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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)

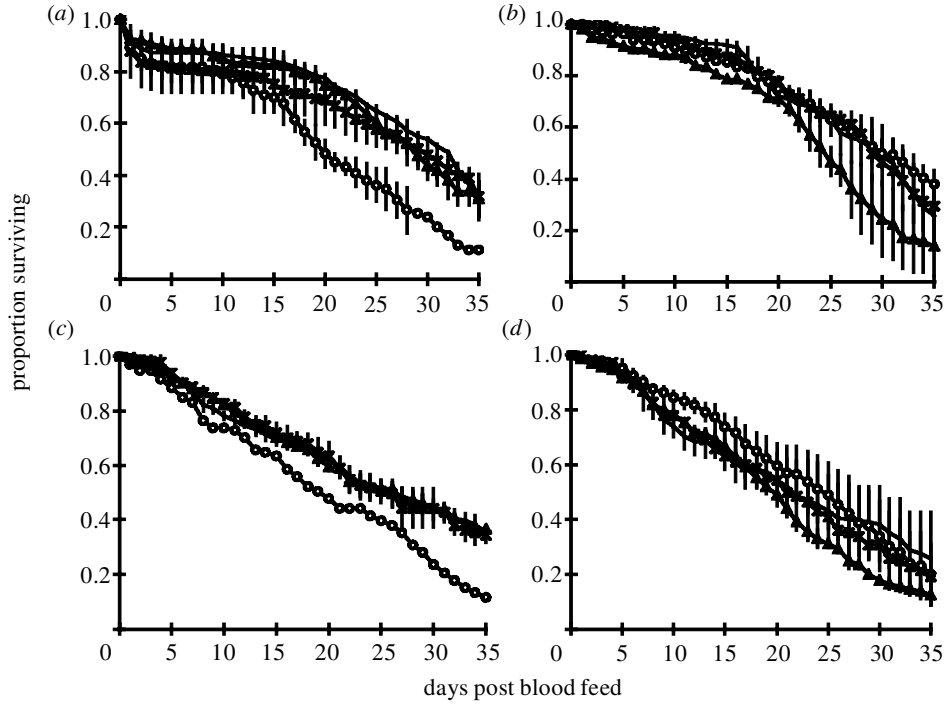

Figure 1. Survival curves for female *A. stephensi* mosquitoes infected with different genotypes of *P. chabaudi.* (*a*) Glucose water deprivation—block 1, (*b*) glucose *ad libitum*—block 1, (*c*) glucose water deprivation—block 2, (*d*) glucose *ad libitum*—block 2. In (*a*) and (*b*) each line represents 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

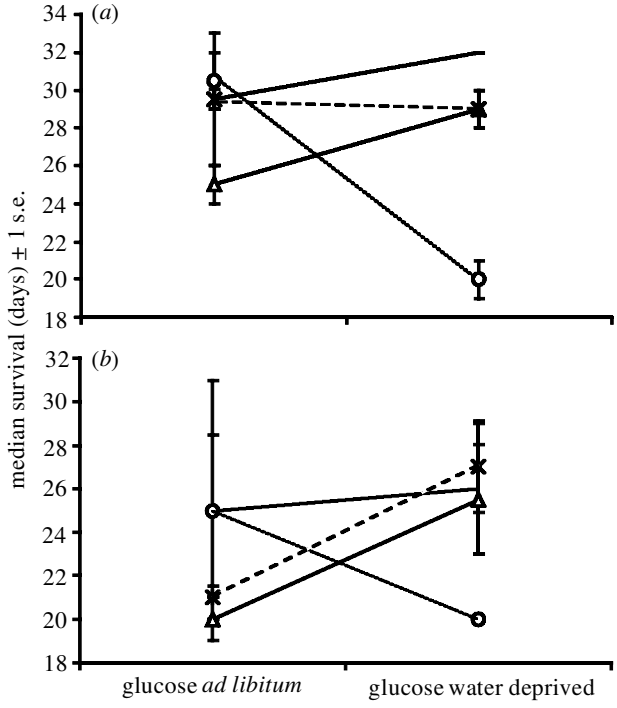

Figure 2. The mean value of the median survival of mosquitoes infected with different genotypes of *P. chabaudi*, and maintained with glucose water provided *ad libitum* or every second day. (*a*) Block 1, (*b*) block 2. Points are treatment means (± s.e.); each point is based on two replicates, except 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.

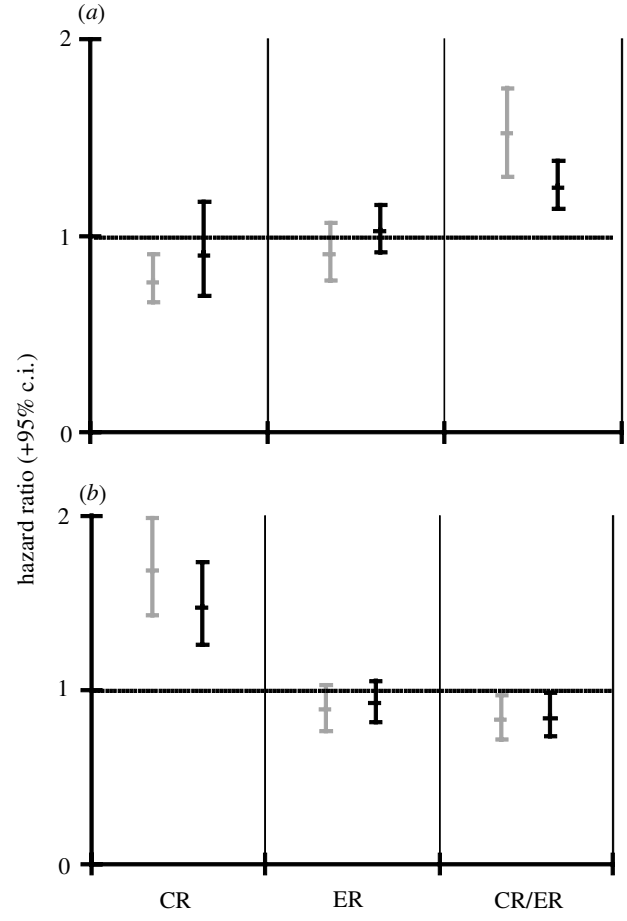

Figure 3. The mean and 95% confidence intervals of the hazard ratio for each infection group in standard glucose water deprivation conditions. (*a*) Glucose water *ad libitum*, (*b*) glucose water deprivation conditions. The grey lines are for block 1 and the black lines for block 2.

