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MOSQUITO MORTALITY AND THE EVOLUTION OF MALARIA VIRULENCE

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Abstract.—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 *P. 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 anemia in infected mice were monitored for 16–17 days before *Anopheles stephensi* mosquitoes were allowed to take a blood meal from them. Infection virulence in mice was positively correlated with transmission to mosquitoes (infection rate) and weakly associated with parasite burden (number of oocysts). Mosquito survival fell with increasing oocyst burden, but there was no overall statistically significant relationship between virulence in mice and mosquito mortality. Thus, there was no evidence that more virulent strains are more lethal to mosquitoes. Both vector survival and fecundity depended on parasite clone, and contrary to expectations, mosquitoes fed on infections more virulent to mice were more fecund. The strong parasite genetic effects associated with both fecundity and survival suggests that vector fitness could be an important selective agent shaping malaria population genetics and the evolution of phenotypes such as virulence in the vector.

Key words.—*Anopheles stephensi*, evolution of virulence, malaria, *Plasmodium chabaudi*, vectors.

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Theoretical studies have shown that natural selection will favor the evolution of increased pathogen virulence when there is a positive genetic correlation between disease severity and transmission success (Anderson 1982; Bremermann and Pickering 1983; Sasaki and Iwasa 1991; Frank 1992; Bull 1994; Read 1994; Ebert and Herre 1996; Frank 1996). By virulence, we refer to the harm inflicted on 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 sublethal symptoms. Positive correlations between virulence and transmission could arise if, for instance, parasite replication determines the number of infective propagules that will be produced to infect new hosts. At the same time, virulence should also increase with replication rate because disease severity is likely to increase with the number of parasites in the host. Thus, hosts in which parasite replication is high should be both good transmitters and experience the highest amount of illness.

Empirical studies suggest that positive relationships between pathogen virulence and transmission exist in many pathogen taxa (e.g., Diffley et al. 1987; Turner et al. 1995; Lipsitch and Moxon 1997; Ebert 1998; Mackinnon and Read 1999a,b; Weiss 2002). Given these virulence-associated transmission benefits, the question arises as to what limits continual evolution toward higher virulence. Conventional wisdom postulates that the source of selection against increasing virulence is host death. Here we consider a different possibility. Many parasites have indirect (multihost) life cycles. If there are positive correlations between parasite virulence in successive hosts, mortality induced by a parasite in one could host could select for reduced virulence in another.

We investigate this issue in the context of malaria. Several studies of rodent malaria indicate that *Plasmodium* virulence in mice is positively correlated with transmission success to mosquitoes (Mackinnon and Read 1999a,b, 2003). This correlation arises because both the severity of sublethal symptoms (anemia, weight loss) and the risk of host death increase with the density of asexual parasites in mouse blood, a trait that is also positively linked with the production of mosquito-infective transmission stages (gametocytes: Mackinnon and Read 1999a,b; Mackinnon et al. 2002). There is, necessarily, a lack of comparable studies of human malaria parasites, but 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). Thus, more virulent parasites apparently have a transmission advantage and should therefore be favored by natural selection.

Why, then, does malaria not generate higher mortality? Host death is one possible source of selection against higher virulence. Certainly, the transmission cost of inducing host death for the rodent malaria parasite *P. chabaudi*, a species that shares many life-history traits with the human parasite *P. falciparum* (Cox 1988), is high: infections that killed their laboratory mouse host had approximately 75% lower lifetime transmission potential than those in which the mouse survived (Mackinnon et al. 2002). However, a selection experiment on this same parasite showed that even when parasites from 50–75% of the most virulent infections were not allowed to transmit (mimicking a mortality cost of transmission), virulence continued to increase (Mackinnon and Read 1999b). The case fatality rate of *P. falciparum* in humans in Africa is possibly as high as 10% but most probably 1% or less

(Snow et al. 1999; Trape et al. 2002). It remains to be determined whether such low mortality rates are sufficient to select against more virulent *P. falciparum* in humans.

There are a number of other candidate sources of selection against malaria virulence (e.g., Ebert 1998; Day 2001), but to our knowledge the role of vectors has never been explored. This may be because vectors are generally assumed to be unaffected by the pathogens that require them for dispersal (Ewald 1983, 1994), a supposition that has been supported in some studies of malaria-vector interactions (e.g., Chege and Beier 1990; Robert et al. 1990; Gamage-Mendis et al. 1993). However, other studies have found that *Plasmodium* can reduce both vector fecundity and longevity (Hacker 1971; Hacker and Kilama 1974; Freier and Friedman 1976; Hogg and Hurd 1995a,b, 1997; Koella et al. 1998; Anderson et al. 1999; Koella 1999; Anderson et al. 2000; Ferguson and Read 2002a,b), and these virulence effects can differ between parasite genotypes (Ferguson and Read 2002a). There is also parasite genetic variation for virulence in the vertebrate host (Carlson et al. 1990; Rowe et al. 1997; Taylor et al. 1997; Mackinnon and Read 1999a; Chotivanich et al. 2000; Ariey et al. 2001; Ofosu-Okyere et al. 2001; Timms et al. 2001; de Roode et al. 2003). If this virulence variation in the two hosts is correlated, parasite-induced mortality in the mosquitoes could constrain the evolution of parasite virulence in the vertebrate host. Virulence correlations in the vector and vertebrate could arise in two ways. First, the increased transmission associated with high levels of *Plasmodium* virulence in vertebrates (Mackinnon and Read 1999a,b; Mackinnon et al. 2002) could generate high parasite burdens in mosquitoes. In at least some studies, mosquito mortality increases with the number of oocysts, the parasite life stage attached to the midgut wall (Klein et al. 1986). Thus, highly infectious malaria strains may be more lethal to mosquitoes. 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 anemia, 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 define virulence in the vector as a reduction in mosquito fecundity and/or survival that arises as a consequence of feeding on infected blood. Because malaria parasites are transmitted directly from vector to vertebrates and never horizontally, mosquito survival is a much more important determinant of parasite fitness than mosquito fecundity. However, our aim is to provide a comprehensive description of malaria parasite virulence in mosquitoes and how this relates to virulence in the vertebrate host, so we report parasite effects on vector fecundity as well as survival.

