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GENETIC RELATIONSHIPS BETWEEN PARASITE VIRULENCE AND TRANSMISSION IN THE RODENT MALARIA *PLASMODIUM CHABAUDI*

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Abstract.—Many parasites evolve to become virulent rather than benign mutualists. One of the major theoretical models of parasite virulence postulates that this is because rapid within-host replication rates are necessary for successful transmission (parasite fitness) and that virulence (damage to the host) is an unavoidable consequence of this rapid replication. Two fundamental assumptions underlying this so-called evolutionary trade-off model have rarely been tested empirically: (1) that higher replication rates lead to higher levels of virulence; and (2) that higher replication rates lead to higher transmission. Both of these relationships must have a genetic basis for this evolutionary hypothesis to be relevant. These assumptions were tested in the rodent malaria parasite, *Plasmodium chabaudi,* by examining genetic relationships between virulence and transmission traits across a population of eight parasite clones isolated from the wild. Each clone was injected into groups of inbred mice in a controlled laboratory environment, and replication rate (measured by maximum asexual parasitemia), virulence (measured by live-weight loss and degree of anemia in the mouse), and transmission (measured by density of sexual forms, gametocytes, in the blood and proportion of mosquitoes infected after taking a blood-meal from the mouse) were assessed. It was found that clones differed widely in these traits and these clone differences were repeatable over successive blood passages. Virulence traits were strongly phenotypically and genetically (i.e., across clones) correlated to maximum parasitemia thus supporting the first assumption that rapid replication causes higher virulence. Transmission traits were also positively phenotypically and genetically correlated to parasitemia, which supports the second assumption that rapid replication leads to higher transmission. Thus, two assumptions of the parasite-centered trade-off model of the evolution of virulence were shown to be justified in malaria parasites.

Key words.—Malaria, *Plasmodium chabaudi*, transmission, virulence.

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The harm done by infectious organisms to their hosts (virulence) is a problem confronted daily, either because of our own afflictions or because of the health of animals we rely upon for food, fiber, or company. Darwinian medicine, the application of evolutionary theory to disease control (Ewald 1980, 1994; Williams and Nesse 1991; Read et al. 1998), has promised much for the understanding of virulence and spread of pathogens, but there are very few data to support any of the numerous models on which this philosophy is based. In particular, there is a paucity of information on the within-species genetic relationship between parasite fitness and virulence, which is defined here as the reduction in host fitness caused by the parasite. Because many models assume this is the key relationship driving the evolution of virulence, it is important to evaluate genetic correlations between virulence and transmission in major disease-causing organisms.

Several classes of models have been proposed to explain why parasites are generally virulent (reviewed by Ewald 1983, 1994; Bull 1994; Levin and Bull 1994; Read 1994; Ebert and Herre 1996; Frank 1996). The favored hypothesis is that greater within-host replication is associated with higher between-host transmission; because transmission is a direct determinant of parasite fitness, high replication rate is thus expected to be favored by natural selection. However, one cost of this rapid replication may be higher virulence, with parasite transmission being reduced by host death. This view is sometimes called the "adaptive trade-off" hypothesis: Natural selection should optimize parasite replication rates in the face of a trade-off between higher transmission and increased risk of no transmission due to the unavoidable side-effect of greater replication, that is, virulence (Bremermann 1980; Levin and Pimentel 1981; Anderson and May 1982; Bremermann and Pickering 1983; Ewald 1983; May and Anderson 1983).

Some empirical support for the trade-off hypothesis has been found in viruses of rabbits and birds (Fenner et al. 1956; Swayne et al. 1996), bacteria in mice and termites (Greenwood et al. 1936; Anderson and May 1982; Jones et al. 1996), protozoans of guinea pigs and cattle (Diffley et al. 1987; Matsui et al. 1996), microsporidians of the water crustacean *Daphnia* and mosquitoes (Ebert 1994; Agnew and Koella 1997), phage and plasmids of bacteria (Bull et al. 1991; Turner et al. 1998), and various fungal, viral, and bacterial pathogens of plants (reviewed by Power 1992). However, there are just as many examples that do not fit this hypothesis (reviewed by Lipsitch and Moxon 1997), and large systematic studies within species, especially of parasites of valuable mammals, have not been conducted. Broad patterns across parasite species may also be consistent with trade-off models (Ewald 1983, 1988, 1991, 1994; Herre 1993; Clayton and Tompkins 1994) although interpretations of some of these data are controversial (Read et al. 1998).

However, the trade-off hypothesis has rivals. For example, in some cases pathogens appear to be virulent because they find themselves in inappropriate host tissue and this can cause unusual levels of damage (Levin and Svanborg Éden 1990). Alternatively, virulence may be caused by new mutations that sweep through the within-host population in an epidemic fashion, but that may not ultimately survive because of their detrimental effect on host survival (Levin and Bull 1994). A

TABLE 1. Details on the source of clones of *Plasmodium chabaudi* used in the experiments.

Clone	Isolate	Species found in original isolate	Replicates in which clone was included	No. passages between natural host and replicates 1, 2, 3, 4
AD	2AD1	*P. chabaudi, P. yoelii*	1–4	6, 9, 7, 8
AJ	AJ	*P. chabaudi*	1–4	6, 9, 7, 8
AQ	2AQ	*P. chabaudi, P. yoelii*	1–4	7, 9, 9, 10
AS	2AS	Unrecorded	3–4	—, —, 8, 9
AT	4AT	*P. chabaudi*	1–4	7, 9, 9, 10
BC	1BC	*P. chabaudi, P. yoelii*	1–4	6, 9, 7, 8
CW	2CW	Unrecorded	1–4	6, 9, 6, 7
ER	56L	Unrecorded	3–4	—, —, 5, 6

further reason why the trade-off hypothesis may not always be relevant is that, for some parasites, transmission and disease symptoms in hosts may be independent of parasite replication rate. In their review of empirical data on virulence, Lipsitch and Moxon (1997) highlight some important examples of disease organisms that appear to fit these alternative models.

Thus, it is clear that there is no single explanation for the level of microparasite virulence; this is undoubtedly because parasites and hosts adopt a wide range of strategies for dealing with each other. In this study we examine the adaptive trade-off hypothesis as the evolutionary basis for virulence of one of the major pathogens of humans, the malaria parasite. The overall aim of our experiment was to examine the two fundamental assumptions underlying the trade-off hypothesis, namely, that high replication rate leads to higher virulence and that higher replication leads to higher transmission. This was done using the rodent malaria *Plasmodium chabaudi* infecting inbred laboratory mice, an experimental system often used as a model for the virulent human malaria, *P. falciparum* (Cox 1988). By measuring virulence and transmission of eight parasite clones isolated from the wild, the across-clone genetic relationships between replication rate, virulence, and transmission were estimated.

