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Infection, Genetics and Evolution 1 (2002) 287–296

Infection, Genetics and Evolution

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Virulence in rodent malaria: host genotype by parasite genotype interactions

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Received 7 November 2001; received in revised form 12 March 2002; accepted 20 March 2002

Abstract

In an effort to understand what limits the virulence of malaria parasites, we infected inbred mice of three genotypes (C57Bl/6J, CBA/Ca and DBA/2) with one of two parasite lines of the rodent malaria *Plasmodium chabaudi*. One of these parasite lines had been serially passaged through C57Bl/6J mice and had evolved higher asexual growth rate, virulence and transmission in the process. The other parasite line was the unadapted ancestral line which had low virulence. In all three host genotypes, the C57Bl/6J-adapted parasite line was more virulent than the ancestral line thus indicating that trade-offs in virulence between alternative host genotypes had not placed strong constraints on the evolution of high virulence in this system. By examining the infection dynamics for fitness-related components—asexual parasite population growth, transmission and virulence—we revealed alternative possible explanations for what sets the upper limit to virulence in nature. The total number of transmission forms (gametocytes) produced during the infection, a measure of parasite Darwinian fitness, was four-fold higher in mice that survived the infection than those which died. Among mice that survived, total gametocyte production was greatest in the host genotype that suffered intermediate levels of morbidity (anaemia and weight loss). Thus, there were transmission costs of high virulence that were partly due to host death (as most theoretical models of virulence evolution assume), but perhaps partly due to some factor related to high morbidity. Both mortality and morbidity-related factors might therefore influence the upper limit on virulence of malaria parasites.

Keywords: Virulence; Malaria; *Plasmodium chabaudi*; Evolution; Parasite fitness; Host–parasite genotype interactions

1. Introduction

Many theoretical models for the evolution of parasite virulence (defined in these models as the infection-induced host mortality rate) assume that virulence confers both benefits and costs to parasite fitness (total lifetime transmission). Parasites that extract more resources from their host, it is argued, produce more transmission forms per unit time, and for a longer period (the benefits). However, they do so at an increasing risk of host death, which cuts short the infectious period (the cost). Thus, host death is assumed to act as a brake on natural selection for ever-increasing virulence (Levin and Pimentel, 1981; Anderson and May, 1982; Bremermann and Pickering, 1983; May and Anderson, 1983; Frank, 1996).

Data to support this widely discussed evolutionary theory are scarce. Evidence is mounting to support the assumption that transmission rate is positively and genetically related to virulence (Anderson and May, 1982; Diffley et al., 1987; Turner et al., 1995; Ebert and Mangin, 1997; Lipsitch and Moxon, 1997; Mackinnon and Read, 1999a,b). But despite its logical appeal, data to support the second assumption—that host death incurs a cost to lifetime transmission success—are rare (Fenner et al., 1956; Fenner et al., 1957). Moreover, other factors might constrain virulence evolution in nature. After continual propagation in novel hosts or in vitro cell cultures, parasites almost invariably increase in virulence and growth rate in these novel environments, but show decreased virulence in their original hosts (Ebert, 1998, 2000). This is consistent with two constraints on virulence other than host death. First, where there are two stages to the parasite's life cycle—one for asexual growth and one for the production of sexual stages for transmission to new hosts (as in malaria parasites)—life history theory predicts a trade-off between parasite growth and reproduction (Sasaki and Iwasa, 1991; Stearns, 1992; McKenzie and Bossert, 1998). In serial passage experiments, the constraints of producing transmission forms and host death are usually absent. Second, the expression of virulence may be highly host genotype-specific such that parasite genotypes causing high virulence in one host genotype may cause low virulence in another, and vice versa, to give a 'cross-over'

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1567-1348/02/$ – see front matter
PII: S1567-1348(02)00039-4

interaction. This could explain the attenuation of virulence in the parasite's natural host as a result of serial passage in novel hosts (Ebert, 1998, 2000). In nature, virulence might therefore be limited because host genetic diversity prevents the parasite from achieving high host-specific virulence (Ebert, 1994; Dybdahl and Lively, 1998; Lively and Dybdahl, 2000; Regoes et al., 2000; Carius et al., 2001; Woolhouse et al., 2001).

In our previous studies on malaria parasite virulence (taken from hereon to include host morbidity as well as mortality) using the rodent malaria *P. chabaudi* in C57Bl/6J mice, host death was not the primary limitation on parasite virulence (Mackinnon and Read, 1999b). Despite strong divergent selection within an avirulent parasite clone for causing higher or lower probability of host mortality, both the high and low selection lines evolved to cause much higher morbidity than the ancestral line. Thus, strong between-host selection against host death failed to prevent the parasite from evolving higher virulence. We assume that this was due to the overriding strength of within-host selection. Furthermore, when the same selection regime was applied to a virulent clone, morbidity levels were unaltered, thus suggesting some regulatory factor governing maximum morbidity (Mackinnon and Read, 1999b). Mortality rates (4% over all lines and generations) were insufficient to explain this upper limit on morbidity. There was also no apparent trade-off between growth and reproduction: transmission increased with virulence (Mackinnon and Read, 1999a,b).

