly (e. g. Ahmed *et al.* 2002), would not be advantageous (why mount a costly immune response if infected with a benign strain?).

7.4 Epidemiological and Evolutionary Implications

The possible epidemiological consequences of parasite-induced survival reductions have been discussed in the preceding chapters. To briefly review, the parasite genetic effects I observed are strong enough to curtail the percentage of mosquitoes that reach the sporozoite stage by approximately 12%, and cause a loss

of 3-4 transmission opportunities throughout the infectious period (based on the survival difference between most and least virulent clones in Chapter 6). The epidemiological implications of reduction of this magnitude should be large, and suggest that differential vector mortality could play an important role in structuring the genetic composition of *Plasmodium* populations

It is uncertain whether the survival differences I have described in the laboratory would occur in nature. The mean value of median mosquito longevity in my experiments ranged between 24-27 days. This is 3-5 times longer than the predicted value of 5 and 8 days for *A. gambiae* and *A. funestus* respectively under natural field conditions (Chege and Beier 1990). The parasite-specific mortality differences we see in the laboratory could be inconsequential in the field, where relatively small parasite effects might be overwhelmed by those imposed by predators and the environment. Alternatively, the more intense mortality risks placed on mosquitoes in nature could magnify the pathogenic effects of particular parasite genotypes, increasing the cost of infection and widening the selective advantage between virulent and avirulent strains and/or mixtures. Further study of mosquito survival and impact of infection multiplicity under natural conditions are needed to resolve these hypotheses.

Plasmodium–induced reductions of vector fecundity will have weaker effects on malaria epidemiology than reductions of vector survival. However, fecundity reductions could have important consequences for the evolution of vector-parasite interactions. For example, infections that reduce vector fecundity may select for parasite evasion measures (behavioural or immunological), which could ultimately reduce parasite prevalence in vectors and vertebrates. Could any of the fecundity

associations described in thesis create selection for such evasion measures? Certainly, the fact that infection of any sort disrupts the efficacy of blood meal conversion into eggs (Chapter 6) suggests that a generalised anti-parasite response could be beneficial. However, there was variation between parasite clones for their effect on fecundity; with some clones having no effect relative to the controls. In such cases, an evasive response would be favoured only if it was specific to certain parasite genotypes or strain combinations, a phenomenon that has never been documented in *Anopheles.*

Finally, the experiments presented here give only one half of the story on genetic regulation of virulence in vector-parasite interactions. I used a diverse array of parasites in these experiments, but the vectors came from a single population with a similar genetic background. Studies in other invertebrate-pathogen systems suggest that the observed level of parasite virulence is highly dependent on host genetic variation as well as parasite genotype (Carius *et al.* 2001). Although similar studies have yet to be undertaken in *Anopheles,* there is evidence that susceptibility to *Plasmodium* can vary significantly both between (Chege and Beier 1998) and within vector species (Yan *et al.* 1997). The elucidation of possible vector genotype-by-parasite genotype interactions poses considerable experimental challenges, but is a necessary prerequisite for prediction of how malaria virulence evolve will in its vectors.

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Appendix 1: Statistical tables for Chapter 6

Transmission measure	Minimum Model	r^2	Factor	F	DF	P
Total gametocyte production (all mice)	Clone	0.30	Clone	2.98	6	0.02
			Error		41	
			Total		47	
Total gametocyte production (survivors)	Clone + Virulence	0.48	Clone	6.27	6	<0.01
			Virulence	4.54	1	0.04
			Error		28	
			Total		35	
Oocyst infection rate	Feed day + Virulence	0.26	Feed day	6.52	1	0.02
			Virulence	5.38	1	0.03
			Error		32	
			Total		34	
Oocyst burden	Clone	0.36	Clone	4.21	6	<0.01
			Error		28	
			Total		34	
Blood meal size	N/A		Total		34	

Table A2.1: Test statistics for the minimal statistically significant models of various measures of *Plasmodium chabaudi* transmission to mosquitoes. The maximal model for all transmission measures included mouse virulence score and parasite clone, and in the case of oocyst infection rate, burden, and blood meal size, the day of blood feeding was also tested. All these main effects (virulence, clone, feed day) and their higher order interactions were evaluated, and non-significant terms were sequentially eliminated. 'N/A' in the model column indicates that none of the main treatment effects or their interactions were significantly associated with the transmission measure.

Table A2.2: Test statistics for the minimal statistically significant models of various measures of mosquito survival. Measures where 'all' are specified indicates that both *P. chabaudi* infected and control mosquitoes were included in the analysis. For all other measures, just mosquitoes that fed on *P. chabaudi* infected blood were examined. The maximal model for survival measures of all mosquitoes included infection status, feed day, glucose treatment, blood meal size, red blood cell density, blood meal size, body size and all feed day interactions. The maximal model for survival measures from infected mosquitoes included parasite clone, virulence score, glucose treatment, feed day, gametocyte density, red blood cell density, oocyst infection rate, mean oocyst burden, blood meal size, body size and all their feed day interactions. Non-significant terms were sequentially eliminated to yield a minimal significant model as presented above. 'N/A' in the model column indicates that none of the main treatment effects or their feed day interactions were significantly associated with the transmission measure.

Mosquito survival measure	Minimum Model	r^2	Factor	F	DF	P
Median Survival (all)	Infection status + Glucose treatment + Feed day + (Infection status*Glucose treatment)	0.13	Infection status	0.25	1	0.62
			Glucose treatment	1.05	1	0.31
			Feed day	6.33	1	0.02
			Infection*Glucose	3.78	1	0.06
			Error		37	
			Total		41	
Median survival (just infecteds)	Clone + Oocyst burden	0.31	Clone	2.65	6	0.04
			Oocyst burden	6.65	1	0.02
			Error		27	
			Total		34	
Proportion alive at day 14 (all)	N/A				41	
Proportion alive at day 14 (just infecteds)	Clone + Feed day + Oocyst burden + (Oocyst burden*Feed day)	0.39	Clone	3.03	6	0.02
			Feed day	5.93	1	0.02
			Oocyst burden	11.62	1	<0.01
			Oocyst*Feed day	6.46	1	0.02
			Error		25	
			Total		34	

Mosquito fecundity Measure	Minimum Model	r^2	Factor	F	DF	P
Fecundity (all)	Infection status + Blood meal size + (Infection status*Blood meal size)	0.16	Infection status	5.27	1	0.03
			Blood meal size	5.03	1	0.03
			Infection*Blood	5.53	1	0.02
			Error		38	
			Total		41	
Fecundity (just Infecteds, with BC)	N/A				34	
Fecundity (just Infecteds, without BC)	Clone + Virulence	0.22	Clone	2.58	5	0.05
			Virulence	3.87	1	0.06
			Error		23	
			Total		29	

Table A2.3: Test statistics for the minimal statistically significant models of mean mosquito fecundity. Measures where 'all' are specified indicates that both *P. chabaudi* infected and control mosquitoes were included in the analysis. For all other measures, just mosquitoes that fed on *P. chabaudi* infected blood were examined. The maximal model for fecundity of all mosquitoes included infection status, feed day, blood meal size, red blood cell density, blood meal size, body size and all feed day interactions. The maximal model for survival measures from infected mice included parasite clone, virulence score, feed day, gametocyte density, red blood cell density, oocyst infection rate, mean oocyst burden, blood meal size, body size and all their feed day interactions. Non-significant terms were sequentially eliminated to yield a minimal significant model as presented above. 'N/A' in the model column indicates that none of the main treatment effects or their feed day interactions were significantly associated with the transmission measure.

Appendix 2: Publications arising from this work

Chapters 2 and 3 of this thesis have been published. Details of publication and copies of reprints are included here.

Chapter 2 **Why is the effect of malaria parasites on mosquito survival still unresolved?** 2002. H. M. Ferguson and A. F. Read. Trends in Parasitology 18: 256-261

Chapter 3 **Genetic and environmental determinants of malaria parasite virulence in mosquitoes.** 2002. H. M. Ferguson and A. F. Read. Proceedings of the Royal Society of London Series B – Biological Sciences 269: 1217-1224

Why is the effect of malaria parasites on mosquito survival still unresolved?