METHODS

Isolation of Parasite Clones from the Wild

Plasmodium chabaudi isolates were collected from their natural host (*Thamnomys rutilans*) from the Central African Republic in 1969 and 1970 and stored in liquid nitrogen until this experiment began. To obtain clones (lines of parasites derived from a single parasite) from these field samples, we injected each isolate (each from a different host) into groups of 14–30 mice with an inoculum expected to contain an average of one parasite (Beale et al. 1978). This resulted in zero to two mice per group developing an infection (an average of 9%). Assuming a Poisson distribution for the number of parasites infecting the mice, it was highly probable that these successful infections were established by a single parasite to produce a clonal population. The possibility of multiple parasites founding a clone can only be ruled out by extensive genotyping, and this was not done. Only one clone from each isolate, which themselves had previously been shown to be genetically distinct using electrophoretic enzyme analysis (Carter 1978), was allowed in the experimental panel of clones to minimize the chances that clones were genetically identical. Some of the isolates from which clones were derived comprised more than one species of parasite, the predominant one being *P. chabaudi*, with occasional *P. yoelii*. Therefore, after cloning, the species of each clone was confirmed as *P. chabaudi* by morphology (Carter and Walliker 1975).

Because of the need to store enough parasite material for experiments, it was necessary to passage the clones serially during the cloning phase, that is, transfer asexual stage parasites from one mouse to another. This involved mixing 5–10 μl of blood from the tail of an infected mouse (usually before the peak of the infection) with 0.1 ml of citrate saline (0.85% NaCl, 1.5% trisodium citrate) and injecting it into the peritoneal cavity of an uninfected mouse. The number of passages per clone (Table 1) was kept to a minimum to avoid somatic mutations accumulating in the parasites, which may render the parasites unable to transmit through mosquitoes (Wéry 1968), and to minimize the amount of selection that may occur in this artificial laboratory system. One of the clones (ER) was not obtained directly from the isolates in the natural host, but instead was derived from a clone that had been stored in the laboratory of R. Hamers in Belgium between 1969 and 1983 and was then transmitted through mosquitoes in our laboratory. Table 1 summarizes the laboratory history of the clones, including the number of asexual passages between isolation from the wild and use in these experiments.

Mice used during the cloning phase were a mixture of inbred C57BL/6J and outbred MF1 lines. However, there was no confounding of mouse genotype with clone, so that any host genotype-specific selection should have been randomized across clones. Mice were housed at 21°C (± 1°C) and fed on SDS rat and maintenance diet with 0.05% para-aminobenzoic acid (PABA) added to their drinking water to assist with parasite growth (Jacobs 1964).

Experimental Procedures and Measures of Virulence and Transmission

Mice used for the experiments were female inbred C57BL/6J (B and K Universal, Hull, England), aged between five and eight weeks at the beginning of the infection with a maximum range in age of one week within each experimental group. Infections were established by injection of parasites in a volume of 0.1 ml into the peritoneal cavity of the mouse. Inocula were prepared by taking blood from the tail vein of the donor mouse and diluting it in medium suitable for par-

TABLE 2. Summary of the experimental design. Days postinfection on which measurements were made are shown as black boxes.

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

asite maintenance (50% heat-inactivated calf serum, 50% Ringer solution [27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl], with 20 units of heparin/ml mouse blood) to the appropriate concentration for the inoculum size. The required volume of blood was calculated from the blood cell density and parasitemia in donor mice calculated immediately before experimental inoculations.

After infection, virulence and transmission were monitored in individual mice using the following procedures. Parasite replication rate was assessed by counting the proportion of red blood cells (RBC) infected by asexual parasites (parasitemia) on Giemsa-stained thin blood smears. Approximately 500 cells in two to three separate fields of vision on the microscope were counted for each smear. Loss of body weight of the mouse during "crisis," the period of infection when parasitemia maximizes and is then rapidly reduced, was considered to be an indicator of the damage to host fitness caused by the parasite and thus a reasonable measure of virulence. Mice were weighed to an accuracy of 0.01 g at two- to four-day intervals at the beginning and end of infection, and daily during the peak of the asexual infection. Anemia, another measure of virulence, was assessed by measuring RBC density every two to four days in a diluted sample of blood using flow cytometry (Coulter Electronics, Luton, England). Density of gametocytes, the blood stage parasites that are transmissible to mosquitoes, was obtained by counting the number of RBC containing mature gametocytes (distinguishable from asexual parasites by their morphology and presence of pigment as detected by polarized light) in the same thin blood smears used for counting asexual parasites. Between 30 and 100 fields were scanned for each measurement, and the average number of cells per field estimated from the counts of asexual parasites. Infectivity to mosquitoes was assessed by allowing 30–50 one- to three-day-old *Anopheles stephensi* mosquitoes to take a blood meal from anesthetized and immobilized mice between 1900 and 2000 h. Groups of mosquitoes that had fed on the same mouse were stored in individual pots at 25°C and fed on glucose at a concentration of 10% with 0.05% PABA. Mosquitoes that had fed partially or not at all were removed from the pot immediately after the experimental feed. After eight to nine days, the survivors were then killed with chloroform and their midguts dissected to count the number of mosquitoes with oocyst infections. *Anopheles stephensi* is not the natural vector for *P. chabaudi*, but experience with a wide range of *P. chabaudi* clones has shown it to become infected reliably using this experimental feeding procedure.

Experimental Design

The experimental design is summarized in Table 2. Four experimental replicates of infections were performed and measures of virulence and transmission were obtained from individual mice throughout their infections. In replicates 1 and 2, six clones and an uninfected control were used, and in replicates 3 and 4, an additional two clones and no control were used. Four mice per clone were used in each of replicates 1 and 2, and two per clone in each of replicates 3 and 4 giving a total of 12 mice per clone. The sizes of inocula were 10^6, 10^4, 10^5, and 10^5 in replicates 1 to 4, respectively. Parasites for inoculating mice in replicates 1 and 3 were obtained from frozen stocks that had been serially passaged once or twice.

TABLE 3. Definitions, transformations, and summary statistics (mean and standard deviation of raw data, excluding control mice) of traits used in the statistical analysis. PI, postinfection; RBC, red blood cell.

Variable (y)	Definition and units of measurement	Transformation of variable y	No. of mice	Mean	Standard deviation
Virulence					
Maximum parasitemia (%)	Maximum proportion of cells infected with asexual parasites (no./100 RBC)	arcsine $\sqrt{(y/100)}$	73	21.2	10.9
Day PI of maximum parasitemia	Day PI of above	None	73	9.8	2.0
Minimum live-weight (g)	Minimum live-weight reached by mouse around the peak of infection (g)	None	79	18.0	2.2
Day PI of maximum weight	Day PI of above	None	79	10.8	2.4
Minimum blood density ($10^9 \times$ RBC/ml)	Minimum RBC density around peak of infection, i.e., maximum anemia	$\log_{10}(y) - 9$	77	1.54	1.11
Day PI of minimum blood density	Day PI of above	None	77	11.0	2.2
Parasitemia at maximum gametocytemia (%)	Parasitemia on the day of maximum gametocytemia	$\log_{10}(y)$	71	3.8	6.4
Transmission					
Maximum gametocytemia (no./1000 rbc)	Maximum proportion of cells infected with gametocytes	$\log_{10}$ (arcsine $\sqrt{[y/10]}$)	71	0.62	0.82
Day of maximum gametocytemia	Day PI of above	None	71	13.5	2.1
Total no. gametocytes ($\times 10^6$/ml)	Absolute no. of gametocytes produced during the whole infection	$\log_{10}(y)$	62	5.62	7.29
Infectivity (%)	Proportion of mosquitoes infected, adjusted for day of feed and then averaged for each mouse.	Logit	30	17.1	23.4

Parasites for inoculating mice in replicates 2 and 4 were obtained after serial passage one to three times from single mice used in replicates 1 and 3, respectively. For each experimental replicate, clone-groups were inoculated and measured in random order.