Our definition of virulence, a reduction in mosquito survival or fecundity after feeding on infected blood, is inclusive of whether mosquitoes went on to develop parasites (midgut oocysts) or not. The reason for doing so is that there is no reason to assume that only mosquitoes with oocysts have been detrimentally affected by parasitism. Several properties of infected blood such as anemia and antibodies influence mosquito fitness (Almeida and Billingsley 1998; Taylor and Hurd 2001), and all mosquitoes feeding on infected hosts are exposed to these factors. Also, we cannot be certain that a mosquito without oocysts was not infected with earlier stages that failed to develop. Indeed, the absence of oocysts may be due to an immune response of the mosquito against earlier parasite stages, a phenomenon that is known to reduce mosquito fitness (Ahmed et al. 2002). This potential cost of parasitism, along with those arising from early parasite stage damage to the midgut (Han et al. 2000), would be hidden if only oocyst infected mosquitoes were considered. Thus, given there are potentially multiple causes of *Plasmodium* virulence in mosquitoes, the most conservative means to estimate virulence is to follow the fitness of all mosquitoes feeding on infected blood.

To our knowledge, this is the first comparison of parasite virulence in the two obligate hosts of *Plasmodium*. Indeed, we know of only one other comparison of the virulence of different life stages of any pathogen or parasite that have multihost life cycles: across five strains of schistosome worms, virulence in laboratory mice was negatively but not significantly related to the survival of the snail vectors they infected (Davies 2000; Davies et al. 2001).

Materials and Methods

Mouse Infection

Seven different clones of *P. chabaudi* known as AJ, AQ, AS, BC, CW, CR, and ER were used (Mackinnon and Read 1999a). Clones are asexually replicated lineages derived from a single ancestral parasite obtained by serial dilution (Beale et al. 1978). These clones were chosen because their behavior has been extensively studied in laboratory mice where they are known to generate infections of varying severity (Mackinnon and Read 1999a).

Groups of seven female mice (C57BL/6J, Harlan, Oxon, England) were injected with 10^5 parasites of one of the seven clones or were sham injected with the parasite-free diluent to act as controls ($n = 56$). From the first day of injection (day 0), daily measurements of weight and anemia (red blood cell density) were taken from all mice. Red blood cell density was estimated by diluting a 2-μl sample of tail blood in 80 ml of Isoton (Beckman-Coulter, High Wycombe, U.K.) and running the resulting solution through a coulter cell 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 (gametocytemia). 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 parasite density from day 5 to day 17 (few parasites detected before day 5). One mouse in the AJ treatment did not develop any parasites after being injected and was eliminated from the analysis.

Measures of Parasite Virulence in Mice

The proportion of mice that died during the course of acute infection (days 0–14 postinoculation) were computed for each parasite clone. Additionally, four measures of virulence were defined for each mouse that survived until the time of blood feeding: maximum percentage weight loss from weight on day 0, maximum percentage 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 between days 1–16 postinoculation), and cumulative total red blood cell loss (sums of difference between starting red cell density and red cell density during each day of the infection, days 1–16). The latter two measures were selected to reflect infection chronicity, as their value increases with the time taken for an animal to regain normal weight and red cell density.

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 (see Ferguson and Read 2002a). Groups of 280 similarly aged pupae were placed in each of 48 net-covered emergence cages (16 × 16 × 16 cm). 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 43 of the original 56 mice were available for blood feeding ($n_{day16} = 23$, $n_{day17} = 20$). 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 h before being allowed to blood feed on mice. One anaesthetized mouse was placed on each cage, and mosquitoes were allowed to feed for 20 min (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 × 2.5 cm) covered with mesh. These mosquitoes were used in the fecundity experiments and the remaining mosquitoes were used for survival experiments (see below).

Prior to blood-feeding, mosquitoes in one cage had a very poor emergence rate and exceedingly high mortality, yielding less than half of the average number of mosquitoes that emerged in the other cages. We decided to proceed with the infection of mosquitoes in this cage (with the AQ clone), after which atypical mortality continued (mortality by day 9: this cage = 100%, all other infected cages = 11.2%, SE = 1.9%). We believe mosquitoes in this case were predisposed to high mortality before the application of the infection treatment, possibly as a result of bacterial contamination in the pupal bowl, and their data were discarded from these analyses.

Mosquito Survival

On each blood 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). With the exception of the ER-clone, there were at least two replicates of each parasite clone and glucose water treatment combination. Because 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). In 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 dissected to assess oocyst presence and their wings measured as an index of body size (see Ferguson and Read 2002a). Survival monitoring was terminated 45 days after the blood feed, when all remaining survivors were killed, counted, and measured.

Blood Meal Size and 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 three-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, Gillingham, U.K.). Solutions with an absorbance of less than or equal to 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.

After the three-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 (days 4–6 postinfection). Mosquitoes were subsequently moved into new tubes for one to two days (days 7–8 post blood feeding) before being killed with chloroform, examined for oocysts, and having a wing measured.

Statistical Analysis

The two aims of our statistical analysis were to investigate the relationship between the virulence of *P. chabaudi* infection in mice and their transmission to mosquitoes and to test whether the virulence of *P. chabaudi* infections in mice is correlated with their virulence in mosquitoes. The four measures of parasite virulence in surviving mice (maximum percentage weight loss, maximum percentage red blood cell density loss, cumulative total weight loss, and cumulative total red blood cell density loss) were combined in a principal components analysis to produce one representative measure of mouse infection virulence (Eisen and Schall 2000; Mack-

innon and Read 2003). Data collected from mice that died before day 16 postinfection were not included in this analysis.

Relationships between mouse infection virulence score and three measures of parasite transmission: total gametocyte production over days 5–17 (defined above), oocyst infection rate and mean oocyst burden (number of oocysts per infected mosquito) were examined using General Linear Models (GLM; SPSS 1995). Associations between parasite virulence and infectivity were tested both at a phenotypic (across all infections) and genetic level (across all parasite clones). Genetic correlations between parasite virulence and infectivity were tested by assessing the correlation between mean clone virulence and mean clone values of all transmission traits (total gametocyte density, mosquito infection rate, and oocyst burden). All mean clone values were adjusted for feed-day effects prior to analysis. This was done by using the least square means value of transmission traits for each clone (obtained from a statistical model that included feed-day as a covariate), and not the uncorrected raw means, in the correlation.