Here we investigated the possibility that host genotype by parasite genotype cross-over interactions constrain virulence by infecting mice of less resistant genotypes with the evolved, virulent parasite line and its ancestral control line. The infection dynamics, and consequential mortality, morbidity and transmission, were examined in order to understand the basis of potential constraints on virulence evolution.

2. Materials and methods

2.1. Mouse and parasite genotypes

We used the rodent malaria, *P. chabaudi*, as a model for our studies because of its similarity in life history and genetic characteristics to the most virulent of the human malarias, *P. falciparum* (Cox, 1988). The natural host of *P. chabaudi* is the thicket rat, *Thamnomys rutilans*, a sibling genus of the laboratory mice used in these studies (Ellerman, 1940). In this experiment, three mouse host genotypes were each infected with one of two parasite lines. This gave six experimental groups, each comprised of five female, 6-week-old mice (Harlan, UK). Host genotypes were C57Bl/6J, DBA/2 and CBA/Ca. These inbred genotypes are known to be relatively resistant to *P. chabaudi* malaria (Stevenson et al., 1982) and are genetically different from each other, including at the major histocompatibility locus (Lyon and Searle, 1989). The two parasite genotypes used in this experiment were the adapted, virulent line (denoted CW-V) that had evolved to grow well in C57Bl/6J mice by serial passage (11 passages, each with 12 replication cycles of 24 h each), and its ancestral line (denoted CW-0) which was relatively avirulent (Mackinnon and Read, 1999b). The lines had been stored in liquid nitrogen and underwent, for maintenance reasons, a further 4 and 3 passages, respectively, in C57Bl/6J mice prior to being used to infect the mice in this experiment.

2.2. Infections

Each mouse was infected with 10^5 parasites obtained from female C57Bl/6J donor mice which had themselves been infected from frozen aliquots of infected blood 13 days previously. Measures of asexual parasite density, gametocyte density, red blood cell (RBC) density and mouse live-weight were taken every 2–3 days until Day 22 post-infection (PI) using routine procedures (Taylor et al., 1998).

2.3. Statistical analyses

All analyses were performed using the SAS statistical analysis software (version 6.1, SAS Institute, Cary, NC, USA). Three sets of response variables relating to asexual parasite load, gametocyte production (transmission potential) and virulence, were analysed for the effects of host genotype, parasite line and the interaction between them by analysis of variance fitting these terms as fixed effects. Where data comprised repeated observations on the same mouse, an additional random effect for mouse was also fitted. Parasite numbers were expressed in terms of the proportion of cells infected (asexual parasitaemia or gametocytaemia) and in terms of parasite densities (calculated from the product of the proportion of cells infected and RBC density). The proportion of asexual parasites that produced gametocytes (conversion ratio) was calculated for each day of sampling using the method described in Buckling et al., (1999). In addition to the values on individual days, maximum values, average values and areas under the parasite number by time curves were calculated to represent summary measures of the infection. Log_{10} transformations were applied to gametocyte densities, gametocytaemias and conversion ratios in order to normalise the data. Mortality was analysed by logistic regression to allow for its binomial nature, or using Fisher's exact test. The two measures of morbidity that we analysed were the maximum amount of live-weight lost during the infection, and the maximum reduction in RBC density, both calculated as differences between their minima and the values at the beginning of the infection. The days on which these minima occurred were also analysed as response variables using logistic regression. If the mouse died during the course of the experiment, all the data available until the time of death were included in the analysis unless stated otherwise.

We also analysed for differences between the mice that survived and the mice that died by fitting a linear model with survival category as a fixed effect, with or without additional fixed effects for host genotype, parasite line and their interaction. Further, we investigated the relationships among traits, e.g. between transmission and virulence traits, using regression analyses. Evidence of trade-offs between traits was sought by fitting a quadratic as well as a linear term for the regressor in the model. Fixed effects for host genotype and parasite line were not included in the latter models because we wanted to examine the general relationship between pairs of traits across a wide range of virulence levels, as afforded by the different host and parasite genotypes, and because of lack of statistical power to detect within-group relationships among traits.

3. Results

At the start of the experiment, CBA/Ca mice were on average slightly heavier (22.7 ± 0.53 g, $P = 0.07$) and DBA/2 mice were significantly lighter (18.5 ± 0.53 g, $P < 0.01$) than C57Bl/6J mice (20.7 ± 0.53 g). Hosts genotypes had similar mean RBC densities at the beginning of the infection ($P > 0.10$). There were no significant parasite line effects on these initial measures ($P > 0.10$).