In replicates 1 and 2, measurements of virulence were taken more often than in replicates 3 and 4, where transmission was measured more intensively. Measurements of asexual parasitemia, live-weight loss, anemia, and gametocytemia were made on each mouse at approximately the same time each day between 0900 and 1300 h (because these parasites have a synchronous 24-h cycle) at two- or three-day intervals until the infections had almost been cleared by the host's immune system. Assessment of infectivity to mosquitoes took place on days 12 to 15 postinfection (PI) to obtain a measure of total infectivity in the lifetime of an infection; these days correspond to the period during which the bulk of gametocytes are produced in *P. chabaudi* infections (Taylor et al. 1997). In replicates 1 and 2, each mouse was subjected to mosquito feeding only once (two mice per clone on days 12 and 13 PI in replicate 1 and days 14 and 15 PI in replicate 2), and in replicates 3 and 4, each mouse was used twice (one mouse per clone per day on days 12, 13, 14, or 15 PI).

Statistical Analyses: Data Transformations and Clone and Replicate Effects

The traits analyzed fell into two categories, virulence traits (parasitemia, live-weight, anemia) and transmission traits (gametocytemia, infectivity to mosquitoes). (Note that parasitemia is not a measure of virulence per se but instead is a measure of the parasite's replication rate; however it is included among the virulence traits for convenience). Within each category, there were two types of traits, the maximum value (or minimum for weight and red blood cell density) of the trait during the infection and the day on which these maxima/minima occurred, that is, the timing of the infection pattern. Thus, the data under analysis comprised only one measurement per mouse for each trait, rather than repeated measures per mouse. Because some traits were measured two or more days apart, it was probable that in some mice the peak value was not observed. This would cause some inaccuracy in measuring both the levels and timing of the peaks.

Before statistical analysis, the data were transformed to bring the distributions close to normal so that the statistical assumptions of the analyses were justified. This was not necessary for live-weight, nor was it done for infectivity to mosquitoes, which was analyzed taking into account the binomial error structure of the data (see below). It was necessary to apply two transformations for maximum gametocytemia, because the first transformation (arcsine square root) failed to normalize the data. Trait definitions, abbreviations, and their transformations are given in Table 3.

For all traits except infectivity, a linear model with factors for experimental replicate and clone was fitted to the data by least-squares techniques using PROC GLM of the statistical

package SAS (SAS Institute 1990). A clone-by-replicate interaction was not fitted because two of the eight clones were not measured in replicates 1 and 2. For analyses of minimum live-weight and minimum blood cell density, a covariate for the average of the two minimum live-weights (on consecutive days) or the average of two minimum blood densities measured early in the infection (day 0–6) was also fitted to adjust for the mouse's initial value of these variables. Significance levels for each effect were determined by *F*-tests, and least-squares means of clone and replicate effects were computed.

Infectivity to mosquitoes was analyzed by fitting a model with factors for experimental replicate, clone and day of feed (day 12, 13, 14, or 15) and all two-way interactions to data that comprised the number of mosquitoes infected as a proportion of the number of mosquitoes fed on each mouse on each day. This was done using the PROC GENMOD procedure (SAS Institute 1990), which accounts for the binomial error structure of the data using the logit transformation. Clone and interactions with clone were then dropped from the model and the Pearson residuals from this analysis (on the logit scale) were then averaged for each mouse to obtain a single datapoint per mouse, as for other traits. These mouse means were used for the variance component analyses described below.

Statistical Analyses: Repeatabilities and Correlations

For each trait, estimates of the total phenotypic variance (σ_P^2) between-clone variance (σ_C^2) and within-clone variance (σ_E^2) were estimated by maximum likelihood using the PROC MIXED procedure of SAS (SAS Institute 1990). For this, the same models described above were fitted except that clone was fitted as a random effect rather than as a fixed effect. The proportion of total variance attributable to between-clone variance measures the repeatability of the trait. This is the correlation between repeated measures of a clone both within an experimental replicate and across experimental replicates that had been established by asexual passage, that is, with no sexual recombination. The covariance, rather than the variance, between replicates was estimated to allow the between-clone variance to be negative in cases where repeatability was low. For infectivity data, residuals on the logit scale were analyzed.

Estimates of phenotypic correlations (r_P), genetic correlations (r_G) and residual (within-clone) correlations (r_E) among the traits were obtained by performing bivariate analysis of variance, fitting replicate as a fixed effect and clone as a random effect using PROC GLM with the MANOVA and RANDOM options (SAS Institute 1990). As a check, approximate estimates of genetic correlations were also obtained by computing the correlations between the clone least-squares means. In the context of this experiment, where traits were repeatedly measured in experimental replicates established by passage of asexual parasites, residual correlations measure within-clone relationships both between and within experimental replicates. Genetic correlations measure between-clone relationships among traits, and phenotypic correlations measure relationships within mice across clones, after adjusting for means of replicates for both.

Confidence intervals around repeatabilities and correlations were obtained by stochastic simulation. One thousand replicates of the data were simulated according to the actual data structure using estimates from analyses of the real data as parameter values; the analyses described above were run on each replicate. This yielded a distribution around each parameter estimate from which the probability that the bounds on the estimate included zero could be found. No adjustment of significance levels was made for multiple testing because methods for doing so are variable and somewhat unsatisfactory, especially for correlated estimates (Rothman 1990).

RESULTS

Across all experiments, 88 mice were measured for their infection patterns of which eight were uninfected controls. One mouse in the BC clone group failed to develop an infection and so its data were excluded from the analysis. Ten of the infected mice died during the course of the experiment. All of these deaths occurred under anesthetic during the mosquito feeds late in the infection and would probably not have occurred without this stress. An analysis of variance showed that mice that died under anesthetic had significantly higher ($P < 0.05$) parasitemias on the day before death (days 12–19 PI), but not significantly higher weight loss, anemia or parasitemia earlier in the infection. This indicated that the probability of host death (virulence) was correlated to high parasitemia.

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

Typically, maximum parasitemias of 21% were reached on during days 8–10, followed by rapid weight loss and anemia the next two days, after which a peak of gametocytes was reached between days 12 and 16. During this latter phase, parasitemias were around 4%.

Effects of Experimental Replicate and Size of Inoculum

Figure 1 shows live-weights, parasitemia, and gametocytemia over the course of infection averaged for each experimental replicate. On average, peak parasitemias occurred on days 7, 9, and 11 PI for inocula of 10^6, 10^5, and 10^4, respectively, followed by minimum weights one day later, peak anemia (minimum blood cell density) two days later, and peak gametocytemia on days 12, 13, and 14 PI. Thus, a 10-fold reduction in the size of inoculum roughly corresponded to a one- to two-day delay in the course of infection. Care is needed in interpreting these differences, however, because comparisons were not contemporaneous (i.e., inoculum size was confounded with replicate) and were biased by day of sampling, which differed among replicates. Nevertheless, least-squares estimates of replicate effects (Table 4) showed a clear and significant delay in the infection as inoculum size decreased, suggesting that higher establishment rates of the parasite led to more rapid onset of symptoms.