Heather M. Ferguson and Andrew F. Read

Despite almost a century of effort, the question of whether malaria parasites kill their mosquito vectors remains open. Some direct comparisons of the longevity of infected and uninfected mosquitoes have found malaria-induced mortality, whereas others have not. Here, we use meta-analysis to show that, overall, malaria parasites do reduce mosquito survival. However, mortality effects are more likely to be detected in unnatural vector–parasite combinations and in studies of longer duration. Until these factors are systematically investigated, no firm generalities are possible.

During the two weeks that malaria parasites (*Plasmodium* spp.) take to complete development in the mosquito, they can cause substantial damage (Box 1). However, conventional wisdom postulates that natural selection will favour parasites that do not influence vector survival because a parasite that kills its vector will kill itself. Although this view of virulence evolution has been rejected on theoretical grounds (selection maximizes fitness not life expectancy [1,2]), it still pervades the malaria literature [3–6]. The evidence for it is contradictory. Some indirect field data support the idea [7] whereas others do not [8]. Direct laboratory comparisons of the survival of malaria-infected and uninfected mosquitoes have also produced conflicting results, with some reporting reduced mosquito survival [9–17] and others finding vector survival to be unaffected [18–30] (Table 1). This inconsistency is surprising: if *Plasmodium* does impose a direct cost on mosquito survival, why is it not consistently found in controlled laboratory experiments? Understanding the reason for this variation is crucial not only to answer the general question of whether malaria parasites are detrimental to their vectors, but also to identify the conditions under which vector survival could limit the epidemiology and evolution of malaria.

We conducted an analysis of 22 previously published laboratory studies (Table 1) to determine the overall direction and magnitude of *Plasmodium* effects on mosquito survival, and to test whether the variation in outcomes could be explained by experimental design. We used quantitative meta-analytical methods [31,32], which require that summary statistics and sample sizes are given for each study so that a standard measure of statistical effect size can be computed. This information was missing from several of our studies [10,12,18–21,23–26,29]. Consequently, meta-analysis was conducted on only 11 of the original 22 studies (Table 2). In total, these 11 studies provided information on 24 separate experiments.

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Overall effect

The proportion of studies that have found a statistically significant detrimental effect of *Plasmodium* is similar to the proportion that have not (41% and 59%, respectively; Table 1). For all accounts of reduced survival to have arisen by chance alone (Type 1 errors), there would need to be ~360 unpublished studies with null results, in addition to about nine showing increased survival. Given the experimental effort involved in survival studies and the novelty of showing that malaria is a longevity enhancer, this degree of under-reporting seems unlikely. The lack of any studies showing a significant positive influence of *Plasmodium* is interesting because, if parasites can manipulate mosquito longevity, they might be expected to enhance it (at least during the time when oocysts are growing [33]) – by, for instance, reducing fecundity and hence the longevity costs of reproduction [34]. The absence of this effect suggests that no such manipulation is occurring.

Quantitative analysis shows that, in 22 out of 24 experiments, *Plasmodium*-infected mosquitoes had poorer survival than their uninfected counterparts (Table 3) and that overall, malaria does reduce mosquito survival. The mean effect size was similar when experiments were treated as independent units and when analysis was conducted on the average effect size of all experiments in a study. Of the 24 experiments that we examined, there was no relationship between effect size and sample size ($P = 0.26$, $n = 24$).

What factors influence experimental outcome?

To include as many data as possible, all further meta-analyses were conducted at the level of individual experiments.

Choice of species

Of the 22 studies that we identified, ten were studies of natural combinations of vector and parasite species, ten were of unnatural combinations and, for two, the natural vector is unknown (Table 1). Of the ten studies that used an unnatural vector–parasite combination, seven found that infection decreased mosquito survival; none of the ten studies that used natural associations reported a significant effect, although all studies exhibited a tendency towards poorer survival in the infected group (Table 2). The magnitude of *Plasmodium* effects on mosquito survival is substantially greater when novel pairings are used (Table 3, $P = 0.04$, $n = 22$).

This analysis supports the notion that *Plasmodium* is harmful only in novel vector species, an idea that is often proposed to explain the lack of

http://parasites.trends.com 1471-4922/02/$ – see front matter PII: S1471-4922(02)02281-X

Box 1. Mechanisms by which *Plasmodium* can damage its vector

Tissue damage
Mosquito midguts are perforated when ookinetes pass through them [a]. In addition to the physical damage, this perforation might increase susceptibility to bacterial infection or invasion by other parasites during subsequent feeding [b,c].

Physiological disruption
Levels of aminopeptidase, a digestive enzyme, are reduced in mosquitoes with oocysts [d].

Resource depletion
Infected mosquitoes have lower concentrations of amino acids in their haemolymph than those that are uninfected, and their midguts use eight times as much glucose [e,f].

Cost of immunity
Mosquitoes can mount a diverse array of immune responses when invaded by pathogens [g]. *Plasmodium* infection elicits the transcriptional activation of at least six different immune markers in the human malaria vector *Anopheles gambiae*, particularly when parasites are invading the midgut and salivary glands [h,i]. The production of these molecules could be energetically costly and divert resources away from growth and maintenance. Some mosquitoes kill oocysts by melanotic encapsulation, a process that is known to reduce mosquito ovary size and protein content when directed against filarial worms [j]. Mosquitoes selected to be refractory to *Plasmodium* have also been shown to have poorer fitness than susceptible mosquitoes in the absence of infection [k].

Behavioural modification
Infected mosquitoes have less salivary apyrase (a platelet inhibitor) [l]. Consequently, these mosquitoes spend more time feeding, probe more often [m,n], are more persistent in biting [o] and feed more often [p] than uninfected mosquitoes. These changes in behaviour are likely to increase the risk of infected mosquitoes being detected and killed while feeding.

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virulence in studies of natural infections [27,30]. Assuming that the unnatural combinations used in the laboratory are a random sample of novel pairings, is there an *a priori* reason to expect them to be more virulent? It is often assumed that only maladapted parasites are virulent, so that virulence is high in host–parasite associations that have not co-evolved. Yet neither theory nor empirical studies support this: both increased and decreased virulence can arise from novel host–parasite pairings [35–37]. Certainly, there are many accounts of increased virulence in novel associations [38,39], but ancient virulent associations are common and avirulent interactions are less likely to be noticed [36].

Although a reduction in mosquito survival is more commonly reported in unnatural *Plasmodium*–vector combinations, there are examples of novel combinations that did not result in virulence [26]. Furthermore, a range of outcomes can be found for the same vector–parasite combination, with different studies reporting different effects (e.g. *Plasmodium berghei* in *Anopheles stephensi* reduced survival [12] or did not [26]). Comparative studies, in which the effect of malaria parasites is assessed simultaneously in natural and novel vectors in the same laboratory, are crucial to determine the relevance of co-evolutionary history to mosquito–malaria interactions. We know of no such studies.

Length of study

Mosquitoes cannot transmit malaria until approximately two weeks after infection, when the parasite has transformed into a sporozoite and invaded their salivary glands. Natural selection should minimize virulence, at least until sporozoites have developed. Once sporozoites have developed, natural selection will favour parasites that can increase the biting rate of their vectors, possibly at the expense of longevity [33,40]. This prediction has received empirical support from the observation that sporozoite-infected mosquitoes are more persistent feeders and have greater feeding-associated mortality than their uninfected counterparts [41,42].