3.1. Host genotype and parasite line effects

Fig. 1 shows the average infection patterns through time for each host genotype by parasite line combination, and

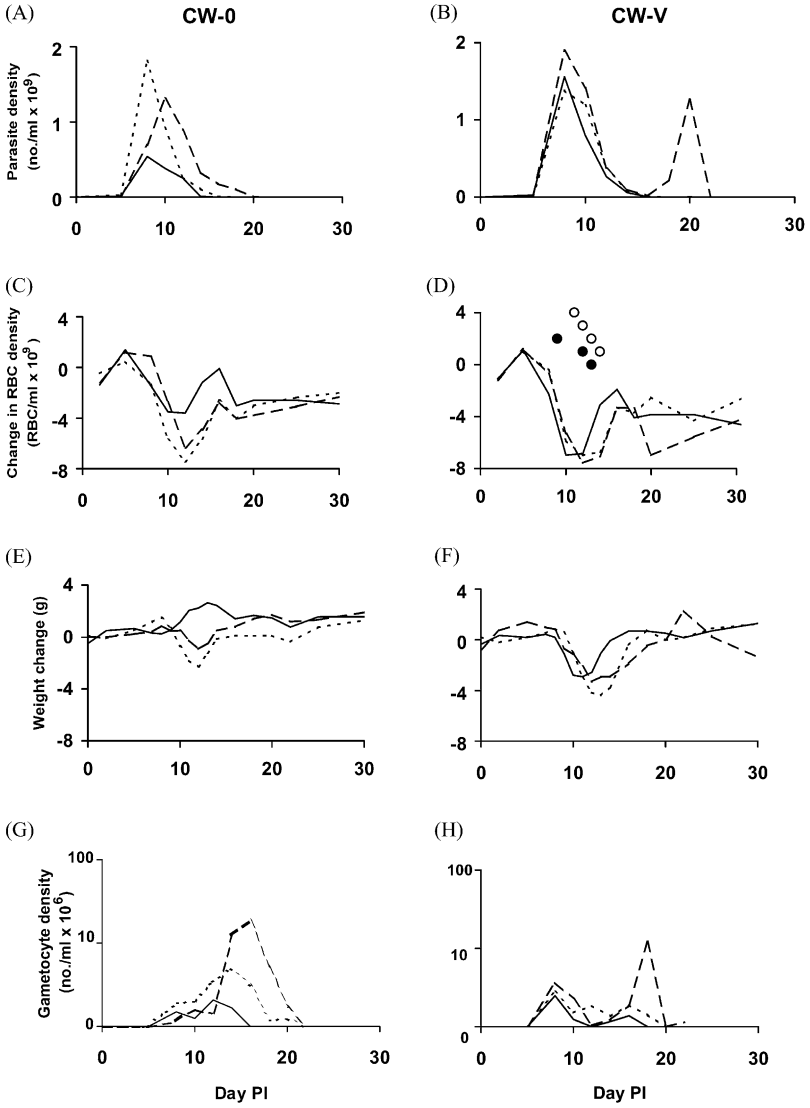

Fig. 1. Mean infection profiles for three mouse genotypes (C57Bl/6, solid line; CBA, short dash line; DBA/2, long dash line) infected with either an avirulent, ancestral line of parasites (CW-0, left side) or a mouse-adapted line of parasites (CW-V, right side). (A and B) Asexual parasite densities. (C and D) Deviations in RBC densities from initial values. (E and F) Deviations in mouse live-weights from initial values. (G and H) Gametocyte densities. Data from mice that died are included in the means until their death occurred. Days of death are marked in D by closed and open circles for DBA/2 and CBA/Ca mice, respectively.