There were no significant differences between replicates ($P > 0.05$) in the amount (rather than timing) of weight loss,

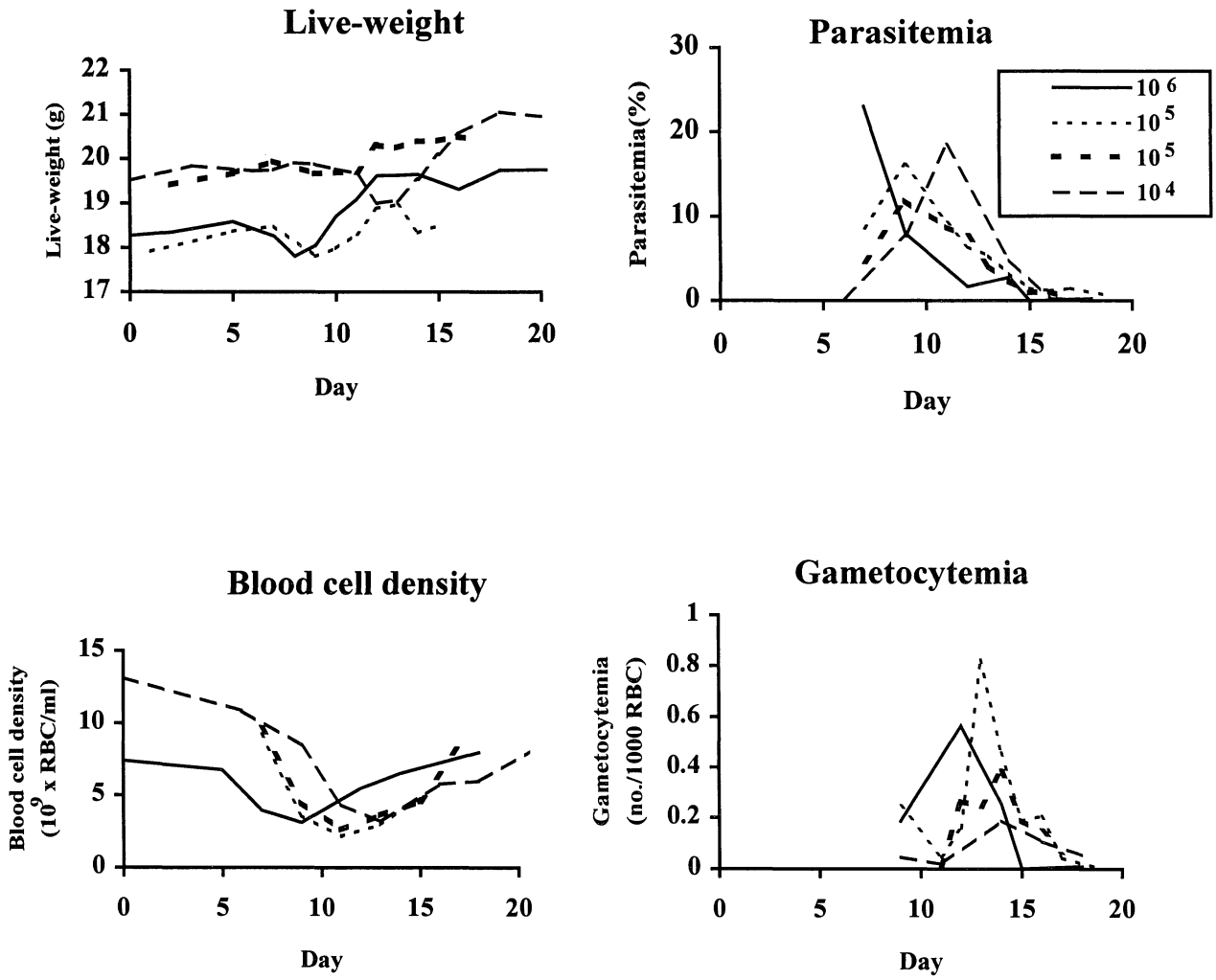

FIG. 1. Average infection patterns of groups of mice infected with *Plasmodium chabaudi* in experiemental replicates started with inocula of either 10^4, 10^5, or 10^6 parasites per mouse, averaged over all clones.

anemia, peak parasitemia, or total number of gametocytes, but there was a significant difference between replicates in gametocytemia and parasitemia at peak gametocytemia. These differences did not rank consistently with inoculum size, and for gametocytemia they were different for the two replicates initiated with 10^5 parasites. Differences were therefore probably an artifact of the incomplete sampling regime (i.e., peaks may have been missed), or some other unexplained condition affecting parasite growth, for example, the concentration of PABA supplied in the water.

In summary, the comparisons between replicates suggested that inoculum size altered the time course of infections, but did not affect virulence or transmission.

Clone Effects on Virulence and Transmission Traits

Significant differences among clones were found for all traits relating to levels of parasite virulence and transmission (Table 5). For example, some clones (CW and AS) were quite avirulent, having 8% peak parasitemia, causing no live-weight loss, and transmitting to 1–3% of mosquitoes, whereas some clones were highly virulent (AT, BC, and ER) reaching greater than 24% parasitemia, causing 15% live-weight loss, and transmitting to 12–28% of mosquitoes. Clones of medium virulence were also found (AD, AJ, and AQ).

Repeatabilities for most traits relating to levels of infection were moderate (between 30% and 45%; Table 6), thus indicating that these were stable and repeatable characteristics of the clones when measured across different experimental replicates and mice. An exception to this was infectivity for which the repeatability was low because of a strong replicate-by-clone interaction ($P < 0.01$). This was due to three clones with high infectivity in replicate 3 having very low infectivity in replicate 4 (Table 5), which gave a between-replicate correlation (repeatability) of -0.35. However, within replicates 3 and 4 (both started with 10^5 parasites), the repeatability of clones was 0.65 and 0.88, respectively. Repeatability of the level of asexual parasitemia at the time of peak gametocytemia was zero. This was possibly due to the low mean (4%), in which case between-clone differences would have been difficult to detect, and due to the high amount of variation between mice near the end of the infection.

In contrast to the wide among-clone variation in peak val-

TABLE 4. Effects of initial inoculum size (replicate) on virulence and transmissibility traits. Figures are replicate least-squares means and standard errors after back-transformation to the original scale. PI, postinfection; RBC, red blood cell. $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; n.s., $P > 0.05$.