The virulence of *P. chabaudi* infections in mosquitoes was assessed by their survival and fecundity. Two measures of survival were computed: median survival time per blood feed and the proportion of mosquitoes in each cage surviving until day 14 after each blood feed. Mosquitoes are vertebrate-infective after 14 days, when the *Plasmodium* sporozoites are fully developed and present in mosquito salivary glands. Both measures were obtained from Kaplan-Maier estimates of the survival distribution in each cage (SPSS 1995). We used GLM to test 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, day of blood feed (16 or 17), and glucose water treatment (SPSS 1995). Because the main effect of parasite clone and infection status cannot be tested simultaneously, two separate GLM models were tested for each survival measure that contained either infection status or clone, plus the remaining three main effects. Maximal models included all main effects and their interaction with blood-feeding day; nonsignificant 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 six additional infection explanatory variables we collected (gametocyte density on feed-day, oocyst infection rate, mean oocyst burden, anemia, mean blood meal size, and mosquito body size) could explain variation in vector survival or fecundity, either when considered independently or added to the minimum statistically significant model of main treatment effects. Two types of phenotypic correlations were calculated: relationships across all infections and relationships across infections within a clone treatment (where the effect of clone was significant). Genetic correlations between parasite virulence in mice and mosquitoes were conducted by correlation analysis of mean clone virulence and mean survival time and fecundity of the mosquitoes they infected. As with our analysis of genetic correlation between transmission traits, all mean clone values of mosquito fitness traits were adjusted for feed-day effects prior to correlation analysis.

Before analysis, all proportion data (gametocytemia, parasitemia, mosquitoes that laid eggs, mosquitoes surviving until day 14, mosquitoes infected) were arcsine–square root transformed to improve their fit to a normal distribution, and values of total gametocyte density, gametocyte density on the day of blood feeding, mean oocyst burden, and maximum asexual parasite density were log transformed. Throughout, means ($\pm$ SE) are reported.

Results

Virulence in Vertebrate Host

Plasmodium chabaudi infection induced substantial morbidity and mortality in mice (Fig. 1). Over the 16-day monitoring period, infected mice lost 3–30% of their initial body weight and 27–87% of their red blood cells. Eleven of the initial group of 48 infected mice died within two weeks of infection (Table 1). Six of these 11 were euthanized because, based on experience, they appeared likely to die within the following 24 h. All deaths occurred between days 8 and 16 postinfection. The proportion of mice that died during infection varied between parasite clones ($\chi^2_6 = 13.11$, $P = 0.04$, Table 1), as did the severity of symptoms in surviving mice (Table 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 ($P < 0.01$ in both cases).

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 percentage weight loss $r = 0.92$, maximum percentage 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 ($r = 0.65$, $P = 0.11$, Fig. 2a), suggesting that this morbidity measure (PC1) also represents mortality risk.

Virulence in Vertebrate Hosts and Transmission to Vectors

We observed a general tendency for measures of parasite transmission (oocyst infection rates and burden) to rise with the clone's virulence rank (Figs. 2b–c). One consistent exception to this trend was the BC clone, which was highly virulent but produced few gametocytes. We believe this clone may be an outlier: 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 1999a) 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. All analyses of virulence and transmission were conducted both with and

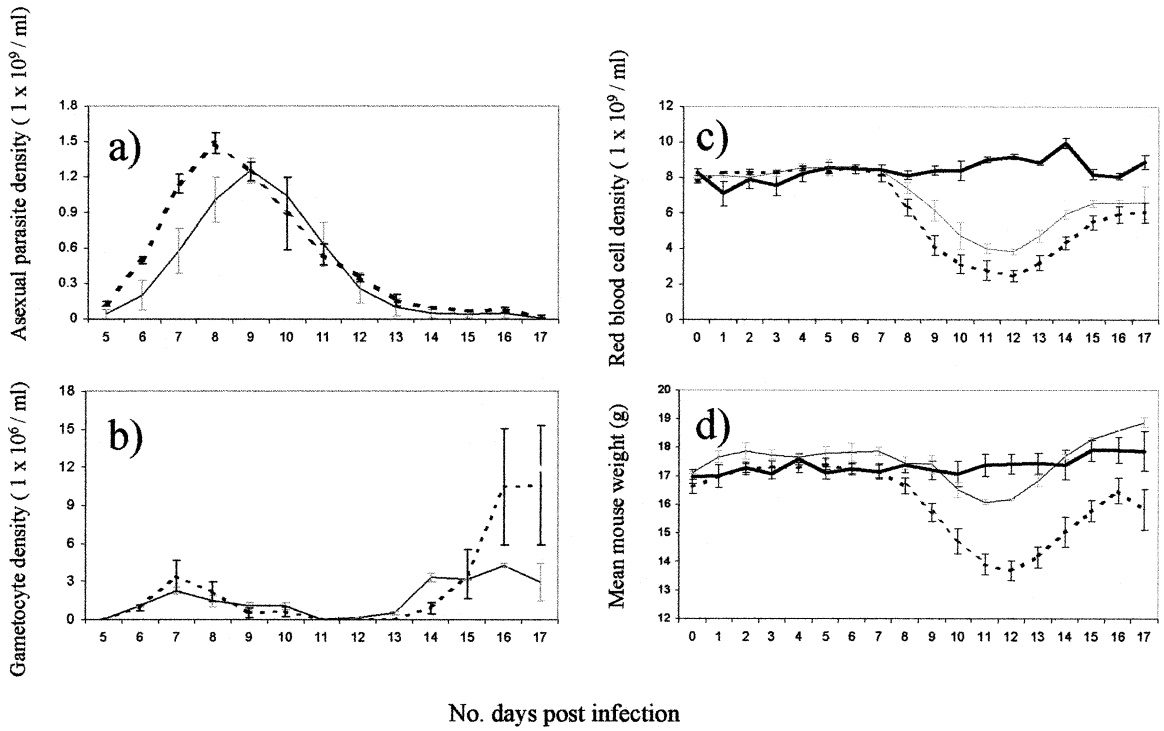

FIG. 1. Kinetics of asexual parasite density (a), gametocyte density (b), red blood cell density (c), and weight (d) in laboratory mice infected with clones of *Plasmodium chabaudi* that differ in virulence. Solid, thick black lines give data for uninfected control mice (c, d). The thin solid lines give the mean dynamics for the two low virulence clones (AS and CW), and the dotted lines give the mean dynamics for clones with medium to high virulence (AJ, AQ, BC, CR, and ER). Data on individual clones are given in Table 1.