Even though only one of the studies in our meta-analysis allowed host biting after initial infection [16], longevity effects were more likely to be detected in

Table 1. Studies investigating the effects of *Plasmodium* on vector survival[a]

Parasite	Vector	In the wild? [b]	Study length (days)[c]	Evidence [d]	Statistics reported? [e]	Control type[f]	Refs
Vector survival reduced							
Plasmodium praecox	*Culex fatigans*	?	5	E	N*	U	[9]
Plasmodium rhadinurum	*Aedes aegypti*	N	3	E	N	U	[10]
	C. fatigans	N	3	E	N	U	[10]
Plasmodium cathermerium	*Culex pipiens*	?	20	E	N*	U	[11]
Plasmodium berghei	*Anopheles stephensi*	N	14	E	–	U	[12]
Plasmodium cynomolgi	*Anopheles dirus*	N	65	E	Y	U	[13]
P. cynomolgi	*A. dirus*	N	65	E	Y	U	[14]
Plasmodium yoelii	*A. stephensi*	N	6	E	Y	U	[15]
P. yoelii	*A. stephensi*	N	6–18	E	Y	U	[16]
Plasmodium chabaudi	*A. stephensi*	N	35	E	Y	U	[17]
Vector survival unaffected							
Plasmodium vivax	*Anopheles punctipennis*	Y	–	O	N	–	[18]
Plasmodium falciparum	various *Anopheles* spp.	Y	–	O	N	–	[19]
P. vivax	*A. punctipennis*	Y	17	O	N	–	[20]
P. falciparum	*Anopheles crucians*	Y	17	O	N	–	[20]
P. vivax	*Anopheles maculipennis*	Y	–	O	N	–	[21]
P. vivax	*A. maculipennis*	Y	7–19	E	N*	U	[22]
P. vivax	*Anopheles quadrimaculatus*	Y	–	O	N	–	[23]
Plasmodium gallinaceum	*Aedes aegypti*	N	7	E	N	U	[24]
P. gallinaceum	*A. aegypti*	N	8	E	N	U	[25]
P. berghei	*A. stephensi*	N	20	E	N	U	[26]
P. falciparum	*Anopheles funestus*	Y	30	E	Y	S	[27]
	Anopheles gambiae	Y	30	E	Y	S	[27]
P. falciparum	*A. gambiae*	Y	5	E	Y	U	[28]
P. falciparum, P. vivax	*Anopheles tessellatus*	Y	14	E	N	U	[29]
P. falciparum	*A. gambiae*	Y	5	E	Y	S	[30]

[a]All studies involve experimental blood feeds except Refs [27,30], which used wild-caught mosquitoes.
[b]? indicates that the natural vector is unknown; Y indicates that the species combination is known to exist in the wild (according to Ref. [54]) and N indicates that it is not.
[c]– indicates that the study length is unclear from the relevant paper.
[d]This column indicates whether the conclusions were based on anecdotal observation (O) or experimental testing (E).
[e]– indicates that effect size was not obtainable from reported statistics; Y indicates that statistical analysis was given; N indicates studies without statistical analysis and N* indicates that no statistical analysis was given, but that raw data were given; in these cases, we did an appropriate test.
[f]S indicates that the control group was mosquitoes without sporozoites; U indicates that the control was mosquitoes fed uninfected blood and – indicates that no control was used.

studies that lasted until the sporozoite stage or longer. Effect size was positively related to study length ($P = 0.001$), with the overall effect size being greater for studies ending after sporozoite invasion than for studies that ended before sporozoite invasion (Table 3). These time-dependent effects could arise because sporozoites cause more physiological disruption than oocyst or ookinetes, because parasites alter mosquito behaviour (coincidentally increasing mortality), or simply because small differences in daily survival are more easy to detect over long time periods. Regardless of the mechanism, this analysis strongly suggests that virulence will be underestimated by studies that end before the completion of *Plasmodium*'s extrinsic incubation period.

Dose effects

Malaria biologists have long argued that *Plasmodium* is harmful to mosquitoes only when parasite load is very high [15,21]. This idea has been used to dismiss the possibility that parasites could limit vector populations because few infected mosquitoes carry more than one to two oocysts in the wild [27]. However, the absence of high oocyst burdens could also be due to the mortality of heavily infected mosquitoes [7]. In the laboratory, variable effects of parasite dose have been reported. In one study, mortality increased with the density of asexual parasites, but only at one of two temperatures [12]. In other studies, mortality was unrelated to gametocytaemia [11,17], and oocyst burdens have been correlated with mortality in some [14,16] but not all [17] studies. We know of no studies comparing sporozoite load with mosquito survival.

It is difficult to assess the importance of parasite dose to effect size in our sample of experiments because estimates of blood-stage and sporozoite densities were mostly absent. Some analysis was possible for oocyst burden, a parameter that was reported in five studies (12 experiments) [13–17]. In this sample, there was no relationship between effect size and mean oocyst burden ($P = 0.76$, $n = 12$). Clearly, the data are far from definitive, but they do suggest that oocyst burden is not a large, universal predictor of harm.

Environmental conditions

Mosquito longevity varies with temperature [9,12], season [21], diet [43] and larval density [44,45].

Table 2. Studies used in the meta-analysis of the effects of *Plasmodium* on vector survival[a]

r	n	Oocyst load	Mean temperature (°C)	Mean humidity (%)	Refs
0.187	99	–	23	90	[9]
0.387	2692	–	25	83	[11]
0.686	720	–	26	67	[13]
0.195	280	<10	26	67	[14]
0.705	360	10–40			
0.827	480	41–70			
0.907	320	>71			
0.259	207	>75	27	80	[15]
0.056	101	>75	27	80	[16]
0.048	93	>75			
0.032	116	4.4			
0.137	100	4.4			
0.286	100	4.4			
0.010	1527	17.2	27	70	[17]
0.058	1814	44.6			
–0.048	60	–	24	75	[22]
–0.001	100				
0.028	40				
0.060	50				
0.449	60				
0	1128	–	25.3	65.5	[27]
0	1221				
0.005	1600	–	28	80	[28]
0.047	967	2.4	24.5	80	[30]

[a]The Pearson correlation coefficient (*r*) represents the standardized effect size of *Plasmodium* on mosquito survival. We assigned a positive value to the effect size if the survival of uninfected mosquitoes was higher than that of infected mosquitoes. Effect sizes were obtained by converting the one-tailed *p* value for the test of differences in survival between infected and uninfected mosquitoes into a standard normal deviate (*z*) and dividing by the square root of the sample size [55]. For studies that consisted of several different experiments, separate *r* values were computed for each. Mixed effects analysis was used to evaluate the relationship between effect size and experimental design parameters. *n* is the total number of mosquitoes used in each experiment. Oocyst load is the mean number of oocysts on the midgut of an infected mosquito.

Table 3. Mean effect sizes of *Plasmodium* on mosquito survival[a]

Sample	Mean *r*	95% confidence interval of *r*	Number of experiments
All experiments	0.287	0.136–0.470	24
Studies (experiments pooled within a study)	0.259	0.102–0.447	11
Experiments that used natural associations	0.061	–0.004–0.170	9
Experiments that used unnatural associations	0.436	0.201–0.705	13
Experiments that ended before sporozoite invasion	0.129	0.055–0.218	10
Experiments that ended after sporozoite invasion	0.395	0.147–0.664	14

[a]If there was no effect of malaria infection on longevity, effect size would be indistinguishable from zero. Positive effect sizes indicate that mortality is increased by infection. Confidence intervals were obtained by bootstrapping. Statistical analysis was conducted on *Z*-transformed values of the Pearson correlation coefficient (*r*), which represents the standardized effect size of *Plasmodium* on mosquito survival. Results were calculated using the program METAWIN (Sinauer Associates) [55].

The particular rearing, climatic and nutrient regimes used in different labs could therefore influence survival. Furthermore, this variation is likely to influence the ability of mosquitoes to tolerate parasitism. Several studies have shown that *Plasmodium*-induced mortality varies with environmental conditions such as temperature [9,12], diet [17], adult density [11] and bacterial infection [26]. The survival studies that we examined were performed over a wide range of temperatures (21–30°C) and humidities (57–90%). Across experiments, there was no obvious relationship between temperature and effect size ($P = 0.42$, $n = 24$), but there was for humidity ($P = 0.04$, $n = 24$), with less *Plasmodium*-induced mortality at higher humidities. Experiments are required to assess whether this association has any causal basis.