	Replicate mean				Standard error of mean	Significance of replicate effects
Inoculum size (parasites per mouse)	10^6	10^5	10^5	10^4		
Replicate number	1	3	4	2		
Virulence						
Maximum parasitemia						
value (%)	22.0	20.1	15.0	19.5	1.8	n.s.
day PI	7.7	10.1	10.4	11.2	0.4	***
Minimum weight						
value (g)	18.1	18.1	18.4	17.5	0.4	n.s.
day PI	8.7	11.1	10.8	12.7	0.5	***
Minimum blood density						
value (RBC $\times$ 10^9/ml)	1.37	1.02	1.67	1.14	0.3	n.s.
day PI	9.2	11.4	10.9	13.1	0.5	***
Parasitemia at maximum gametocytemia						
value (%)	0.8	2.6	2.1	0.4	0.4	*
Transmission						
Maximum gametocytemia						
value (no./1000 RBC)	0.33	0.70	0.29	0.19	0.3	**
day PI	12.2	13.1	13.2	14.9	0.4	***
Total no. gametocytes						
value (no. $\times$ 10^6/ml)	3.78	4.60	1.78	2.14	1.7	n.s.
Infectivity						
value (%)	—	—	19.9	16.0	7.0	n.s.

ues reached during the infection, there was no detectable among-clone variation in timing of the infection (Tables 5,6). This suggests that timing is more related to stochastic events such as number of parasites in the initial inoculum that become established rather than to the intrinsic rate of growth of the parasite. Alternatively, the inaccuracy of measuring traits related to timing may be responsible for the large amount of within-clone variation relative to among-clone variation. Repeatabilities for timing traits were all less than 7% (Table 6). Therefore, correlations with other traits were not estimated.

Phenotypic and Genetic Relationships among Traits

Phenotypic, genetic, and residual correlations among traits are shown in Table 7 and some key relationships are illustrated in Figures 2 and 3. In these figures, data are on the untransformed scale, after adjustment for replicate and day effects. Infectivity data were pooled across replicates despite the replicate-by-clone interaction. There was a strong relationship between peak parasitemia early in the infection and the two virulence measures, weight loss and anemia (Table 7, Fig. 2A), suggesting that virulence was a consequence of fast early parasite replication rates. (The phenotypic and genetic correlations between peak parasitemia and parasitemia two days earlier were 0.67 and 0.80, respectively [data not shown], indicating that peak parasitemia is a good measure of unconstrained replication rate.) These relationships held at both the genetic and nongenetic levels (i.e., across clones and within mice infected with the same clone). Correlations among transmission traits were also strong, especially at the genetic level, thus validating the use of pretransmission forms (gametocytes) in the host as indicators of actual transmission to the vector (Table 7, Fig. 2B).

Correlations between early parasitemia and transmission traits were positive (Table 7, Fig. 3A,B), especially at the genetic level, showing that higher replication rates were linked to higher levels of transmission. Confidence intervals obtained by stochastic simulation showed that these correlations were significant only for infectivity, despite the poor repeatability of infectivity across replicates. Correlations between gametocyte production and the traits reflecting virulence, weight loss and anemia, were low, indicating that gametocyte production is not directly influenced by the amount of damage incurred to the host. Instead, asexual parasitemia at the time of peak gametocyte production was moderately related to gametocyte output at the phenotypic and genetic levels, suggesting that transmission success depends on the persistence of the infection after the crisis period. The high genetic correlations underlying this relationship (Table 7) are somewhat unreliable because the amount of between-clone variance in this trait was very low (Table 6). Despite low correlations between virulence measures and gametocyte production, there was a significant ($P < 0.05$) genetic relationship between virulence and infectivity to mosquitoes (Table 7, Fig. 3C).

In summary, estimates of phenotypic and genetic corre-

TABLE 5. Effects of clone on virulence and transmissibility traits. Figures are least-squares means after adjusting for experimental replicate and have been back-transformed to the original scale. Significance tests are indicated by asterisks. RBC, red blood cell. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Trait	Clone mean									Standard error of clone mean	Significance of clone differences
	AD	AJ	AQ	AS	AT	BC	CW	ER	Control		
Virulence											
Maximum parasitemia											
value (%)	19.3	19.2	23.3	8.4	27.5	27.6	8.6	24.0	—	5.2	**
day	10.4	9.4	9.7	10.2	8.8	9.8	10.2	9.5	—	0.8	n.s.
Minimum weight											
value (g)	18.2	17.8	18.6	19.5	16.9	16.9	19.4	16.8	20.1	0.7	***
day	10.9	10.9	11.0	10.3	10.0	10.9	10.8	11.3	—	1.1	n.s.
Minimum blood density											
value (RBC $\times$ 10^9/ml)	1.68	0.98	1.19	2.79	0.77	1.03	2.07	0.78	10.53	0.58	***
day	11.7	10.8	10.8	11.5	10.0	11.1	11.3	12.0	—	0.9	n.s.
Parasitemia at maximum gametocytemia (%)	1.6	1.0	2.2	0.5	0.9	1.6	0.6	2.9	—		
Transmission											
Maximum gametocytemia											
value (no./1000 RBC)	0.40	0.14	1.17	0.23	0.29	0.26	0.18	0.81	—	0.46	***
day	13.5	14.4	13.2	13.8	14.7	13.1	13.2	13.9	—	0.9	n.s.
Total number of gametocytes (no./10^6 RBC)	2.31	0.84	9.52	1.85	3.21	1.94	1.48	13.96	—	2.24	***
Infectivity											
value (%) Overall	22.2	12.6	29.2	5.4	18.0	18.4	7.5	24.6	—	13.4	n.s.
Replicate 3	18.6	24.8	58.7	13.6	36.0	15.1	10.2	4.1			
Replicate 4	25.7	1.1	12.3	0.0	6.4	55.2	2.6	39.6			

TABLE 6. Estimates of variance components and repeatability (intraclass correlation) of virulence and transmissibility traits in eight clones of *Plasmodium chabaudi* measured in four replicate experiments. Estimates are on the transformed scale. Standard errors and significance tests of the hypothesis that repeatability is zero were obtained by stochastic simulation based on the observed data structure and estimates. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Trait	Variance component			Repeatability	Standard error of repeatability
	Between clones	Within clones	Total		
Virulence					
Maximum parasitemia (×100)	0.90	1.08	1.97	0.45***	0.12
Day of maximum parasitemia	0.12	1.89	2.01	0.06*	0.08
Minimum weight	1.00	1.30	2.30	0.44***	0.11
Day of minimum weight	−0.15	3.44	3.29	−0.05	0.06
Minimum blood density	0.0278	0.0634	0.0915	0.30**	0.11
Day of minimum blood density	0.01	2.46	2.56	0.04	0.07
Parasitemia at maximum gametocytemia	−0.016	0.702	0.686	−0.02	0.06
Transmission					
Maximum gametocytemia (×100)	0.075	0.156	0.231	0.32**	0.12
Day of maximum gametocytemia	0.28	2.98	3.26	0.09	0.13
Total number of gametocytes	0.126	0.228	0.354	0.36**	0.13
Infectivity	0.389	3.833	4.222	0.09	0.18

TABLE 7. Phenotypic, genetic, and residual correlations among virulence and transmissibility traits (coded 1–7) in *Plasmodium chabaudi* infections in inbred mice. Correlation estimates are shown above the diagonal, and standard errors are shown below the diagonal. Asterisks indicate the probability that estimates differ from zero by chance. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