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.

In surviving mice, total gametocyte production varied across parasite clones ($F_{6,28} = 7.11$, $P = 0.01$). Including data from mice that died prior to blood feeding did not qualitatively change the relationships between parasite clones ($F_{6,41} = 2.98$, $P = 0.02$), probably because mice that died prior to blood feeding did not have a lower total gametocyte production than those that survived ($F_{1,46} = 1.50$, $P = 0.23$). Thus, clones producing the most gametocytes in survivors retained an overall advantage even when the potential cost of death was considered.

Across all infections, there was no relationship between total gametocyte production from days 5 to 17 and virulence ($F_{1,34} = 0.61$, $P = 0.44$; without BC: $F_{1,29} = 2.03$, $P = 0.17$). There was a tendency for total gametocyte production to rise with virulence across infections within clones ($F_{1,28} = 3.49$, $P = 0.07$). There was no significant genetic correlation between virulence and total gametocyte production, with the mean virulence of each clone being unrelated to total gametocyte production (with BC: $R = 0.11$ $P = 0.82$; without BC: $R = 0.34$, $P = 0.51$). Similar results were obtained when the data was grouped into virulence categories (high: AJ, AQ, BC, CR, ER; low: AS, CW). Although gametocyte densities during the last two days of infection (days 16, 17) tended to be greater in the high-virulence category than in the low-virulence category (Fig. 1), overall there was no significant difference in the total gametocyte production of these two groups ($F_{1,34} = 0.39$, $P = 0.54$).

A higher proportion of mosquitoes became infected from blood feeds on day 16 than from those on day 17 (43.4% vs. 27.2%, $F_{1,32} = 6.52$, $P = 0.02$), probably due to the higher gametocyte densities of infected mice on which mosquitoes fed on day 16 ($F_{1,34} = 4.05$, $P = 0.05$). Accounting for this variation between days, there was a positive phenotypic relationship between mouse virulence score and mosquito infection rate ($F_{1,32} = 5.37$, $P = 0.03$; Fig. 2b). A positive genetic correlation was evident between mean virulence of each clone and mosquito infection rate, but this was only statistically significant when the BC clone was excluded (with BC: $R = 0.59$, $P = 0.17$; without BC: $R = 0.89$, $P = 0.02$; Fig. 3).

Mean parasite load in mosquitoes (oocyst burden) varied between clones ($F_{6,28} = 4.21$, $P < 0.01$). Across all infections, there was a tendency for mean oocyst burden to increase with infection virulence, but not significantly so ($F_{1,33} = 2.68$, $P = 0.11$; Fig. 2c). Similarly, at a genetic level, the mean virulence of each clone showed a positive yet nonsignificant association with mean oocyst burden (all clones: $R = 0.51$, $P = 0.25$; without BC: $R = 0.71$, $P = 0.11$).

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

TABLE 1. Mean value of virulence and parasite abundance traits for mice infected with different clones of *Plasmodium chabaudi*. Superscripts that differ indicate that the trait was significantly different between clones (Bonferroni adjusted for multiple comparisons, $P < 0.05$). Values were computed only for mice that survived. For maximum weight loss and minimum red blood cell density, the analysis controlled for variation in the starting value of the trait on day 0 (values are least squares adjusted means). Pooled SE gives the standard error of clone values from a common mean, P gives the significance level for the test that the trait differed between clones, and R^2 is the coefficient of determination. The overall virulence score (PC1) is based on a principal components analysis of virulence traits: the higher the PC1 score, the more virulent the clone is in mice.

Trait	Parasite clone									
	AJ	AQ	AS	BC	CR	CW	ER	Uninfected	Pooled SE	P	R^2
% Mortality	33	43	14	28	0	0	57	0			
Maximum parasitemia	$34.2^{a,b}$	$36.3^{a,b}$	28.5^{b}	41.5^{a}	43.1^{a}	24.0^{b}	$36.8^{a,b}$	—	0.02	<0.01	0.53
Day of maximum parasitemia	10.0	9.3	9.8	9.0	10.9	9.9	8.7	—	0.23	ns	—
Maximum parasite density (1×10^9/ml)	$1.96^{a,b}$	$1.67^{a,b}$	$1.77^{a,b}$	2.04^{a}	$1.72^{a,b}$	1.26^{b}	$1.95^{a,b}$	—	0.07	0.04	0.22
Day of maximum asexual density	9.0	7.8	9.3	8.4	9.3	9.1	8.0	—	0.19	ns	—
Total asexual density (1×10^9/ml) × day	6.74^{a}	$5.69^{a,b}$	$5.19^{a,b}$	$6.03^{a,b}$	6.66^{a}	4.77^{b}	7.30^{a}	—	0.20	<0.01	0.42
Maximum weight loss (g)	3.21^{a}	3.90^{a}	1.66^{b}	3.14^{a}	2.97^{a}	1.40^{b}	3.33^{a}	0.17^{c}	0.22	<0.01	0.75
Minimum red blood cell density (1×10^9/ml)	$2.10^{a,b}$	2.01^{a}	3.60^{b}	1.97^{a}	$2.21^{a,b}$	$3.25^{a,b}$	1.74^{a}	6.32^{c}	0.26	<0.01	0.77
Cumulative total weight loss (g × day)	$9.52^{a,b,c}$	$15.22^{a,b}$	$-6.14^{b,c}$	$6.62^{a,b,c}$	$8.03^{a,b,c}$	$-3.69^{b,c}$	$5.15^{a,b,c}$	$-6.12^{b,c}$	1.62	<0.01	0.41
Cumulative total red blood density loss (1×10^9/ml × day)	$23.70^{a,b,c}$	$27.35^{a,b,c}$	$20.82^{a,b,c}$	$31.03^{a,b}$	$34.97^{a,b}$	$23.84^{a,b,c}$	$36.63^{a,b}$	$-1.95^{b,c}$	2.83	<0.01	0.33
Overall virulence score (PC1)	$0.43^{a,b}$	0.89^{a}	-1.05^{c}	$0.48^{a,b}$	$0.39^{a,b}$	$-0.87^{b,c}$	$0.66^{a,b}$	—	0.17	<0.01	0.46