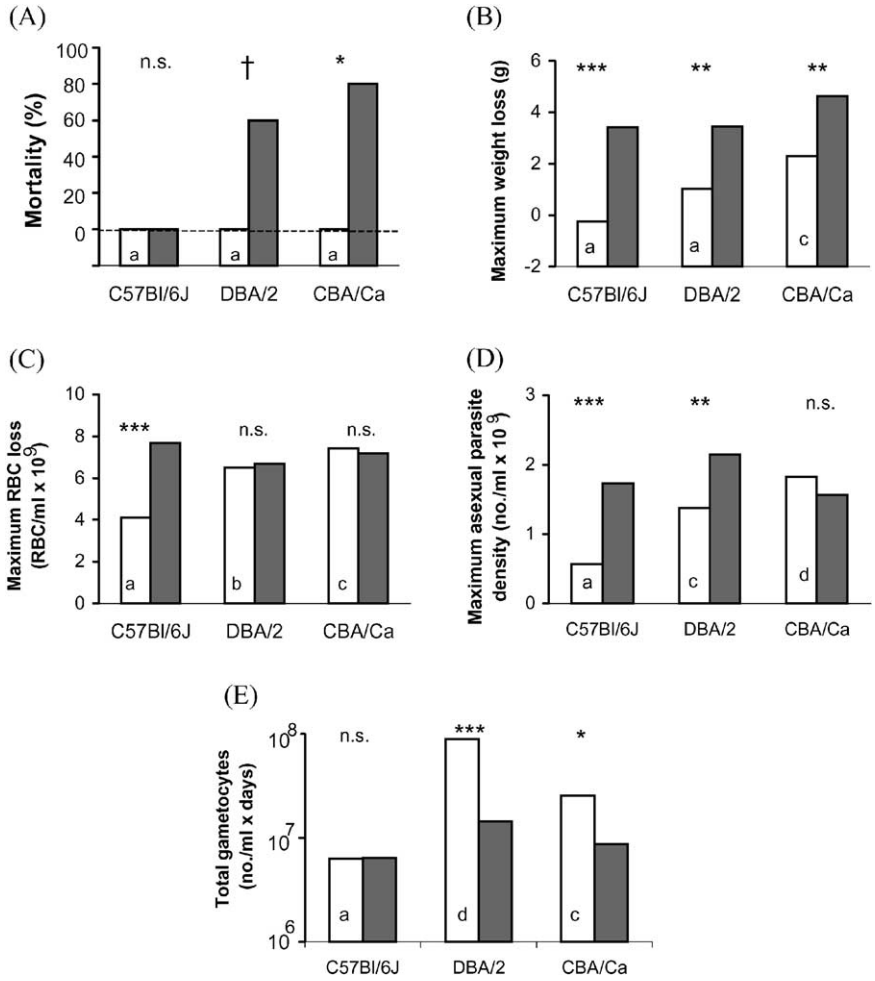

Fig. 2. Mean values of summary measures of the infection from mice infected with the ancestral parasite line, CW-0 (white bars) or the adapted parasite line CW-V (black bars). Symbols above pairs of bars denote significant parasite line differences within host genotypes (n.s., $P > 0.10$; (†) $P < 0.10$; (*) $P < 0.05$; (**) $P < 0.01$; (***) $P < 0.001$). Letters on white bars denote significant differences between host genotypes infected with the CW-0 line relative to the C57Bl/6J genotype (a) $P > 0.05$; (b) $P < 0.05$; (c) $P < 0.01$; (d) $P < 0.001$).

Fig. 2 shows the mean values of summary measures of the infection.

3.1.1. Mortality

During the experiment, 7 out of the 30 mice died as a result of infection: all of these occurred between days 10 and 15 PI. Four of these were euthanased because, based on experience, they were very likely to die within the ensuing 24 h. All of the mice that died were infected with the CW-V line, four in CBA/Ca mice and three in DBA/2 mice (Fig. 2A). Combined, the mortality in these two genotypes (70%) was significantly higher ($P < 0.05$, one-tailed Fisher's exact test) than the zero mortality in the C57Bl/6J genotype when infected with the CW-V parasite line. The average day of death in CBA/Ca mice was 12.5 and in DBA/2 mice was 11.3, which were not significantly different ($P > 0.10$).

Mice that died had similar maximum parasite densities, initial growth rates and maximum RBC loss to those that survived (Table 1). However, they had significantly higher weight loss and maximum parasitaemias, but these differences became non-significant ($P > 0.10$) when host genotype, parasite line and their interaction were included in the model. This suggests that there were host genotype influences on mortality that were not fully explained by weight loss or maximum parasitaemia. Nevertheless, of the seven mice that died, weight was still decreasing on the day of death in six cases, whereas parasite density was still increasing and RBC density still decreasing on or before the day of death in one and two cases, respectively. Thus, continued weight loss appeared to be a better predictor of death than parasite load or RBC loss.

Maximum conversion ratios, total number of gametocytes and total gametocytaemia during the infection were significantly lower in mice that died than in survivors (Table 1). However, there was no evidence that parasites increased their gametocyte production in the mice about to die: gametocyte densities and gametocytaemias for individual days were similar in surviving and non-surviving mice after allowing for

Table 1
Differences in mean values of asexual parasite growth, virulence and transmission traits in mice that survived vs. those that died during infection with *P. chabaudi*

Trait	Mean (S.E.)[a]		Significance[a]	
	Survived ($n = 23$)	Died ($n = 7$)	Unadjusted	Adjusted
Asexual growth				
Parasite density Day 5[b]	0.0157 (0.0031)	0.0144 (0.0057)		
Maximum parasitaemia (%)	22.1 (1.9)	31.2 (3.4)	*	
Maximum parasite density[b]	1.46 (0.13)	1.80 (0.24)		
Total no. parasites[b]	6.42 (0.57)	6.36 (1.03)		*
Virulence				
Maximum weight loss (g)	1.92 (0.37)	4.10 (0.68)	**	
Maximum RBC loss[b]	6.61 (0.37)	6.55 (0.68)		
Transmission[c]				
Total gametocyte density	32.4 (6.8)	8.4 (12.3)	†	*
Total gametocytaemia	5.14 (1.02)	1.22 (1.86)	†	**
Average conversion ratio (%)	1.71 (0.31)	0.91 (0.56)		*
Maximum conversion ratio (%)	10.6 (2.0)	2.2 (3.7)	†	**

[a] 'Adjusted' and 'unadjusted' mean that host genotype, parasite line and their interaction (where significant, $P < 0.05$) were included and excluded from the model respectively. Means are presented on the raw scale and are not adjusted for any fixed effects.
[b] Measured in no./ml $\times 10^9$.
[c] As reflected by gametocyte production. 'Total gametocyte density' and 'total gametocytaemia' are areas under gametocyte density by time and gametocytaemia by time curves between Days 0 and 22 PI, in units of no. gametocytes per ml blood × days × 10^6, and no. of gametocytes per 1000 RBC × days, respectively.
* $P < 0.05$.
** $P < 0.01$.
† $P < 0.10$.

host genotype and parasite line effects (not shown), and average conversion ratios up to the time of death or clearance were, in fact, significantly higher in mice that survived versus mice that died (Table 1). Thus, there was a direct cost of host death to total lifetime transmission, i.e. this cost was not indirectly due to intrinsic differences among host or parasite genotypes in gametocyte production. The magnitude of the cost was large: mice that died produced 26% of the number of gametocytes produced by survivors (average of untransformed data across all genotypes). This cost to lifetime transmission resulted from the fact that the majority of deaths (six out of seven) occurred on or before Day 12 PI, after which the bulk of the gametocytes (83% in survivors) were produced.