Trait	Phenotypic correlations						
	1	2	3	4	5	6	7
Virulence							
1. Maximum parasitemia		−0.71***	−0.60***	0.17	0.18	0.22	0.44*
2. Minimum weight	(0.06)		0.72***	−0.28*	−0.11	−0.12	−0.18
3. Minimum blood density	(0.13)	(0.10)		−0.19	−0.10	−0.02	0.23
4. Parasitemia at maximum gametocytemia	(0.13)	(0.12)	(0.12)		0.36**	0.20	0.32
Transmission							
5. Maximum gametocytemia	(0.13)	(0.13)	(0.13)	(0.11)		0.89***	0.63***
6. Total no. of gametocytes	(0.14)	(0.14)	(0.15)	(0.13)	(0.08)		0.77***
7. Infectivity	(0.13)	(0.19)	(0.18)	(0.16)	(0.06)	(0.08)	
	Genetic correlations						
	1	2	3	4	5	6	7
Virulence							
1. Maximum parasitemia		−0.91***	−0.88*	0.69	0.44	0.38	0.78**
2. Minimum weight	(0.05)		0.90**	−0.40	−0.03	−0.03	−0.70*
3. Minimum blood density	(0.25)	(0.19)		−0.32	−0.12	−0.03	−0.40
4. Parasitemia at maximum gametocytemia	(0.32)	(0.31)	(0.32)		0.79	0.72	0.53
Transmission							
5. Maximum gametocytemia	(0.26)	(0.30)	(0.31)	(0.28)		0.93***	0.83***
6. Total no. of gametocytes	(0.27)	(0.31)	(0.29)	(0.29)	(0.13)		0.91***
7. Infectivity	(0.20)	(0.30)	(0.34)	(0.30)	(0.11)	(0.11)	
	Residual correlations						
	1	2	3	4	5	6	7
Virulence							
1. Maximum parasitemia		−0.53***	−0.40**	0.00	0.00	0.08	0.17
2. Minimum weight	(0.09)		0.61***	−0.29	−0.16	−0.18	0.20
3. Minimum blood density	(0.13)	(0.11)		−0.17	−0.09	−0.06	0.60**
4. Parasitemia at maximum gametocytemia	(0.13)	(0.12)	(0.18)		0.30	0.09	0.15
Transmission							
5. Maximum gametocytemia	(0.14)	(0.13)	(0.14)	(0.12)		0.86	0.46***
6. Total no. of gametocytes	(0.15)	(0.14)	(0.15)	(0.14)	(0.09)		0.59***
7. Infectivity	(0.16)	(0.22)	(0.17)	(0.21)	(0.07)	(0.10)	

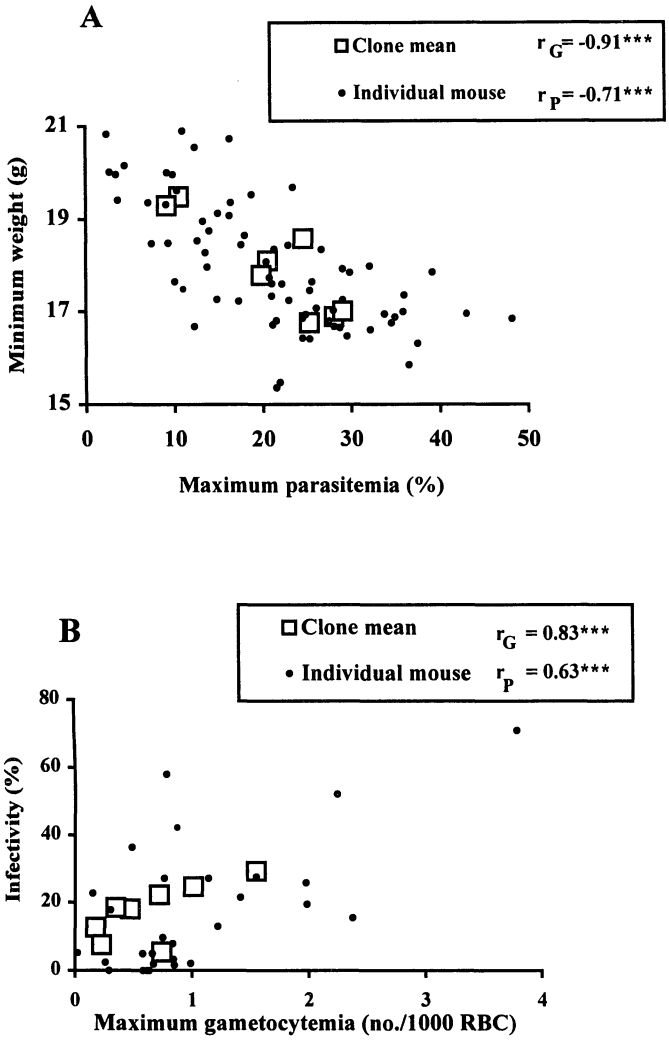

FIG. 2. Relationships among virulence and transmission traits in *Plasmodium chabaudi* infections. (A) Virulence (minimum live-weight) versus replication rate (maximum parasitemia). (B) Infectivity versus gametocytemia. Dots indicate the phenotypic relationship, with each dot representing a measurement on one mouse. Squares indicate the genetic (across-clone) relationship, each square representing the mean of mice infected with the same clone. Phenotypic (r_P) and genetic (r_G) correlations are shown with their significance levels (***$P < 0.001$).