FIG. 2. Phenotypic and genetic relationship between the virulence score of *Plasmodium chabaudi* infections in surviving mice and (a) the proportion of mice that died, (b) infection rate in mosquitoes, and (c) mean oocyst burden in mosquitoes. Small black symbols represent individual infections (phenotypic correlations), and large symbols are the mean trait value of each parasite clone, pooled across feed-days. Regression lines are presented when there were significant phenotypic relationships across infections. In (b), the phenotypic relationship was significant within each feed-day (dark line, day 16; light line, day 17). All error bars are standard errors except where indicated on the *y*-axis (a).

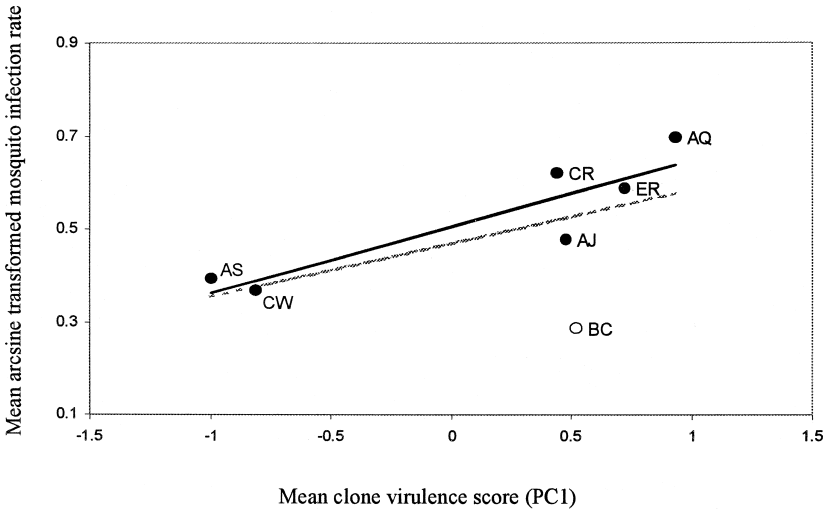

FIG. 3. Genetic correlations between the virulence of *Plasmodium chabaudi* clones in mice and their mean infectivity to mosquitoes. The relationship between mean clone virulence and infectivity was statistically significant only when day the BC clone was excluded from analysis (association shown by black line, nonsignificant trend across all clones shown by gray, broken line). Mean clone values of virulence and infectivity have been adjusted for feed-day effects.

experiment, with the average median survival (across cages) being 24.2 days (range = 13–33 days). A total of 520 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 (Figs. 4a, b). The mean virulence score associated with each clone in mice was not correlated with their effect on mosquito median survival or the proportion surviving until day 14 (median survival: $R = 0.48$, $P = 0.28$; proportion surviving until day 14: $R = 0.13$, $P = 0.78$). Similarly, across infections (phenotypic correlation), virulence score (PC1) could not explain significant variation in mosquito survival (median survival: $F_{1,32} = 0.93$, $P = 0.34$; proportion surviving until day 14: $F_{1,32} = 0.01$, $P = 0.93$). In no case did the effect of mouse virulence on vector survival vary between feed-days (feed-day × median survival interaction not significant for both median survival and proportion surviving until day 14), nor did the exclusion of the BC clone qualitatively change these results. Furthermore, the explanatory power of virulence for mosquito survival did not increase when infection rate was included as an additional predictor variable (significance of virulence in a model including infection rate: median survival: $F_{1,31} = 1.94$, $P = 0.17$; proportion surviving until day 14: $F_{1,31} = 0.31$, $P = 0.58$).

Other Predictors of Vector 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 impact of *P. chabaudi* infection on mosquito survival varied with glucose-water treatment. 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: $F_{1,37} = 3.78$, $P = 0.06$; Fig. 5).

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 ($F_{6,27} = 2.65$, $P = 0.04$; Fig. 6) and mean oocyst burden ($F_{1,27} = 6.43$, $P = 0.02$; Fig. 7a). 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 (feed-day × oocyst burden interaction: $F_{1,25} = 6.46$, $P = 0.02$), appearing to be more crucial for mosquitoes infected on day 16 than day 17 (day 16: $P = 0.01$, day 17: $P = 0.32$; Figs. 7b, c). This discrepancy between days may be due to the fact that oocyst burdens were generally higher from feeds on day 16 than day 17 ($\text{mean}_{16} = 23.0 \pm 7.8$ vs. $\text{mean}_{17} = 11.21 \pm 4.7$; $F_{1,33} = 3.73$, $P = 0.06$).

Plasmodium Virulence in Vertebrate Hosts and Vector Fecundity

A total of 320 of 395 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. When BC was included in the analysis, there were no relationships between the mean fecundity of infected mosquitoes and mouse virulence score (across infections: $F_{1,33} = 0.27$, $P = 0.61$; across clones: $F_{6,28} = 1.27$, $P = 0.30$). However, in the absence of BC, there were significant positive relationships between virulence and fecundity within clone groups (effect of virulence controlling for clone differences: $F_{1,23} = 3.87$, $P = 0.06$; effect of clone controlling for virulence: $F_{6,23} = 3.58$, $P = 0.05$; Fig. 4c). This phenotypic relationship between virulence and fecundity was unaffected by feed-day (feed-day main effect and its interaction with virulence not significant both with and with-