Diet is another factor that could influence mosquito response to *Plasmodium* but, because there was little replication of dietary conditions between studies, we did not conduct any quantitative analysis of this variable. Quantitative analysis was also not possible for larval density because this trait was mentioned in only five studies (and the effect size was available for only three of them). It is interesting to note, however, that the mean larval density used in the three studies that found *Plasmodium* to be virulent [15–17] was almost five times that of the two that did not [24,25] (mean larval density in studies that showed no effect = 113 larvae per litre, mean larval density in studies that showed an effect = 555 larvae per litre). This variation is likely to be important because even a fourfold increase in larval density can generate a significant decrease in adult body size [45], a prime determinant of mosquito survival [46]. Small mosquitoes produced from high-density larval conditions might have fewer resources to combat losses imposed by parasites. Experimental tests of the relationship between larval density and susceptibility to *Plasmodium* would be valuable.

Choice of control

Early research on *Plasmodium*–mosquito associations was mostly observational. Several papers published before 1950 did not use an uninfected control but asserted their results anecdotally [18,21,23]. Despite this, these studies are still cited as evidence for the neutrality of *Plasmodium* on their vector (e.g. Refs [13,27,28]). Among the studies with controls, there is still an important distinction in the type: (1) mosquitoes fed on uninfected blood or (2) mosquitoes without parasites regardless of infection status of earlier bloodmeals. Field-based studies must use the latter because it is not possible to ascertain the feeding history of wild-caught mosquitoes [27,30]. However, using this type of control group could be misleading if pathology associated with infection arises not from parasite development but from the toxicity and/or poor nutrient quality of infected blood. If mosquitoes fed on infected blood have lower survival even when oocysts and sporozoites do not develop, field-based studies might underestimate the effect of malaria on vector survival. Our sample has too few studies that have used the 'no sporozoite' design ($n = 2$) to draw any conclusions.

Genetic diversity

Field studies of *Plasmodium falciparum* and *Plasmodium vivax* have shown that each species harbours an enormous amount of genetic diversity for traits that mediate infection (e.g. merozoite surface

proteins) [47]. However, most laboratory studies of vector–parasite interactions use only one species of inbred mosquito and one genetically homogeneous *Plasmodium* strain. Studies of other invertebrates and their parasites have shown that susceptibility to infection depends on parasite genotype [48,49]. These results have recently been corroborated for malaria, when we showed that the survival of mosquitoes infected with *Plasmodium chabaudi* varied with parasite genotype [17]. One genotype was apparently benign, whereas another reduced mosquito survival (these effects are averaged in Table 2). Thus, a single study captures the contrasting conclusions of different studies. Results from field studies are not limited by genetic homogeneity because wild-caught mosquitoes are infected with many different parasite genotypes [50]. However, averaging the consequences of infection over many parasite genotypes might obscure important variation in pathogenicity.

Conclusions

We have identified several possible reasons why some studies have found that vectors are killed by malaria parasites whereas others have not. Studies reporting detrimental effects typically involve experiments of longer duration and unnatural vector–parasite combinations, and there might also be an effect of humidity. However, it is not easy to disentangle these various factors because they are confounded in studies to date. For example, the longest studies have been done on unnatural vector–parasite combinations (mean of 35.9 days for unnatural combinations vs 15.1 days for natural combinations, $F_{1,20} = 5.38$, $P = 0.03$), and the laboratory reporting the strongest effects [13,14] is also the one that maintained its mosquitoes in the driest atmosphere. Rather than overextend the statistics of meta-analysis, we suggest that experimental manipulations in a single laboratory are required to disentangle these factors and others, such as the role of genetic diversity.

In any case, are data on mosquito longevity in laboratory cages relevant to the natural transmission setting? Critics have suggested that the frequent use of unnatural vector–parasite combinations will generate artificially high virulence [30], and that laboratory experiments exclude possible indirect costs of infection such as increased risk of predation and feeding-associated mortality [42]. Our meta-analysis is certainly consistent with the first objection, although further experimental tests are required to separate the effects of co-evolutionary history and experimental design.

The importance of the second issue (failure to incorporate indirect effects) is unclear. Certainly, mosquitoes face a diverse array of environmental and biotic hazards in the field, most of which are eliminated within laboratory cages. Host defensive behaviour, for example, is a potentially significant source of mortality for mosquitoes [51], yet none of the studies that we have reviewed has allowed the possibility of post-infection anti-vector behaviour. Indirect mortality costs are probably higher for infected mosquitoes. It has been shown that, under natural field conditions, mosquitoes with sporozoites have greater feeding-associated mortality than those without sporozoites [42]. Laboratory results support this observation, confirming that infected mosquitoes are more persistent feeders [41] and have poorer flight ability [52,53], a trait that might reduce their ability to evade a defensive behaviour.

Short of unethical mark–recapture experiments of experimentally infected mosquitoes, it will be difficult to estimate the relative importance of the longevity effects detected in the laboratory and those that are a result of natural host feeding. The most plausible way forward is for experiments in the laboratory to incorporate these other possible sources of mortality. We know of only one study in which there was simultaneous estimation of the survival of a population when kept in cages and living free [27]. Interestingly, this study found survival to be higher in the free-living population, suggesting that the protective benefits of being in a cage might be outweighed by other factors. Only in the laboratory can mortality be properly examined over a range of conditions, such as variation in number of blood meals and environmental stress. Setting laboratory results into the context of the mortality experienced during a natural transmission cycle will require substantially more research, in both the laboratory and the field.

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Genetic and environmental determinants of malaria parasite virulence in mosquitoes

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Models of malaria epidemiology and evolution are frequently based on the assumption that vector–parasitic associations are benign. Implicit in this assumption is the supposition that all *Plasmodium* parasites have an equal and neutral effect on vector survival, and thus that there is no parasite genetic variation for vector virulence. While some data support the assumption of avirulence, there has been no examination of the impact of parasite genetic diversity. We conducted a laboratory study with the rodent malaria parasite, *Plasmodium chabaudi* and the vector, *Anopheles stephensi*, to determine whether mosquito mortality varied with parasite genotype (CR and ER clones), infection diversity (single versus mixed genotype) and nutrient availability. Vector mortality varied significantly between parasite genotypes, but the rank order of virulence depended on environmental conditions. In standard conditions, mixed genotype infections were the most virulent but when glucose water was limited, mortality was highest in mosquitoes infected with CR. These genotype-by-environment interactions were repeatable across two experiments and could not be explained by variation in anaemia, gametocytaemia, blood meal size, mosquito body size, infection rate or oocyst burden. Variation in the genetic and environmental determinants of virulence may explain conflicting accounts of *Plasmodium* pathogenicity to mosquitoes in the malaria literature.

Keywords: *Plasmodium chabaudi*; *Anopheles stephensi*; disease vectors; parasite genetic diversity; mixed infections; survival

1. INTRODUCTION

The basic reproductive rate of many infectious diseases is critically dependent on the lifespan of their hosts (Anderson 1982). For successful transmission to new vertebrate hosts, malaria-infected mosquitoes must survive at least as long as the extrinsic incubation period within the vector, which can be two weeks or more. Several authors have argued that this means there will be strong selection pressure on malaria parasites (*Plasmodium*) not to reduce vector survival (Ewald 1994; Dye & Williams 1995; Koella 1999). However, the evidence that malaria parasites are indeed benign in their vectors is mixed. Some indirect field data support the notion of survival costs to infection (Lyimo & Koella 1992), whereas others do not (Lines *et al.* 1991). There are substantially more data from experimental infections, but these too are contradictory. Several studies have reported reductions in vector longevity (Gad *et al.* 1979; Klein *et al.* 1982, 1986; Maier *et al.* 1987) while others have not (Sinton & Shute 1938; Freier & Friedman 1987; Chege & Beier 1990; Robert *et al.* 1990; Hogg & Hurd 1997).