3.1.2. Asexual parasites

There were no host genotype or parasite line effects on early growth rate as measured by parasite density on Days 5 and 8 PI ($P > 0.10$, data not shown). However, there were clear differences between the host genotypes in maximum parasite density (Fig. 2D) and maximum parasitaemia (data not shown) when infected with the CW-0 line. CBA/Ca mice had the highest densities, followed by DBA/2 and C57Bl/6J. These host genotype effects disappeared when mice were infected with the CW-V line, which produced higher asexual parasite loads on average, giving an overall interaction significance level of $P < 0.01$. These results suggest that the higher asexual growth capacity of the evolved CW-V line was sufficient to overcome the basal differences between host genotypes in resisting the slow-growing CW-0 parasite clone, and that there was some upper bound on parasite density beyond which this capacity could not be expressed. This upper bound was not host death because only one of the seven mice that died did so before peak parasite density was observed. Total number of asexual parasites produced to Day 22 PI showed similar patterns (Fig. 2F) to maximum levels (interaction term, $P = 0.08$).

3.1.3. Morbidity

Profiles of RBC density and live-weights tended to mirror those for asexual parasite densities, although with a 1–2-day delay between maximum parasite density and maximum morbidity (Fig. 1). When infected with the ancestral CW-0 line there were differences between the host genotypes in maximum RBC loss and maximum weight loss, with CBA/Ca suffering the most, followed by DBA/2 and then C57Bl/6J (Fig. 2B and C). These genotype differences were not present when mice were infected with the CW-V line (Fig. 2B and C). This suggests an upper bound to morbidity. Six and two of the seven mice that died had their respective observed minimum weight and RBC density on their day of death, supporting the observation above that weight loss was related to the probability of dying, while minimum RBC density was either not directly related to mortality, or was regulated by other factors.

After the acute phase of the infection (i.e. post-peak parasitaemia), all surviving mice recovered to approximately equal weights and RBC densities (Fig. 1), but there were

too few surviving mice to analyse for parasite line by host genotype interactions in persistence of the infection.

3.1.4. Gametocytes

When data from mice that died were excluded, the total number of gametocytes, maximum and average gametocyte conversion ratios were the same in the CW-0 line as in the CW-V line for all three host genotypes ($P > 0.10$), though total gametocytaemia was higher in the CW-0 line than in the CW-V line in DBA/2 and CBA/Ca mice ($P < 0.05$ for both traits). Maximum and average gametocyte conversion ratios were significantly higher in DBA/2 and CBA/Ca mice ($P < 0.01$) than in C57Bl/6J mice, while total gametocyte density and gametocytaemia were higher in DBA/2 mice than the other two host genotypes ($P < 0.01$). Thus DBA/2 mice had the highest transmission potential, while CBA/Ca mice had intermediate levels, and C57Bl/6J mice had the lowest levels whether infected with the CW-0 or CW-V lines, which were approximately the same. However, when data from mice that died were included in the analysis, the CW-V line suffered considerable loss in transmission potential as a result of premature host death (Table 1) in the two susceptible host genotypes.

Gametocyte conversion ratios showed a similar pattern through time in all three host genotypes and in both parasite lines (Fig. 3), though with an approximate 2-day delay in CW-V compared with CW-0 and an earlier clearance in C57Bl/6J mice, followed by CBA/Ca and then DBA/2 mice. When only data with non-zero gametocyte conversion ratios were included in the analyses in order to exclude this clearance effect, host differences became non-significant ($P > 0.10$), though parasite line differences remained ($P < 0.05$). Also when non-zero ratios were excluded, there were no host genotype or parasite line differences on the slope of the log-linear increase between Days 8 and 16, indicating that the factors that lead to a change in conversion ratio through time were qualitatively the same in both parasite lines and in all three host genotypes.