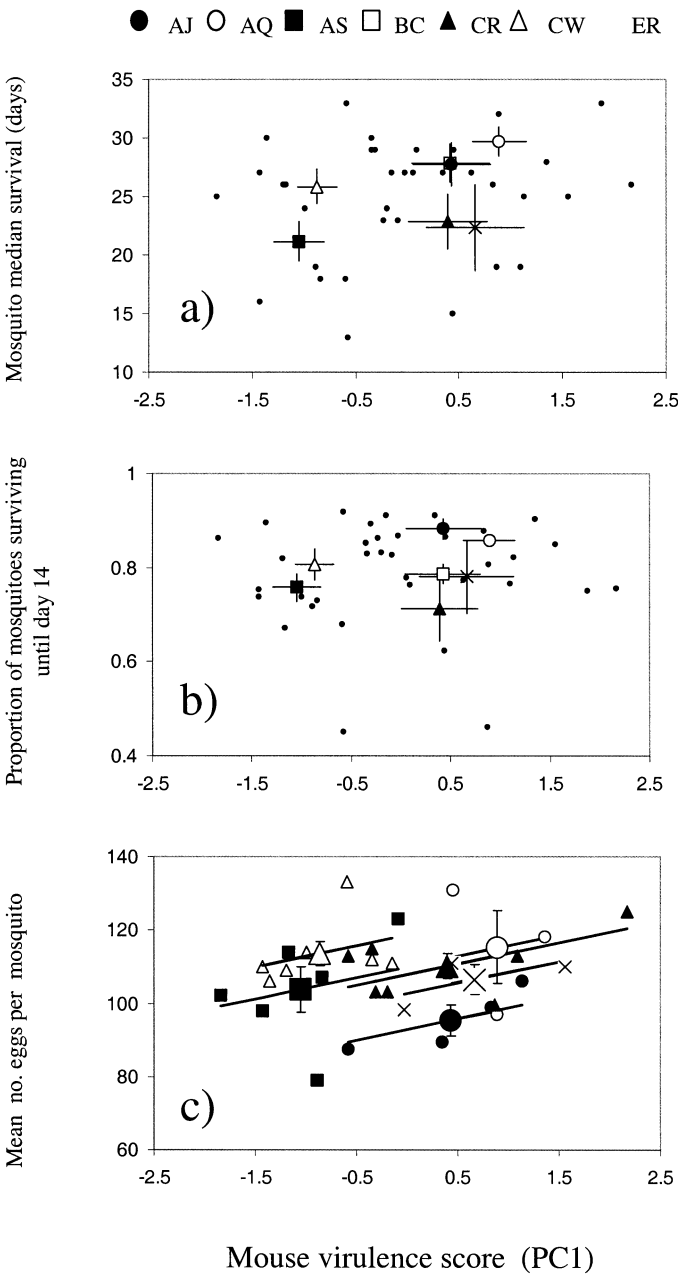

FIG. 4. Genetic and phenotypic relationships between *Plasmodium chabaudi* virulence in mice and three measures of vector fitness: (a) mosquito median survival, (b) the proportion of mosquitoes surviving through the first 14 days of infection, and (c) mosquito fecundity (with BC clone excluded). In the survival graphs (a, b), black dots represent the survival of a group of 60–80 mosquitoes. Regression lines are shown only when there was a statistically significant association between virulence and vector fitness (see text for details). In the fecundity graph (c), black dots represent the mean fecundity of a group of 10 mosquitoes. The large symbols are the mean values for each parasite clone, pooled across feed-days.

out BC). There was no genetic correlation between clone virulence and mosquito fecundity ($R = 0.02$, $P = 0.98$). The statistical significance of virulence to mosquito fecundity was not increased by the inclusion of oocyst infection rate as an additional parameter.

Other Predictors of Vector Fecundity

The role of blood meal size on fecundity differed between infected and uninfected mosquitoes (blood meal size × infection status interaction: $F_{1,38} = 5.53$, $P = 0.02$). The fecundity of uninfected mosquitoes increased with blood meal size, but that of infected mosquitoes did not (Fig. 8). Among infected mosquitoes, none of the nine explanatory variables we measured were related to fecundity when the BC clone was included ($P > 0.25$ in all cases). When the BC clone was excluded, then the minimal model for fecundity included both clone and virulence (which showed a positive relationship, $P = 0.06$; Fig. 4c).

DISCUSSION

Vector Survival

This study confirms that virulence increases parasite fitness in the *P. chabaudi* system: as found previously (Mackinnon and Read 1999a), infections that cause the greatest pathology in laboratory mice have the highest transmission success to vectors. Parasite clones that induced the greatest virulence in mice in terms of weight loss and anemia had higher densities of asexual parasites during the early phase of infection and gave rise to higher infection rates in mosquitoes (Figs. 1, 2b, 3). There was little evidence, however, that this transmission benefit of virulence in mice could be offset by increased mortality in mosquitoes. Neither the virulence of individual infections nor the average disease severity induced by different parasite clones could explain variation in mosquito survival (Figs. 4a, b). There was some evidence of a possible indirect relationship between parasite virulence in mice and mosquito survival: infection virulence in mice tended to increase oocyst burden in mosquitoes, which in turn reduced vector survival. However, the association of virulence in vertebrates to mosquito oocyst burden was relatively weak, so there was no overall correlation between parasite virulence in the two hosts. These results provide little evidence that the evolution of increased virulence in malaria is restricted by vector mortality.

Although there was no relationship between virulence in the vertebrate host and vector survival, *Plasmodium* infection nonetheless affected vector survival. Both parasite infection and parasite clone were predictors of mosquito survival. Pooling across feed-days, the difference in median survival between mosquitoes with the most and least lethal clone to mosquitoes (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 two to three days (Gillies 1953), the loss of three or four transmission opportunities. The clone effects we report 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 we observed could influence not only the frequency of transmission events but also the probability of transmission occurring at all. If replicated in a natural transmission setting, mortality effects of this nature and magnitude could have sizeable effects on malaria epidemiology and evolution.