One explanation for these conflicting accounts is that virulence (here defined as a reduction in survival) is not a fixed property of infection, but varies with parasite genotype. This phenomenon has been documented within vertebrate hosts of *Plasmodium*, where parasite genetic variation is associated with disease severity (Yoeli *et al.* 1975; Carlson *et al.* 1990; Rowe *et al.* 1997; Chen *et al.* 1998; Mackinnon & Read 1999*a*,*b*; Chotivanich *et al.* 2000). However, the relationship between parasite genotype and mosquito survival has not to our knowledge been examined. Genotype-specific reductions in vector survival could arise either as a direct consequence of differential pathogenicity of parasites in the mosquito, or indirectly as a function of parasite-induced changes that reduce the nutritional quality of host blood.

Plasmodium virulence in vectors could also be affected by the genetic diversity of an infection. Some evolutionary theory predicts that virulence should increase as the genetic diversity of parasites in an infection increases (Sasaki & Iwasa 1991; Frank 1992, 1996; Van Baalen & Sabelis 1995), a pattern that has been observed (reviewed by Read & Taylor (2001)). Increased virulence of genetically diverse infections has been attributed to many factors, including the promotion of more virulent strains and overall parasite burdens under competition (Hargreaves *et al.* 1975), collateral damage due to the release of competition-mediated allelopathic substances (Chao *et al.* 2000) and the increased difficulty of immunologically controlling mixed infections (Taylor *et al.* 1998). We know that mixed genotype infections of *P. chabaudi* are more virulent in mice (Taylor *et al.* 1998), and are more infectious to mosquitoes than single genotype infections (Taylor *et al.* 1997). The epidemiological consequences of the enhanced transmission of these mixed infections will depend critically on whether they are also more lethal to mosquitoes.

In addition to parasite genetics, environmental conditions may be an important determinant of virulence: fitness costs are often more evident in harsh conditions (Stearns 1992; Bergelson & Purrington 1996). Many laboratory studies of vector–parasite interactions, however, take place in conditions that aim to maximize vector survival. Consequently, the cost of parasitism may be frequently underestimated. This alone could generate apparently benign vector–parasite associations in some

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laboratory experiments even if virulence in the wild is high. Furthermore, variation in environmental conditions between laboratories may influence the likelihood of detecting parasite-induced mortality—a proposal supported by a meta-analysis of previously published studies of *Plasmodium* virulence in mosquitoes (Ferguson & Read 2002).

The outcome of *Plasmodium*–vector interactions has potentially important implications for malaria epidemiology (Macdonald 1957; Dye & Williams 1995). It is also critical in understanding the conditions that shape virulence evolution (Bull 1994); both in the vector itself and in the vertebrate host. We therefore conducted a laboratory study using the *P. chabaudi*–mouse model to examine whether:

(i) vector survival varies with parasite genotype;
(ii) vector mortality increases with the overall level of diversity in an infection (single versus mixed genotype infections); and
(iii) whether parasites cause greater mortality when vectors are maintained in suboptimal conditions.

2. MATERIAL AND METHODS

Anopheles stephensi larvae were reared in standard insectary conditions of 27 ± 1 °C, 70% humidity and a 12 L : 12 D cycle. Eggs were placed in plastic trays (25 cm × 25 cm × 7 cm) filled with 1.5 l of distilled water. In order to reduce variation in larval growth rate and size at emergence, larvae were reared at a fixed density of 500 per tray (from first day after hatching). Larvae were fed on Liquifry for 5 days and then on ground Tetrafin fish flakes. Larvae took 9–14 days to transform into pupae. On days 10–13 post hatching, groups of 250 pupae were placed in one of 16 emergence cages (16 cm × 16 cm × 16 cm). The adults that emerged ($n = 170$–240) were fed *ad libitum* on a 10% glucose water solution supplemented with 0.05% para-aminobenzoic acid (PABA).

We used two genotypes of *P. chabaudi* known as CR and ER (Beale *et al.* 1978) from the World Health Organization's Registry of Standard Malaria Parasites, University of Edinburgh. These genotypes were chosen because their behaviour has been extensively studied in the vertebrate host, where they are known to generate infections of similar length and parasite density (Taylor *et al.* 1997). These genotypes are clones, which are asexually replicated lineages derived from a single ancestral parasite that was originally isolated by serial dilution. Groups of four mice (C57BL/6J) were infected with 10^6 parasites of CR, 10^6 of ER, 10^6 of a 1 : 1 mix of CR and ER (5×10^5 CR + 5×10^5 ER) or were left uninfected. Previous molecular analyses of parasites in mosquitoes showed that both of these genotypes will transmit from mixed infections in mice (Taylor *et al.* 1997). From the fifth day of infection onwards, thin blood smears were taken on a daily basis. Mosquito feeds took place 14 days after mouse infection, when all infected mice had sufficiently high gametocytaemia (proportion of red blood cells infected with gametocytes >0.1%).

To increase hunger levels, mosquitoes were deprived of glucose for 24 h before feeding on the mice. One anaesthetized mouse was placed on each cage and mosquitoes were allowed to feed for 20 min (16 cages, 85–120 females per cage).

After the feed, half the cages from each parasite treatment were supplied with glucose water *ad libitum*. The others were supplied with glucose water for 24 h every second day until the experiment was terminated. In a pilot study, this feeding regime reduced the survival of uninfected mosquitoes by 50%. Cages were then checked daily and dead mosquitoes were removed. No further blood meals were given after the first infective feed.

Fifteen to twenty females from each cage were killed with chloroform on days 8 and 9 after the blood feed. Their midguts were dissected in a drop of a 0.01 M solution of phosphate-buffered saline and examined under a microscope to assess oocyst prevalence and burden.

The entire experiment was repeated six months later (hereafter called block 2). In this second block, mean blood meal size and anaemia were also estimated. Mean blood meal size was measured indirectly as the mass of haematin excreted by four to five mosquitoes that were taken from each cage immediately after the feed and moved individually into 30 ml tubes for haematin collection (as in Briegel *et al.* 1978). Only mosquitoes that were fully engorged with blood were selected for this analysis. All mosquitoes collected for this analysis excreted haematin, confirming that they had indeed taken a blood meal. Anaemia was measured as the number of red blood cells in a 2 µl sample of mouse blood taken a few hours before the feed. A final difference from the first block was that an error in glucose treatment assignment on the day of the feed meant that only one of the four CR cages was placed under glucose water deprivation conditions, and three of the four ER cages were placed under glucose water deprivation conditions.

In both blocks, survival monitoring continued for 35 days after the feed except in two cases where observation was curtailed due to cage damage (block 1, CR-infected cage under glucose deprivation conditions, stopped on day 29) and problems with glucose water delivery (block 2, ER-infected cage under glucose deprivation conditions, stopped on day 27).

All animal experiments conformed to Home Office Guidelines.

(a) *Statistical analysis*

Our analysis was conducted in two stages to account for survival measurements estimated at the individual level (response variable equals individual's day of death) and at the cage level (response variables: (i) median survival of all mosquitoes in a cage; and (ii) the proportion of mosquitoes in a cage surviving until day 14 post feed). Both the median survival rate and the proportion of mosquitoes surviving until day 14 were obtained from Kaplan–Maier estimates of the survival distribution in each cage (SPSS, Inc. 1995). We chose to examine the proportion of mosquitoes surviving until day 14 as it is an index of survival at the time when mosquitoes are first able to infect new hosts. Sporozoite invasion of the salivary glands can begin as soon as 10 days after an infectious feed (Killick-Kendrick & Peters 1978) and is probably complete by day 14 (R. Carter, personal communication). General linear models were used to evaluate the relationship between each of the two cage-level survival indices and the three main treatment effects of parasite genotype, glucose water treatment and experimental block (SAS Institute, Inc. 1997). Maximal models included all higher-order interactions. Non-significant terms were dropped to yield a minimum model.