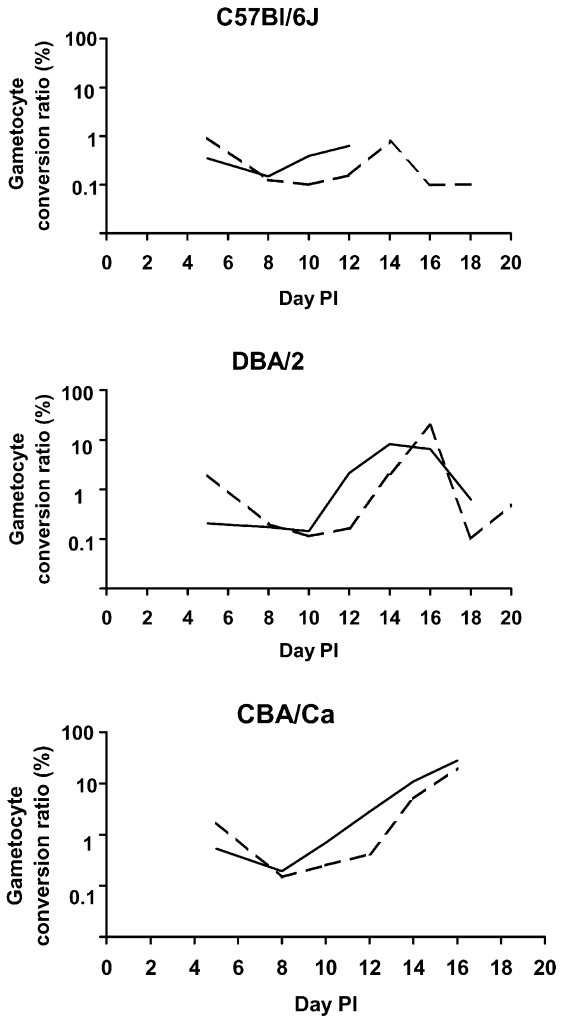

Fig. 3. Mean gametocyte conversion ratios through time in groups of mice of genotype C57Bl/6J, DBA/2 or CBA/Ca infected with an avirulent line (CW-0, solid line) or a virulent, adapted (CW-V, broken line) line of *P. chabaudi*.

3.2. Relationships among traits

In the absence of host death, virulence-related morbidity (weight and RBC loss) was positively related to maximum parasitaemia, with evidence of a 'plateau' (significant negative quadratic effect in addition to a significant linear effect) at high levels of virulence in the case of maximum RBC loss but not weight loss (Table 2). Transmission traits were linearly and positively related to maximum parasitaemia with no evidence of curvilinearity. However, transmission traits showed curvilinear relationships with virulence-related morbidity, especially with RBC loss, with maxima occurring at intermediate levels of virulence, giving a 'humped' relationship rather than a plateau-like relationship (Table 2). These patterns were broadly consistent across transmission traits indicating that these results were not due to auto-correlations generated by their method of calculation. An example of the humped relationship is shown in Fig. 4. The curvilinear components in these relationships existed in the absence of host death and so were generated by other causes: host death merely exacerbated them (Table 2), consistent with earlier results on mortality-related traits (Table 1).

The humped relationship between gametocyte numbers and RBC loss suggests that there is a cost to transmission at high morbidity levels in the absence of host death. As illustrated in Fig. 4, it is clear that this relationship was driven by the higher gametocyte production in DBA/2 mice which also had intermediate levels of morbidity. However, we cannot say whether this higher gametocyte production was due to some host genotype-specific effect, or whether it fitted within a general relationship that was common to all host genotypes and parasite lines. When host genotype, parasite line and their interaction were fitted in the model, there were no relationships among any of the traits. However, when examined within each host genotype separately, the relationships between RBC loss and all the gametocyte

Table 2
Relationships among asexual parasite load, virulence and transmission traits from regression analyses fitting a model with linear and quadratic terms for the regressor

Trait analysed	Regressor	Survivors only		Survivors and non-survivors	
		Linear[a]	Quadratic[a]	Linear	Quadratic
Virulence vs. asexual growth					
Maximum weight loss	Maximum parasitaemia	0.182**	−0.001	0.340**	−0.005†
Maximum RBC loss	Maximum parasitaemia	0.376**	−0.006*	0.371**	−0.006*
Transmission vs. asexual growth[b]					
Total gametocytes	Maximum parasitaemia	0.0822*	−0.0013	0.0709	−0.0013††
Total gametocytes post-crisis	Maximum parasitaemia	0.0832*	−0.0012	n.a.[c]	n.a.
Total gametocytaemia	Maximum parasitaemia	0.0623*	−0.0010	0.0532	−0.0010†
Total gametocytaemia post-crisis	Maximum parasitaemia	0.0635*	−0.0010	n.a.	n.a.
Average conversion ratio	Maximum parasitaemia	0.0445*	−0.0004	0.0480†	−0.0006
Maximum conversion ratio	Maximum parasitaemia	0.0407*	−0.0003	0.0481	−0.0008
Transmission vs. virulence[b]					
Total gametocytes	Maximum weight loss	0.156	−0.048†	0.107	−0.035††
Total gametocytes post-crisis	Maximum weight loss	0.125	−0.049	n.a.	n.a.
Total gametocytaemia	Maximum weight loss	0.114	−0.036†	0.076	−0.026†
Total gametocytaemia post-crisis	Maximum weight loss	0.093	−0.033	n.a.	n.a.
Average conversion ratio	Maximum weight loss	0.159	−0.043	0.126	−0.031
Maximum conversion ratio	Maximum weight loss	0.192	−0.051	0.135	−0.041
Total gametocytes	Maximum RBC loss	0.906	−0.067†	0.634††	−0.046†
Total gametocytes post-crisis	Maximum RBC loss	1.383	−0.103*	n.a.	n.a.
Total gametocytaemia	Maximum RBC loss	0.779	−0.058*	0.469	−0.035†
Total gametocytaemia post-crisis	Maximum RBC loss	0.999	−0.075*	n.a.	n.a.
Average conversion ratio	Maximum RBC loss	0.963	−0.071†	0.635	−0.047†
Maximum conversion ratio	Maximum RBC loss	0.858	−0.063	0.712	−0.052††