The survival consequences of parasite clone were more

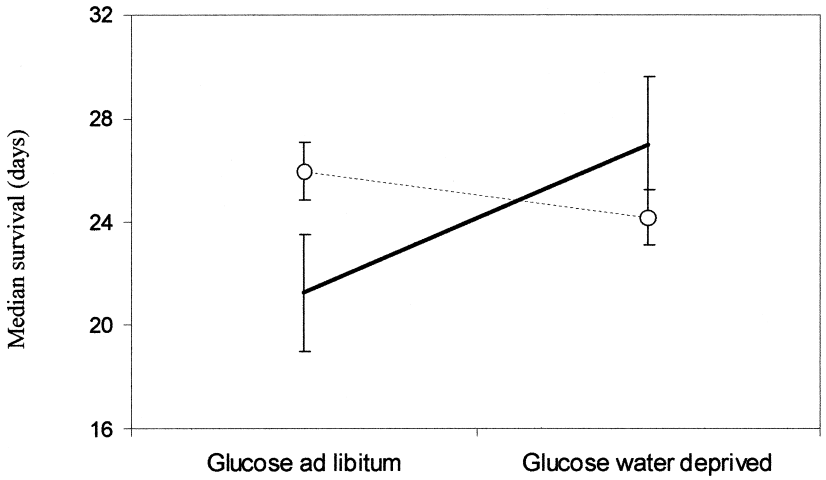

FIG. 5. The mean value of the median survival of *Plasmodium chabaudi*-exposed and -unexposed mosquitoes under two different glucose water regimes. Plotted points are least squares means (± SE) controlling for difference in day of feed (glucose × infection × day interaction, $F_{1,34} = 0.25$, $P = 0.62$). Solid lines are for unexposed mosquitoes, dotted lines are for *P. chabaudi*-exposed mosquitoes (pooled across parasite clones).

direct than those associated with simple infection status. 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 uninfected individuals was also poorer when glucose water was provided freely (Ferguson and Read 2002a). 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). 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 for different parasite clones.

Vector Fecundity

Plasmodium virulence in vertebrates did influence one key component of vector fitness: fecundity. Accounting for variation between parasite clones, the number of eggs laid increased with the virulence of the mouse infection (Fig. 4c). This 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

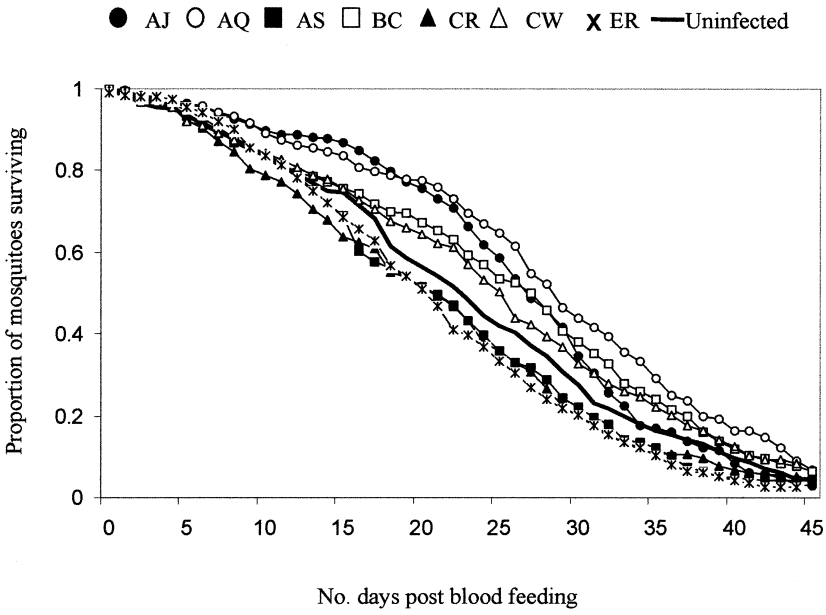

FIG. 6. Survival curves of mosquitoes infected with different clones of *Plasmodium chabaudi*. Each curve represents the mean survival across all cages infected with the same parasite clone.

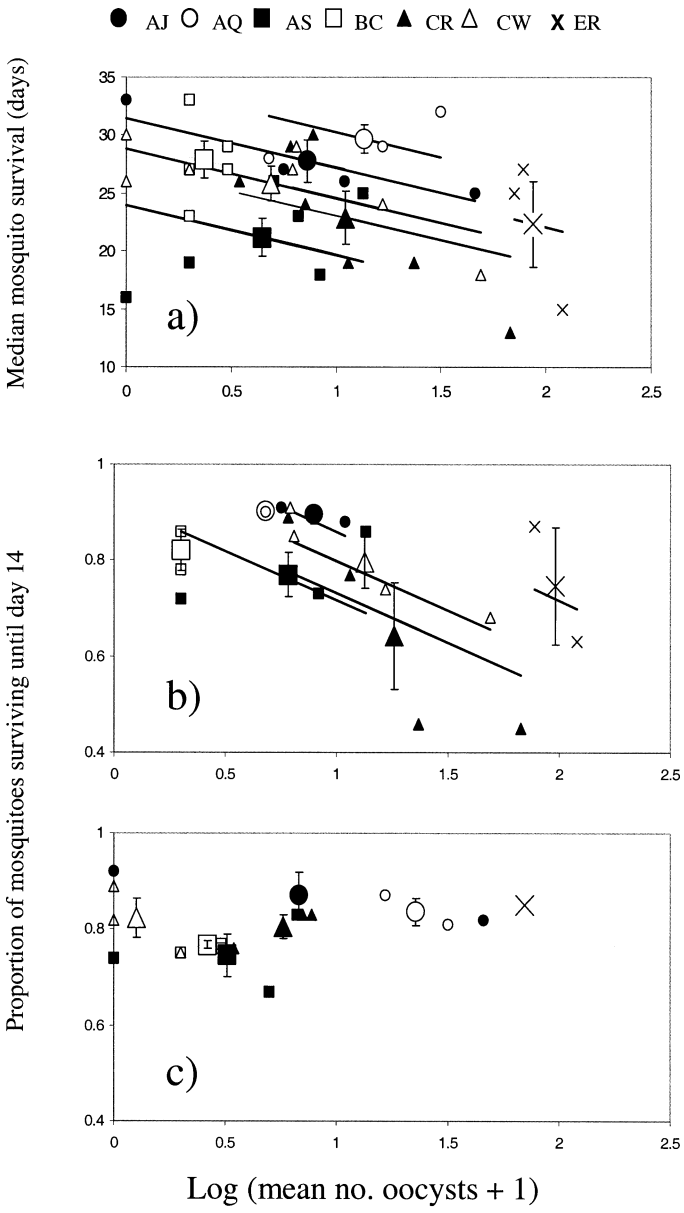

FIG. 7. Relationships between *Plasmodium chabaudi* oocyst burden in mosquitoes and their survival: (a) median survival, (b) the proportion of mosquitoes surviving until day 14 from day 16 mouse infection blood feeds, and (c) the proportion of mosquitoes surviving until day 14 from day 17 mouse infection blood feeds. Regression lines indicate a significant effect of oocyst burden on survival (details in text). Small symbols represent the survival of a group of 60–80 mosquitoes after feeding on one mouse, large symbols are clone means.