To identify the mechanism for any observed genotype or glucose water effects, the other infection covariates measured at cage level (host anaemia and gametocytaemia, mean blood meal size, mean wing length, infection rate and mean oocyst burden)

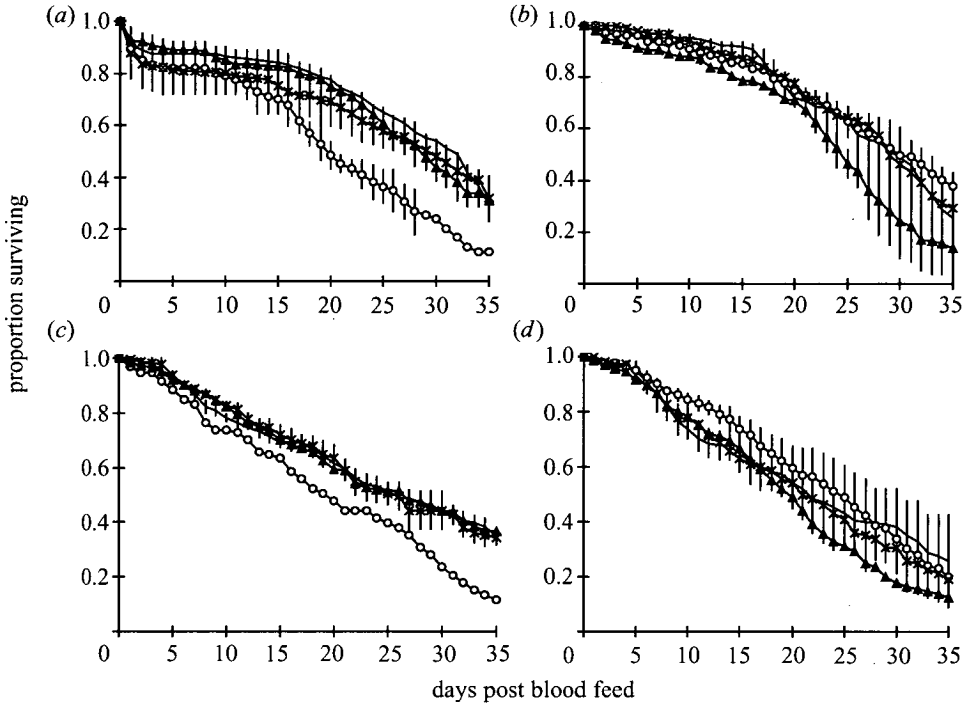

Figure 1. Survival curves for female *A. stephensi* mosquitoes infected with different genotypes of *P. chabaudi*. (*a*) Glucose water deprivation—block 1, (*b*) glucose *ad libitum*—block 1, (*c*) glucose water deprivation—block 2, (*d*) glucose *ad libitum*—block 2. In (*a*) and (*b*) each line represents the mean survival rate across two cages. In (*c*) there are three ER cages and one CR cage, and in (*d*) there is one ER and three CR cages. Bars represent the standard error of estimates between cages (in (*a*) no standard error for CR after day 29 because monitoring was terminated in one of the two cages due to damage). Solid lines without symbols, uninfected mosquitoes; circles, CR-infected mosquitoes; triangles, CR/ER-infected mosquitoes; crosses, ER-infected mosquitoes.

were added individually to the minimum model for the median survival rate. Covariates that eliminated the significance of main treatment effects when added to the minimum model, and were significant in themselves, were identified as potential causal agents of the main treatment effects. Proportion data (alive, infected) were arcsine square-root transformed prior to analysis, and oocyst burdens were analysed as log (number of oocysts + 1).

To estimate the individual cost of infection, survival data were fitted to the Cox proportional hazards model (Collet 1994). A hazard ratio for each infected group was estimated as their instantaneous risk of death relative to that of the uninfected controls. A hazard ratio that is significantly greater than 1 indicates that the infection treatment reduced mosquito survival. Separate models were constructed for each block to examine the consistency of the hazard ratio estimates between both trials.

3. RESULTS

The fate of approximately 3300 mosquitoes was observed. In both blocks, *ca.* 25% survived until the experiments were terminated on day 35 post feed. The midguts of 380 mosquitoes from the infected groups were dissected. On average, 27% were infected in block 1 and 56% in block 2. By contrast to our pilot studies, restricting provision of glucose water to alternate days had no effect on either the median survival rate of mosquitoes fed on uninfected mice, or the proportion of mosquitoes surviving until day 14 ($p > 0.10$ for both indices). This may have been due to improved larval rearing techniques used in the experiments reported here.

Parasite genotype and glucose water availability influenced vector mortality (figure 1). Median survival was determined by the interaction between the parasite genotype and glucose water treatment (figure 2, $F_{3,23} = 4.94$, $p < 0.01$). When glucose water was continuously available, mosquitoes with mixed genotype infections had the poorest survival rate. However, when glucose water was available only on alternate days, it was the CR-infected mosquitoes that had the greatest mortality. This pattern had not achieved significance by day 14 ($F_{3,23} = 2.49$, $p = 0.086$). Parasite genotype × glucose water interactions were repeatable across blocks (figure 2, no block interactions: $p > 0.10$). The minimal model of treatment effects explained 56.2% of the variation in the median survival rate and 68.5% of the variation in the proportion surviving until day 14 (treatment effects: block, parasite genotype, glucose water and glucose water × parasite genotype).

Analysis of the day of death of individual mosquitoes reinforced the conclusions from the cage-level analyses (figure 3), with the parasite genotype by glucose water interaction term significantly improving the fit in the Cox proportional hazard model (block 1, $\chi^2_7 = 57.95$, $p < 0.01$, block 2, $\chi^2_7 = 26.80$, $p < 0.01$). There was no significant between-block variation in the hazard ratios generated for each parasite genotype (Wald statistic = 2.45, d.f. = 3, $p = 0.48$) or the nature of parasite genotype × glucose water interaction (Wald statistic = 3.17, d.f. = 3, $p = 0.37$).

Variation in the proportion of infected mosquitoes (prevalence) and their oocyst burdens could not account for the mortality generated by the experimental treatments

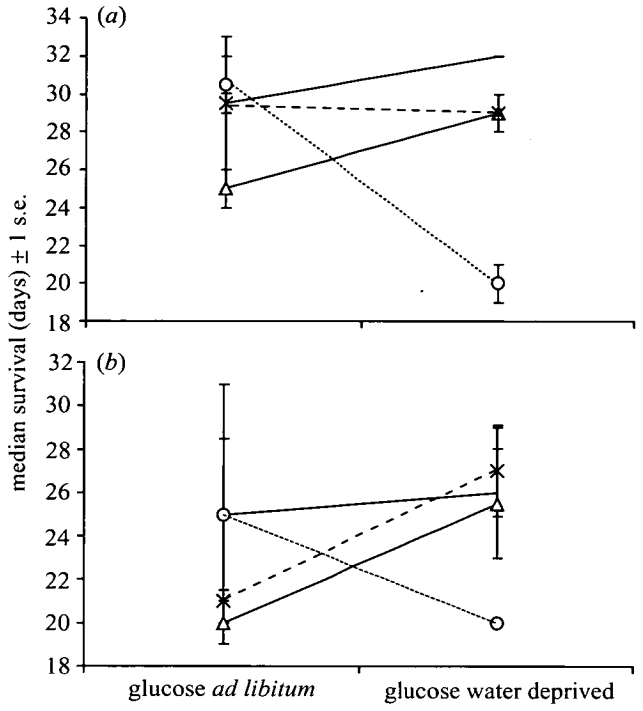

Figure 2. The mean value of the median survival of mosquitoes infected with different genotypes of *P. chabaudi*, and maintained with glucose water provided *ad libitum* or every second day. (*a*) Block 1, (*b*) block 2. Points are treatment means (± s.e.); each point is based on two replicates, except in block 2 where there was only one replicate of CR glucose water deprivation and ER glucose water *ad libitum* treatment (hence no standard error generated). Small dashes with circles, CR; large dashes with crosses, ER; solid lines with no symbols, uninfected mosquitoes; solid lines with triangles, CR/ER-infected mosquitoes.