[a] Significance tests for the linear terms relate to a model without the quadratic term fitted, while significance tests for the quadratic terms relate to a model with both linear and quadratic terms fitted.
[b] Post-crisis values are from Days 12 to 22 PI. 'Transmission' traits are based on gametocyte numbers. See Table 1 for trait definitions and units. Estimates given are on the transformed scale.
[c] n.a. denotes 'not applicable' because data on the trait were available from survivors only.
* $P < 0.05$.
** $P < 0.01$.
† $P < 0.10$.
†† $P < 0.15$.

density and gametocytaemia traits were found to have a significant negative quadratic component (i.e. intermediate maximum) in the DBA/2 mice ($P < 0.05$ and $P < 0.10$, respectively), but not in the other host genotypes.

4. Discussion

The results from this study shed some light on what evolutionary factors regulate maximum virulence in malaria parasites. We found that a parasite line of *P. chabaudi* that had adapted to grow well in a single strain of inbred mouse, C57Bl/6J, also grew faster and caused more mortality and morbidity in two other, less resistant, strains of inbred mice compared with its ancestral control line. Thus, our data do not support the hypothesis that strong host genotype-specific adaptation of parasites leads to maladaptation when infecting alternative host genotypes. Instead, our data suggest that parasite adaptation towards higher virulence in C57Bl/6J mice was general such that any host genotype with lower resistance to one strain of *P. chabaudi* would also be less resistant to other strains. The experimental design of this study does not, however, allow us to rule out some degree of host-specific adaptation in addition to general adaptation: to do so would require adapting parasite lines to all three host genotypes and then testing each line in each host genotype. We can only conclude that any host genotype-specific adaptation that might have occurred was not large enough to overwhelm general adaptation.

We did, however, find support for one of the key assumptions of a large class of virulence evolution models, namely, that host death causes a cost to total lifetime transmission, i.e. parasite fitness. Mice that died had 26% of the transmission potential of those that survived: this was because they died just before the peak of the infectious period. Similarly, in the major human malaria species, *P. falciparum* and *P. vivax*, peak gametocyte production occurs soon after the acute phase and persists for up to many months (Kitchen, 1949). Thus, it is possible that malaria-induced human death rates, which in endemic areas of Africa amount to 10% of

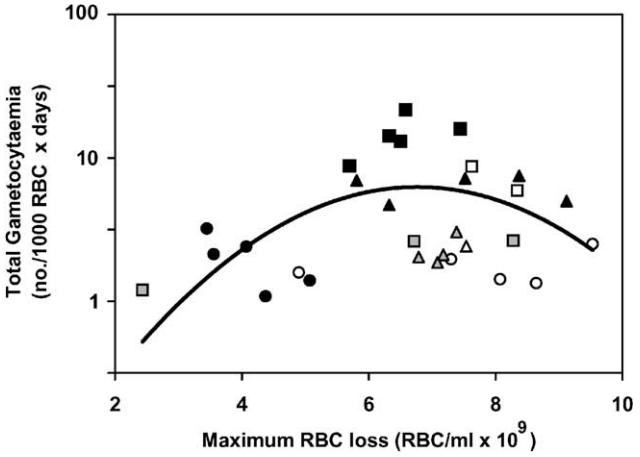

Fig. 4. Curvilinear relationship between a measure of total lifetime transmission and virulence-related morbidity in *P. chabaudi* infections. Maximum reduction in RBC density reached by the mouse is on the *x*-axis; and total gametocytaemia between Days 5 and 22 PI is on the *y*-axis. Each symbol represents a measure on an individual mouse of either genotype C57Bl/6J (circles), CBA/Ca (triangles) or DBA/2 (squares) infected with parasites of either an avirulent unadapted line (CW-0, closed symbols) or a virulent mouse-adapted line (CW-V, open symbols). Symbols in grey indicate that the mouse died before Day 22. The solid line is the least-squares best-fit quadratic relationship between the two traits after excluding data from mice that died.

children under 5 years dying from *P. falciparum* infection (Snow et al., 1999), may be a significant force in limiting the virulence of this major parasite of humans.

Our data also suggest that host death is not the only factor setting the upper limit on disease severity. In the absence of host death, two other constraints on parasite fitness-related traits were apparent. First, we observed an upper limit on morbidity (in the absence of host death). This was evident from (1) the smaller differences among host genotypes when infected with the virulent, evolved line than when infected with the avirulent, ancestral line (Fig. 2) and (2) the plateau-like relationship between morbidity and maximum parasitaemia (Table 2). Results from previous experiments have also indicated such a limit, namely (1) the lack of evolution to higher morbidity during serial passage of a clone that already had high virulence (Mackinnon and Read, 1999b) and (2) the convergence in morbidity levels between a virulent and avirulent clone as parasite inoculation dose increased (Timms et al., 2001). Combined, these suggest that some factor limits parasite densities and hence virulence. This may be RBC supply or some other host-derived factor that neutralises parasite growth such as immunity. An alternative explanation for the smaller differences in morbidity between host genotypes when infected with the virulent line than infected with the avirulent line is that cytokine-mediated, non-specific immunity may have been responsible for controlling peak parasite density in the faster growing line, whereas antibody-mediated immunity which does not become effective until around Day 10 PI (McLean et al., 1982; Jarra et al., 1986; Jarra and Brown, 1989), and which can differ with host HLA type, may have controlled parasite loads in the slower growing line: this explanation is consistent with the difference in timing of peak parasite density (Fig. 1). Whatever the factors regulating peak parasite load, however, the equal maximum morbidity observed in all three host genotypes when infected with the virulent line shows that host death is not the only factor regulating the upper limit of morbidity.