(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,

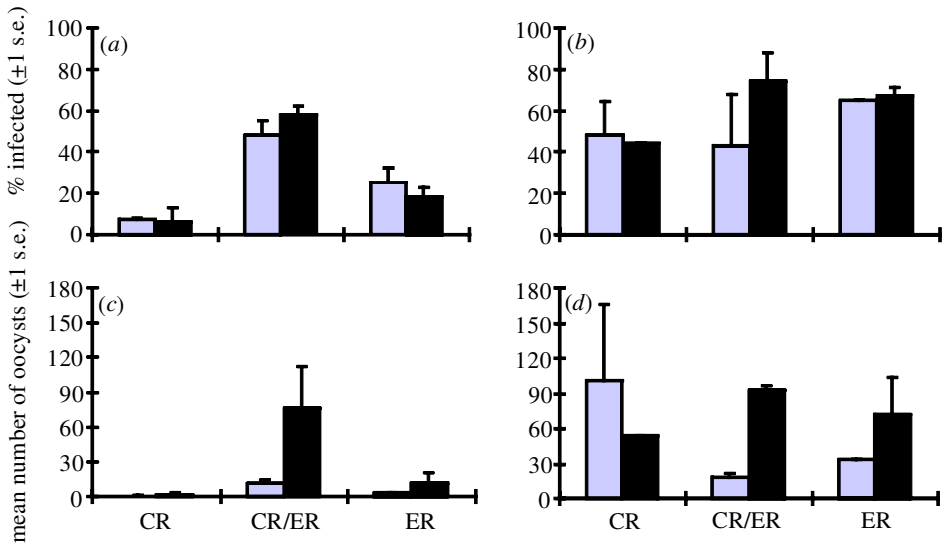

Figure 4. Infectivity and oocyst burdens of different infection treatments in *A. stephensi* mosquitoes. (*a*,*c*) Block 1, (*b*,*d*) block 2. The grey bars are for mosquitoes given glucose water *ad libitum*, and the black bars are for glucose water deprivation conditions. Each bar is based on the mean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

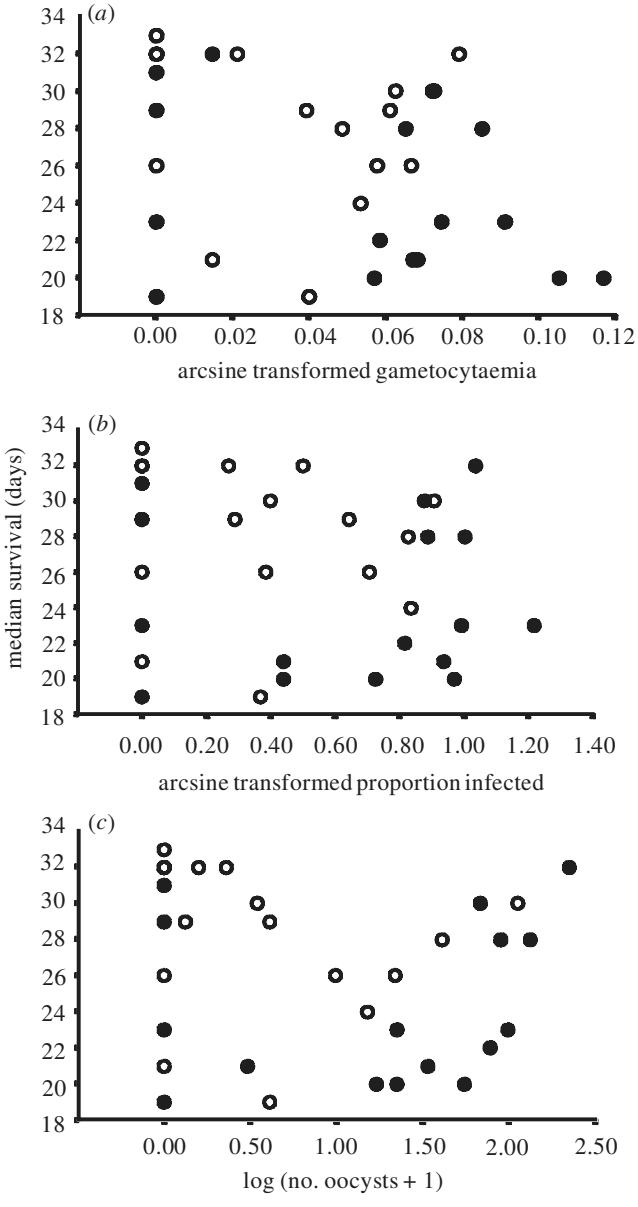

Figure 5. Median survival of female *A. stephensi* mosquitoes as a function of gametocytaemia (*a*), proportion of mosquitoes infected (*b*) and mean oocyst burden (*c*) ($n = 32$). White circles, block 1; black circles, block 2.

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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As this paper exceeds the maximum length normally permitted, the authors have agreed to contribute to production costs.