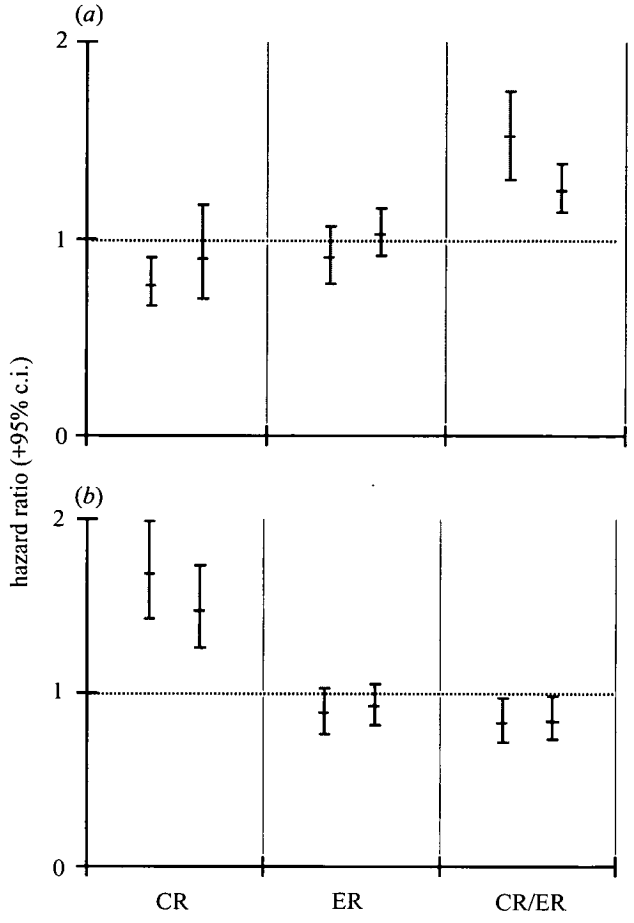

Figure 3. The mean and 95% confidence intervals of the hazard ratio for each infection group in standard glucose water deprivation conditions. (*a*) Glucose water *ad libitum*, (*b*) glucose water deprivation conditions. The grey lines are for block 1 and the black lines for block 2.

(figure 4). Although the interaction between parasite genotype and glucose water had a strong influence on survival, it had no effect on either prevalence or the mean oocyst burden ($p < 0.10$ in both cases). Furthermore, unlike its influence on survival, the effect of parasite genotype on prevalence and oocyst burden was inconsistent between blocks (significant block interactions, $p < 0.05$).

None of the effects of gametocytaemia, mean mosquito body size, oocyst prevalence or oocyst burden were significant when added to the minimal model for the median survival ($p > 0.15$ in all cases, figure 5) and none reduced the significance of the genotype × glucose water interaction ($p < 0.05$ in all cases). Blood meal size and anaemia were measured only in block 2. In that block, there was no evidence that survival was influenced by blood meal size ($F_{1,14} = 1.36$, $p = 0.26$) or anaemia ($F_{1,14} = 0.37$, $p = 0.55$).

4. DISCUSSION

The primary finding of this study was that the vector mortality varies significantly with parasite genotype in an environmental condition-dependent manner. The mixed infections were more virulent when glucose water was provided *ad libitum*, but when mosquitoes were given glucose water only every second day, CR infections were more virulent. The direction of this genotype-by-environment (G × E) interaction was repeatable across two experiments conducted several months apart (figures 1–3).

The interaction between parasite genotype and glucose water was by far the strongest predictor of mosquito mortality, exceeding the effect of all the other parameters we examined, including measures of blood meal quality (size and anaemia of blood meal) and parasite load (gametocytaemia, oocyst prevalence and burden). Under glucose water deprivation conditions in block 1, CR infections caused considerable mortality despite producing significantly lower oocyst burdens (geometric mean ± s.e. = 1.5 ± 1.4) than the other two treatments (11.9 ± 9.8 and 76.0 ± 36.0 for ER and CR/ER, respectively; figure 4). This result contradicts the traditional view that malaria parasites are harmful to mosquitoes only when oocyst burdens are exceedingly high (De Buck 1936; Klein *et al.* 1986). This argument has been used to refute the existence of *Plasmodium*-induced mortality in nature, as most naturally infected mosquitoes carry only one to two oocysts (Chege & Beier 1990). Our results indicate that the determinants of virulence are more complex, and that the detection of parasite-induced mortality may be hindered by focusing solely on parasitic load.

It is difficult to explain why the effects of glucose water on mosquito survival varied across the parasite genotypes. The result for mixed infections is particularly unexpected,

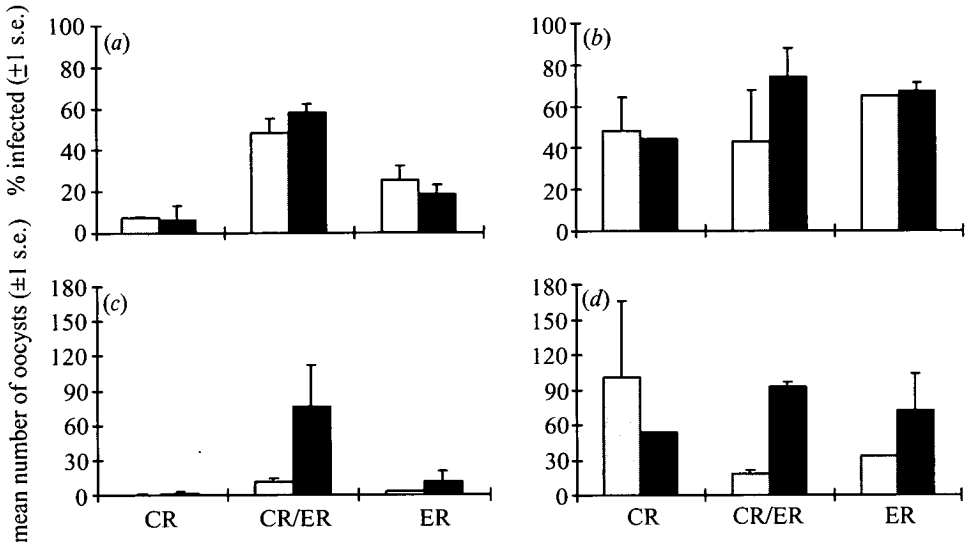

Figure 4. Infectivity and oocyst burdens of different infection treatments in *A. stephensi* mosquitoes. (*a,c*) Block 1, (*b,d*) block 2. The grey bars are for mosquitoes given glucose water *ad libitum*, and the black bars are for glucose water deprivation conditions. Each bar is based on the mean of 18–20 mosquitoes.

as sugar feeding usually enhances mosquito survival (Foster 1995; Straif & Beier 1996; Gary & Foster 2001). We offer the following speculations. First, in both blocks, mosquitoes with mixed infections had significantly higher oocyst burdens when they were maintained on restricted glucose-water. For both the single clones, there were no such differences. Perhaps increasing glucose availability allowed the mixed genotype group to mount an immune response. Activation of the invertebrate immune system can decrease survival (Moret & Schmid-Hempel 2000). Second, *Plasmodium* infection may have altered the sugar-feeding behaviour of mosquitoes in a genotype-specific manner. *Plasmodium* is known to influence the blood-feeding behaviour of their vectors, specifically by increasing the frequency and persistence with which they feed (Wekesa *et al.* 1992; Koella *et al.* 1998; Anderson *et al.* 1999). It is possible that such feeding manipulation extends also to glucose feeding, and that some genotypes promoted a higher intake than others. This could be detrimental if rapid consumption leads to physiological damage, or to increased exposure to bacteria that grow in glucose water (Seitz *et al.* 1987). Third, the G × E interaction could be due to differences in sporozoite development. It is possible that sporozoite load, not oocyst burden, is the prime determinant of mosquito mortality; indeed, mortality differences became apparent only when sporozoites would be in the salivary glands (figure 1). We do not know how or if glucose supply and parasite genotype influenced the number of sporozoites produced by an oocyst, or the ability of these sporozoites to invade the salivary glands. Certainly the efficiency with which oocyst infections develop into salivary gland infections varies (Lombardi *et al.* 1987; Beier *et al.* 1990)—a phenomenon that has been attributed to the genetic diversity of parasites and their vectors (Vaughan *et al.* 1992).