The second constraint on parasite fitness-related traits, other than host death, suggested by these data was a cost of high morbidity to lifetime gametocyte production. (The latter, we assume to represent lifetime transmission on the basis of previous studies that show a high correlation between post-peak gametocyte production and successful transmission to mosquitoes (Buckling et al., 1997; Taylor and Read, 1998; Mackinnon and Read, 1999a)). In this experiment, maximum lifetime transmission stage production occurred at intermediate levels of host morbidity, with declining levels of gametocyte numbers at high levels of RBC loss. In our previous studies, we have only seen positive relationships between gametocyte production and anaemia, but have not seen negative relationships between these traits at high morbidity levels (Mackinnon and Read, 1999a,b). We may have done so here because we used less resistant genotypes, thus extending the range of morbidity and mortality observed. The humped relationship between gametocyte production and morbidity (Table 2, Fig. 4) could be due to some undefined factor promoting gametocyte production that is specific to DBA/2 mice. Alternatively, it may be that the data fell on a curve reflecting a general causal relationship between gametocyte production and morbidity. However, we did not have enough experimental power, or sufficient numbers of susceptible host genotypes, or sufficient overlap between genotypes to distinguish between these hypotheses. In favour of the latter hypothesis was that the humped relationship was also found within the DBA/2 genotype, and that theoretical and experimental studies show that low RBC supply can be partly responsible for limiting asexual population growth rates (Hellriegel, 1992; Yap and Stevenson, 1994; Gravenor et al., 1995; Hetzel and Anderson, 1996). Since gametocyte production depends on asexual parasite density, limits on asexual parasite density might also limit gametocyte density. A humped relationship could also arise if parasites adaptively down-regulate the production of gametocytes during periods of acute anaemia. We did not find this, however, since gametocyte conversion ratios continued to increase in a highly consistent, log-linear fashion during the acute phase in this experiment (Fig. 3) and in previous studies (Buckling et al., 1999). Whatever the causal basis, our data are consistent with a fitness cost of high morbidity in the absence of host death, and with a life history trade-off between growth and reproduction, both of which are potential explanations for why parasites do not evolve higher virulence. More experiments are required to determine whether this result is general across a range of host and parasite genotypes and environmental conditions.

We do not know if the same morbidity-related constraints on virulence observed here apply in the human malaria, *P. falciparum*. Levels of parasitaemia and anaemia are rarely as severe as in the *P. chabaudi*-mouse system. However, absolute values are not important to virulence constraints: what matters is the marginal change in transmission per unit change in virulence or growth. Unfortunately, little is known about the transmission consequences of factors that regulate growth and virulence in malaria parasites of humans. We also do not know whether host genotype by parasite genotype interactions are strong in human malaria, though given that substantial genetic variation in host resistance (Weatherall, 1987) and parasite virulence (Gravenor et al., 1995; Chotivanich et al., 2000) for *P. falciparum* malaria exists, there is certainly the potential for them. In addition to the virulence-limiting factors examined in this study, we also envisage other potential barriers to high virulence in malaria in the field. One of these might be fitness costs to the mosquito of carrying malaria transmission forms (Hurd et al., 1995; Koella et al., 1998; Anderson et al., 2000; Ferguson and Read, 2002). Other possible constraints on virulence evolution include mutation accumulation (Bergstrom et al., 1999; Elena et al., 2001) and spatial structure (Lipsitch et al., 1995; Boots and Sasaki, 1999; Haraguchi and Sasaki, 2000). Nonetheless, the picture building up from our studies is that the genetic differences in virulence among *P. chabaudi* clones as measured in the vertebrate host are consistent across a range of conditions such as dose (Timms et al., 2001), host sex (unpublished data), host immune status (Buckling and Read, 2001), drug treatment (Buckling et al., 1997), the presence of competing parasite genotypes (Read and Taylor, 2001; Taylor et al., 1998; Timms, 2001), and now host genotype. If the virulence property of these parasite lines continues to prove to be robust to the many other factors that determine disease severity, we will have more confidence in the evolutionary theory derived from these empirical studies, such as predictions of the public health consequences of using anti-disease vaccines (Gandon et al., 2001).

Acknowledgements

We thank Heather M. Ferguson, Sylvain Gandon and Lucy Crooks for helpful comments on the manuscript, and B. Chan and J. de Roode for assistance with data collection. The work was supported by the Leverhulme Trust, the BBSRC and the University of Edinburgh. Experimental work on mice was conducted in accordance with the Animal (Scientific Procedures) Act 1986 administered by the Home Office of the U.K. (Personal Licence PIL 60/5649).

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