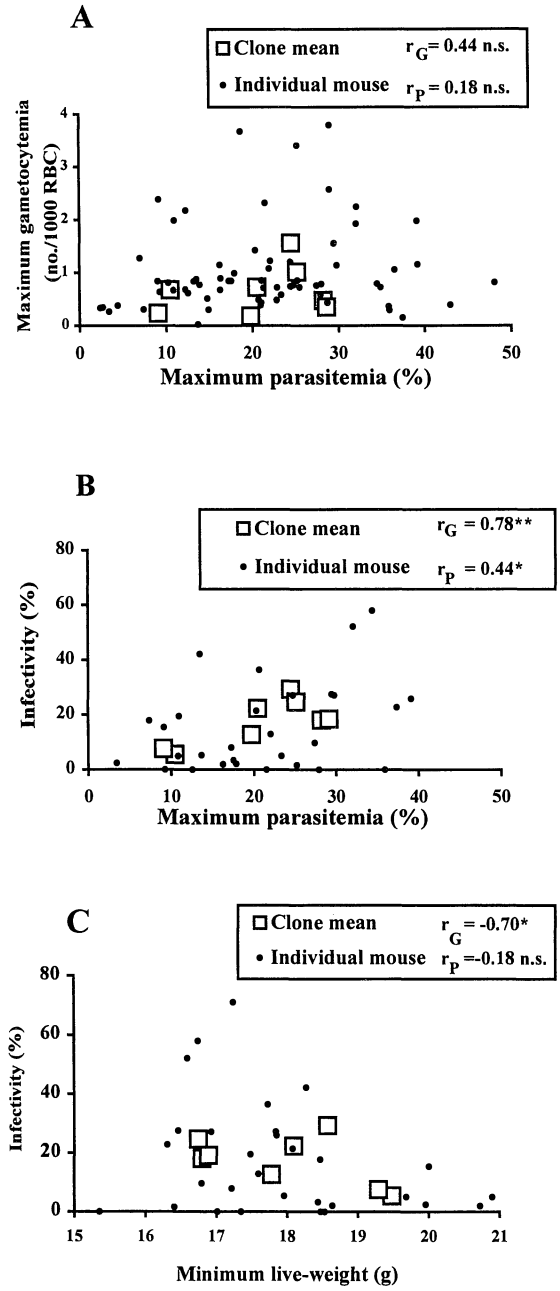

FIG. 3. Relationships between transmission and virulence traits in *Plasmodium chabaudi* infections. (A) Gametocytemia versus replication rate (maximum parasitemia). (B) Infectivity versus replication rate (maximum parasitemia). (C) Infertility versus virulence (minimum live-weight). Dots indicate the phenotypic relationship, with each dot representing a measurement on one mouse. Squares indicate the genetic (across-clone) relationship, each square representing the mean of mice infected with the same clone. Phenotypic (r_P) and genetic (r_G) correlations are shown with their significance levels (n.s., $P > 0.05$; *$P < 0.05$; **$P < 0.01$).

lations suggested that parasite genotypes with the ability to replicate faster in the asexual phase are more able to transmit at higher levels, but also cause higher levels of virulence.

DISCUSSION

Evolutionary Relationships

This study has demonstrated that replication rate, virulence, and transmission are positively genetically correlated in *P. chabaudi* parasites. These observations strongly support one arm of the trade-off hypothesis, namely that virulence does not evolve to zero because of the fitness benefits to parasites in terms of transmission that results from faster within-host replication. The two fundamental assumptions in this hypothesis—that virulence is a by-product of rapid asexual replication, and that faster replication results in higher transmission—were supported by the data. This validates the assumptions of the trade-off hypothesis for a parasite species that shares strong similarities in virulence and life history with one of the most important parasites of humans, *P. fal-*

ciparum. Moreover, these data significantly boost the small amount of empirical evidence upon which a large body of evolutionary theory about parasite evolution is based (e.g., Anderson and May 1982).

The other arm of the hypothesis, namely that selection limits parasite replication rate because of the fitness costs of host death, was not tested here because there was low host mortality in these experiments. However, the few host deaths that did occur were associated with higher parasitemias, and so it is expected that significant host mortality would occur if more susceptible strains of mice were used (Stevenson et al. 1982).

A striking feature of the data from this study was the considerable variation in virulence and transmission among parasite clones that had been isolated from the wild. There are several possible reasons for this. First, the phenotype and, thus, the variation in phenotypes, was probably exaggerated because the laboratory mouse, mosquito, and environmental conditions represented a novel (and relatively uniform) environment to the parasite. It is unlikely that sporozoite-induced primary infections of *P. chabaudi* are so severe in their natural host (Landau and Chabaud 1994), although infections established by blood passage in the natural host can be lethal (Carter and Walliker 1975). Similarly, oocyst burdens in the field are rarely as high as those induced by artificial feeding of mosquitoes in the laboratory (Lyimo and Koella 1992). Second, genetic variation is expected to be maintained by coinfection and superinfection (Nowak and May 1994; May and Nowak 1995) or by strain-specific immunity (Gupta et al. 1994). Third, it is possible that variation between clones was generated in our laboratory during the cloning process. Given the vast numbers of parasites within one host, the potential for genetic change through within-host evolution is high and, in general, multiple passages in the laboratory environment bring about increases in virulence (Dearsly et al. 1990; Mackinnon and Read 1999). One of the strengths of this study, however, is that the clones had been subjected to few and roughly equal numbers of passages in the laboratory since isolation from the wild. A final possibility is that the clones did not differ genetically, but that another organism, such as a bacterium or virus, had become associated with certain clones and not others. These organisms could be cotransmitted with the malaria parasite in the artificial transmission system used here by being simultaneously present in the host's bloodstream. The effects of concomitant organisms in modulating malarial infection patterns is well known for malaria parasites (Baker et al. 1971; Nickell et al. 1987). Because many bacteria contain viruses or plasmids, some of which are virulence factors (reviewed by Levin and Tauxe 1996), it is not unreasonable to suspect that malaria may carry viruses. Viruses have been found in most of the major disease-causing protozoal species and in some cases alter growth characteristics and antigenic expression of the protozoan (reviewed by Wang and Wang 1991). Virus-like particles have been observed in mosquito-stage *Plasmodium* parasites (Dasgupta 1968; Garnham et al. 1962; Davies and Howells 1971; Trefiak and Desser 1973), but there is no evidence of viruses in blood-stage *P. chabaudi* parasites. This is currently under investigation.

Mechanistic Basis of the Observed Relationships

Why does higher replication rate lead to higher virulence and transmission in this particular host-parasite system? Many parasites achieve higher fitness by means other than increasing replication rate, such as through down-regulation of the immune response, immune evasion, and manipulation of host behavior (Wakelin 1996; Hart 1997). Malaria parasites may also employ such mechanisms (Brown and Brown 1965; Day and Edman 1983; Gilbert et al. 1998; Koella et al. 1998), but the results from this study suggest that replication rate is a critical factor in transmission success, with higher virulence as an unfortunate consequence effect. Why?

Indicators of virulence, anemia and weight loss, were strongly associated with parasite growth rate. Although anemia is a direct consequence of the parasite's destruction of host erythrocytes every 24 h upon schizogony and is thereby directly related to parasite density, live-weight loss is probably an indirect consequence of the host's immune response to the parasite through downstream effects of infection such as anorexia, fever, cytokine release, and the energetic costs of mounting an immune response. Thus, the question is whether parasitemia is directly responsible for the cost to the host in a density-dependent way or whether high parasitemia is associated with these host responses through some indirect mechanism. The answer is probably both. In a lucid review of the complex nature of virulence in human malaria, Marsh and Snow (1997) explain how the three major disease syndromes—severe anemia, coma and organ failure, and general pathology such as respiratory distress, aches, and nausea—can be attributed, respectively, to three primary virulence determinants: RBC destruction, infected cell sequestration in vital organs such as the brain, and parasite-induced release of cytokines. These factors interact to produce a complex host response that depends on many other factors, particularly immune priming, thus often masking a direct relationship between parasitemia and pathology (White and Ho 1992). Importantly, however, all three mechanisms potentially underlie the intrinsic genetic link between replication rate and virulence observed in this study. First, rate of RBC depletion is clearly a function of growth rate of the asexual parasite population because the host cell is destroyed at the end of each parasite cycle (24 h in *P. chabaudi*, 48 h in *P. falciparum*). Second, cytoadherence of infected cells in the microvasculature may provide the parasite with a growth advantage through more efficient reinvasion offered by cells in close proximity, by better growth under hypoxic conditions in capillaries, or through reduced death rate by avoiding clearance by the spleen (reviewed by Berendt et al. 1994). Although it is clear that sequestration in critical tissues is related to the probability of host death (Macpherson et al. 1985), none of these potential advantages to parasite growth have been demonstrated conclusively. Similarly, another parasite phenomenon arising from cytoadherence mechanisms—rosetting—correlates with host mortality (Carlson et al. 1990; Rowe et al. 1995), but an advantage to the parasite has yet to be demonstrated (Clough et al. 1998). Third, cytokine induction in response to release of parasite toxins at schizogony may also relate to parasite growth because even though parasite density-dependent release of these cytokines

causes fever, which seems to control parasite growth (reviewed by Kwiatkowski 1995), excessive cytokine production leads to higher general morbidity (Clark et al. 1981, 1989; Grau et al. 1989) and upregulates the dangerous phenomenon of cytoadherence (Schofield et al. 1996). Further studies trying to relate variation between clones in replication rate to variation in any of these mechanisms would help define the root causes of virulence and targets for its control.