that were the most virulent were associated with small blood meals. The most likely explanation is blood meal quality. 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 examinations of the importance of host blood components to vector fecundity and their variation under mild and severe malaria infections would be of great interest. These differences could also explain the clone differences in mean fecundity, which varied by approximately 18% between clones with smallest and greatest effect on egg production (AQ and AJ, respectively).

Like its effect on 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 (Fig. 7). This has also been documented in *P. yoelii nigeriensis* infections of *A. stephensi* (Hogg and Hurd 1995b). As Hogg and Hurd (1995b) pointed out, it seems unlikely that this disruption is due to competition for blood meal resources between *Plasmodium* and mosquitoes, as there is no evidence that parasite load 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).

Conclusions: The Evolution of Plasmodium Virulence

This study found no evidence of a positive relationship between the virulence of *Plasmodium* infections in their vertebrate and vector hosts. Thus, we have no evidence 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 in an animal model in controlled laboratory conditions. *Plasmodium* is known to alter mosquito feeding behavior (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 behavior. In the case of feeding behavior, these alterations have been associated with increased mortality of infected mosquitoes (Anderson et al. 2000). Neither of these two sources of mortality was taken into account in this experiment, and thus their relationship to infection virulence in vertebrates is unknown.

Another factor that may have obscured detection of a relationship between *Plasmodium* virulence in its two hosts is that our measure of the cost of parasitism in mosquitoes may be overly conservative. The survival and fecundity estimates for mosquitoes in infected treatment groups included individuals that did not have oocyst-stage parasites, some of whom may never have actually been infected. It is difficult to quantify how much error this generated because, as discussed previously, we do not know whether oocyst-free mosquitoes were truly uninfected (i.e., never invaded by earlier stages of the parasite that failed to develop). *Plasmodium* numbers can diminish by 100-fold and greater between the early gamete and oocyst stage (Vaughan et al. 1992). Ex-

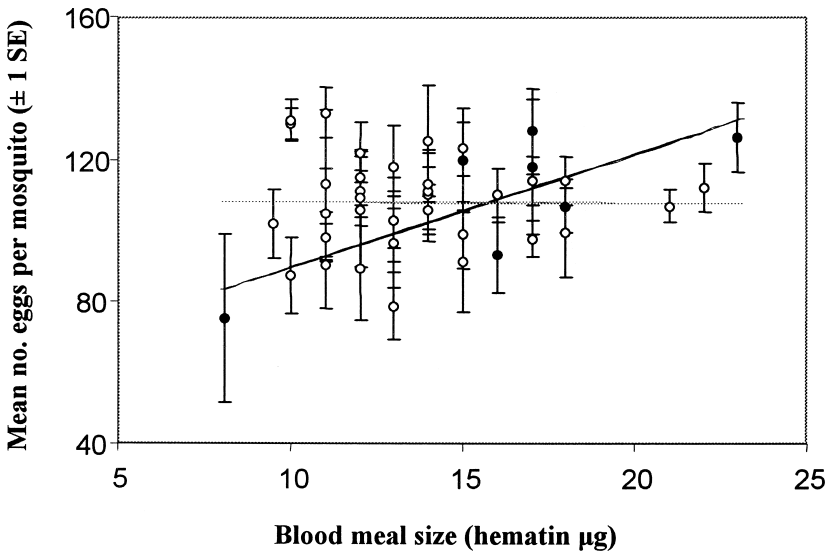

FIG. 8. The relationship between blood meal size and fecundity in mosquitoes fed uninfected mouse blood (closed circles, black regression line) and mosquitoes fed *Plasmodium chabaudi* infected blood (open circles, gray regression line). Bars represent one standard error. The slope of the relationship 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 ($P = 0.92$).

trapolating backward, our oocyst rate of 35.8% suggests that the majority of mosquitoes in infected groups would have had *Plasmodium* gametes and/or ookinetes. Even if this is not the case, the inclusion of exposed but uninfected mosquitoes should not bias our results in a direction that would obscure a positive association between virulence in vertebrate and vector hosts. This is because infection rates were highest in mosquitoes that had fed on mice with virulent infections. If developing an oocyst is more detrimental to a mosquito than simply feeding on infected blood, then mortality estimates from mosquitoes feeding on mice with virulent infections should be less underestimated than those from mosquitoes feeding on mice with low virulence infections, where the lower infection rate would have led to a greater underestimation of mortality. Thus, if present, any such sampling bias should predispose our analysis toward a positive relationship between virulence in the mouse and mosquito, not conceal it. Finally, our estimates of virulence unambiguously test whether parasite-induced changes in mosquito blood meal quality could generate selection against virulence in the vertebrate host of *Plasmodium*. Our results indicate this is unlikely.

Assuming our results do fairly reflect the natural situation, where does this leave us with respect to understanding the limits to malaria virulence in nature? We currently consider it most likely that malaria virulence evolution is being capped by factors such as vertebrate host death and/or the diversity of host genotypes (e.g., sickle-cell trait and blood group; Miller 1988; Riley 1996; Miller et al. 2002) or the availability of resources within hosts such as red blood cells (Mackinnon et al. 2002). Nonetheless, vectors may be an important determinant of virulence in vertebrates, even if not via their survival. For example, we do not know whether virulent and avirulent parasite clones produce sporozoites with the same efficiency or 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, the nature of selection acting on *Plasmodium* virulence in vectors remains to be understood. Conventional wisdom that malaria is avirulent to its vector is clearly wrong (Figs. 3–6).

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