None of these three hypotheses (cost of immunity, feeding rate manipulation or differential sporozoite invasion) are entirely satisfactory as they all rely on some sort of unknown genotype-specific effects. Nonetheless, all three are testable and the subjects of ongoing experiments.

The survival differences we report were not evident within the first 14 days of mosquito infection, and were detected only when survival over the entire 35 day experimental period was considered. Thus, genotype-specific mortality did not influence the proportion of mosquitoes that became infectious; only their survival through the infectious period. If our survival curves (figure 1) are representative of what happens in the field, differences in survival during this period could have a large effect on sporozoite rates in natural populations. The investigation of additional issues such as the importance of subsequent feeds and survival from feeds over the entire infection course will further elucidate the epidemiological consequences of these genotype-specific survival effects.

The mortality effects revealed here may be underestimates for several reasons. First, the survival estimates for mosquitoes in infected treatment groups included individuals that did not actually get infected (figure 4). Second, this experiment necessarily addressed only the direct cost of infection without secondary factors such as the susceptibility to predation and the ability to evade anti-vector behaviour. Sporozoite-infected mosquitoes spend more time feeding, probe more often (Wekesa *et al.* 1992), are more persistent in biting (Anderson *et al.* 1999) and feed more often than uninfected mosquitoes (Koella *et al.* 1998). These behavioural changes will probably increase the mortality of infected vectors (Anderson *et al.* 2000).

Another factor that may have influenced our ability to detect virulence was our blood-feeding procedure. We allowed mosquitoes to take only one blood meal, after which they were maintained exclusively on glucose water. We selected this protocol because we wished to eliminate all factors that could confound our ability to detect pathogenicity arising from the first infectious feed (e.g. differential blood feeding between mosquitoes with different *Plasmodium* infections). Also, we wished to make our results comparable with other studies that have examined the impact of *Plasmodium* on vector survival; most of which have used a similar one blood meal protocol (Buxton 1935; Thompson & Huff 1944; Maier 1973; Gad

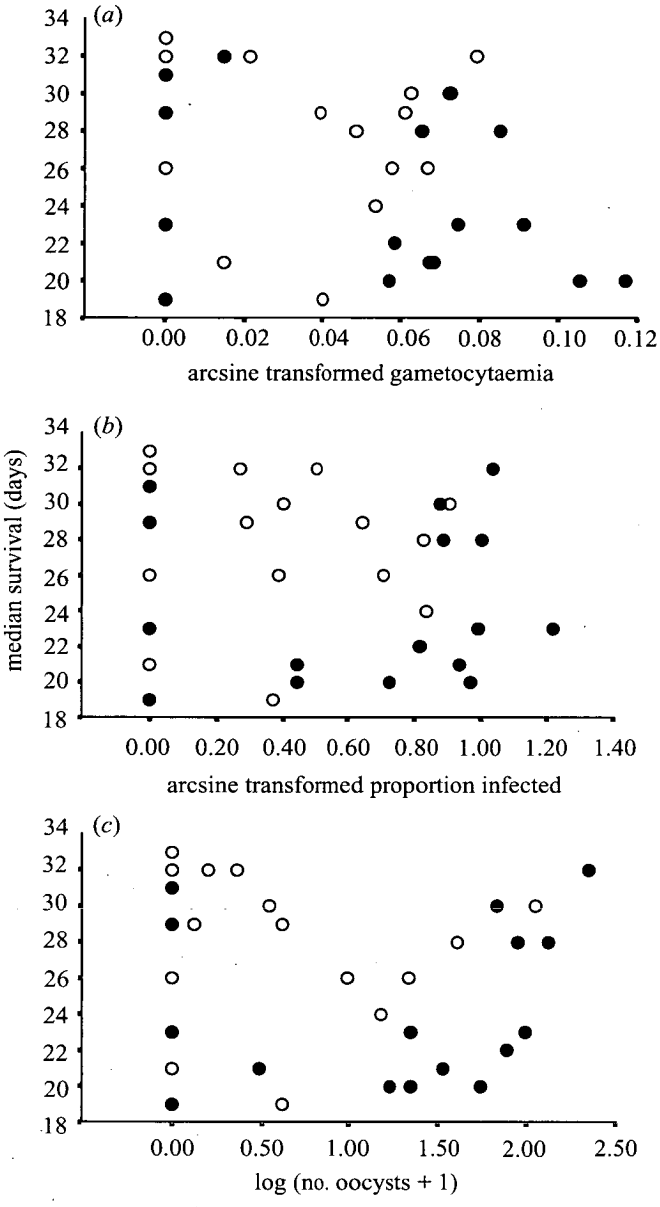

Figure 5. Median survival of female *A. stephensi* mosquitoes as a function of gametocytaemia (*a*), proportion of mosquitoes infected (*b*) and mean oocyst burden (*c*) ($n = 32$). White circles, block 1; black circles, block 2.

et al. 1979; Klein *et al.* 1982, 1986; Freier & Friedman 1987; Maier *et al.* 1987; Chege & Beier 1990; Gamage-Mendis *et al.* 1993; Hogg & Hurd 1995, 1997). However, this feeding regime does not approximate the natural feeding behaviour of *Anopheles* where blood meals are taken every few days.

It is unclear how post-infection blood meals will influence *Plasmodium* virulence. The extra nutrients gained by multiple blood feeds could offset any detrimental effects of parasitism. Alternatively, extra blood meals could increase parasite virulence: they generate higher sporozoite burdens (Vanderberg & Nawrot 1968; Ponnudurai *et al.* 1990), increase the risk of feeding associated mortality (Anderson *et al.* 2000) and increase reproduction (Hurd *et al.* 1995). The survival costs of reproduction are well known (Stearns 1992). To our knowledge there have been no studies of *Plasmodium* virulence under different blood-feeding regimes. As such, further experimentation is required to test whether the genetic variation for virulence we report would be maintained during repeated blood meals. Until this occurs, the epidemiological significance of these results remains unclear.

The genotype-specific mortality reported here was detectable from the examination of just two parasite genotypes. These genotypes represent only a fraction of genetic variation in this *Plasmodium* species (Mackinnon & Read 1999*a*), and are probably more similar than most because they have a common history of laboratory passage, and have approximately equal growth rates in their vertebrate host (Taylor *et al.* 1997). The fact that differences are evident in a survey of only two genotypes indicates that the range of parasite-induced vector mortality may be much greater in the wild. In any case, we have demonstrated that there is genetic variation for virulence in the vector—a necessary prerequisite for virulence evolution. Moreover, widespread G × E interactions of the sort we have discovered could act to maintain diversity in *Plasmodium*.

This study demonstrates the ease with which conflicting accounts of virulence can be generated, even within a single vector and *Plasmodium* species in a laboratory setting. For instance, if we had only worked with ER genotype, we would have concluded, like many others, that malaria is benign in its vector. Similarly, the increased virulence of mixed genotype infections would not have been detected had all mosquitoes been maintained on reduced glucose water. This indicates that the general outcome of vector–parasite interactions can be established only by examination of different parasite genotypes and environmental conditions. This complexity arises even before the role of mosquito genotype has been considered. Susceptibility to *Plasmodium* can vary significantly both between (Chege & Beier 1998) and within vector species (Yan *et al.* 1997). The elucidation of G × E × G interactions poses considerable experimental challenges.

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