The question remains as to why parasite growth rate and transmission were correlated in this study. They need not be; between the period of peak parasite growth rate and peak transmission there was a massive decline in parasite density due to rapid killing by the immune system (Fig. 1). Nevertheless, asexual parasitemia at the time of transmission was strongly correlated with transmission and asexual parasitemia before the crisis less strongly so. Thus, transmission may depend on the ability of the parasite to grow back after the onslaught of the host's immune system. The mechanism that allows early parasite growth may also be one that allows recovery and persistence of the parasite after crisis and, thus, higher lifetime transmission. This may be sequestering ability or some other factor affecting intrinsic growth rate or it may be that greater antigenic diversity can be generated from larger asexual populations. It may also be that clones vary in the rate at which they generate antigenic variation. It has long been argued that constant switching of surface antigens to effect immune evasion, which is found in most malaria species (Cox 1962; Brown and Brown 1965; Brown et al. 1968; McLean et al. 1982, 1986; Hommel et al. 1983; Handunetti et al. 1987; Gilks et al. 1990; Biggs et al. 1991; Roberts et al. 1992), is a strategy to prolong infections (Brown and Brown 1965; Brown 1971). Data on specific immune responses support this view (Brown et al. 1970; David et al. 1983; Udeinya et al. 1983; Gilks et al. 1990). Unfortunately there are currently no data on genetic variation in rates of antigen switching or on relationships between antigenic variation and lifetime transmission. Nevertheless, variation in the rate of switching may also explain why the ability to grow rapidly in the first part of the infection relates to transmission later on. At least in one parasite species (*P. falciparum*) the molecule responsible for antigenic variation (PfEMP1) is the same as the one that enables sequestering (Baruch et al. 1995; Smith et al. 1995; Su et al. 1995). Importantly, this common molecule provides a potential link between parasite growth rate, virulence, and transmission through its dual role in cytoadherence and antigenic variation. This has been proposed previously (Gupta and Day 1996), but quantitative data justifying this link are missing. The present study in *P. chabaudi*, which both cytoadheres and undergoes antigenic variation both early and late in the infection (Gilks et al. 1990; Brannan et al. 1994), is consistent with a link between cytoadherence and antigenic variation and explains why it should be maintained by evolutionary forces. However, the question of whether higher lifetime transmission is a consequence of better growth rate after the crisis due to cytoadherence ability, or of higher survival because of antigenic variation, or both, remains to be resolved.

Alternatively, there may be a less functional link between asexual parasite numbers and transmission. For example, many parasite species have phenotypically variant antigens, but not all species sequester. A third factor may influence both parasitemia and transmission, thus causing an apparent genetic link between these traits. Indeed, the link between parasite growth rate and transmission did not appear to be directly mediated through numbers of gametocytes (Table 7). Furthermore, when infectivity was adjusted for gametocyte number by including the latter as a covariate in the analyses, all correlations between infectivity and parasitemia and virulence traits became only slightly weaker than those with unadjusted infectivity (data not shown). Thus, it seems that the strong across-clone correlations between infectivity and parasite growth rate was not causally related through greater numbers of gametocytes. One possible reason for an indirect across-clone association between replication rate and transmission is that immune killing may be effective against both asexual parasites and gametocytes (or gametes in the mosquito) and that the virulent parasite clones are less immunogenic than others or create a higher level of diversity that confounds the immune system. Alternatively, the effect of rapid asexual replication may be to create an environment more conducive to uptake of gametocytes by the mosquito through, for example, higher anemia.

In conclusion, it seems that parasite genotypes that are able to replicate fast achieve higher transmission. However, this does not seem to be due to a simple causal relationship mediated through higher numbers of gametocytes. A side-effect of faster replication is higher virulence. Closer examination of other properties of the clones such as switching rates of antigenic variants, immunogenicity, cytoadherence ability, intrinsic reproductive rate, and synchronicity should help elucidate the causal basis of these relationships.

Implications for Malaria Control

A motivation for understanding the evolution of virulence is to plan better control strategies in light of knowledge about parasite adaptation. The results we report here demonstrate that the genetic architecture underlying virulence is consistent with that assumed by adaptive trade-off models. But, tempting though it may be (Ewald 1994; Stearns 1999; Dieckmann et al., in press), we believe it is too early to say what, if any, the evolutionary consequences of particular medical interventions would be on malaria virulence. For instance, is virulence an inherent consequence of parasite replication rate or immune evasion? Without knowing that, it is difficult to determine the direction (if any) of selection imposed by vaccination. Another important consideration is the interaction between parasite genotypes within the same host. Genetic diversity per se can affect weight loss and anemia in mice infected with *P. chabaudi* (Taylor et al. 1998). Moreover, many theories suggest that optimal levels of virulence can be affected by within-host relatedness (reviewed by Read et al. 1999). However, at least in *P. chabaudi* in mice, the relative transmission success of competing genotypes can be inversely related to their early performance within a host (Taylor et al. 1997; Taylor and Read 1998). Therefore, the transmission advantages of more rapid within-host replication that we demonstrate here for single clone infections seem to be quite different when infections consist of multiple clones. These complexities and others, such as the degree of

competition between parasite genotypes at the between-host level (Gupta et al. 1994), are scientifically exciting, but until they are fully understood, they caution against premature management advice (Read et al. 1999).

Of course, it may be that such complexities do not matter in the field. Certainly, within-host relatedness is of little consequence in populations where most infections consist of a single clone. In such situations, it may well be possible to extrapolate from the data we report here. An important issue is how well our mouse model captures the human disease situation, and this remains an open issue. Nevertheless, there are some data consistent with our findings. During the era of malaria therapy for curing neurosyphilis earlier this century, when natural transfer of parasites by mosquito feeding and inoculation was regularly practiced in naive and semi-immune patients, it was observed that a widely used strain from Madagascar increased in virulence with a concomitant increase in gametocyte production (James et al. 1936). Furthermore, across the diverse range of strains used, it was observed that the virulence of a strain was directly related to its asexual parasite growth rate (James et al. 1932), although controlled experiments to test this assertion were not conducted. Thus, the results presented in this study, although derived in a highly artificial laboratory environment in a rodent malaria species maintained in unnatural mammalian and vector hosts, are nevertheless consistent with observations made in human malaria by experienced clinicians in a semi-natural environment. Therefore, it appears that the genetic architecture assumed by the trade-off models for the evolution of virulence exists in malaria parasites such that parasite virulence could be driven toward a level that is suboptimal for the host.

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