Virulence and competitive ability in genetically diverse malaria infections

Jacobus C. de Roode*†, Riccardo Pansini‡, Sandra J. Cheesman, Michelle E. H. Helinski§, Silvie Huijben, Andrew R. Wargo, Andrew S. Bell, Brian H. K. Chan, David Walliker, and Andrew F. Read

Institutes of Evolution, Immunology, and Infection Research, School of Biological Sciences, University of Edinburgh, King's Buildings, West Mains Road, Edinburgh EH9 3JT, Scotland, United Kingdom

Edited by Nancy A. Moran, University of Arizona, Tucson, AZ, and approved April 1, 2005 (received for review January 5, 2005)

Explaining parasite virulence is a great challenge for evolutionary biology. Intuitively, parasites that depend on their hosts for their survival should be benign to their hosts, yet many parasites cause harm. One explanation for this is that within-host competition favors virulence, with more virulent strains having a competitive advantage in genetically diverse infections. This idea, which is well supported in theory, remains untested empirically. Here we provide evidence that within-host competition does indeed select for high parasite virulence. We examine the rodent malaria *Plasmodium chabaudi* in laboratory mice, a parasite–host system in which virulence can be easily monitored and competing strains quantified by using strain-specific real-time PCR. As predicted, we found a strong relationship between parasite virulence and competitive ability, so that more virulent strains have a competitive advantage in mixed-strain infections. In transmission experiments, we found that the strain composition of the parasite populations in mosquitoes was directly correlated with the composition of the blood-stage parasite population. Thus, the outcome of within-host competition determined relative transmission success. Our results imply that within-host competition is a major factor driving the evolution of virulence and can explain why many parasites harm their hosts.

competition | evolution | parasite | *Plasmodium* | mixed infection

Explaining virulence is fundamental to understanding the life history of parasites, arguably the most abundant group of creatures on the planet (1). The problem is to explain why parasites, which rely on their hosts for survival and fitness, should cause disease or indeed kill their hosts (2–6). Many explanations of parasite virulence have been put forward (3, 4), but the idea that has received the most attention is that virulence is a consequence of a parasite's efforts to maximize its fitness: parasites require extensive within-host replication to achieve transmission to the next host, but at the same time such replication damages host tissues, increasing the chances of killing the host (2, 3, 7–9). Higher levels of virulence than predicted by this model, however, could arise due to within-host competition between parasite strains (9–14). Many, if not most, parasite infections consist of genetically distinct strains of the same parasite species or contain virulent mutants that have arisen *de novo* (15). It is generally assumed that parasites that exploit their hosts prudently suffer great fitness losses in hosts simultaneously infected with more aggressive parasites. This is because virulent parasites could kill the host or competitively exclude prudent parasites before the latter have realized transmission. Even though host death also reduces the fitness of virulent parasites, prudent parasites suffer disproportionately and are eliminated by natural selection, a process commonly known as "the tragedy of the commons" (16).

Several authors (17, 18) have gone so far as to claim that reducing the number of genetically mixed infections should have beneficial health effects, by reducing the amount of within-host competition and hence reducing the selection for increased virulence. However, despite the large number of theoretical studies addressing within-host competition, there is no experimental evidence that virulent parasites are indeed competitively superior to prudent parasites and that virulent parasites transmit at a higher rate from mixed infections (15), two assumptions critically underlying this theory. The only evidence supporting this claim is indirect; serial passage of pathogens typically increases virulence in the host used for passage (5, 19), and live attenuated vaccines occasionally revert to wild-type virulence (20, 21). Both phenomena could be due to virulent variants rising in frequency, because they have a competitive advantage within hosts, but other explanations are possible. It is possible, for example, that a buildup of genetic diversity *per se* results in higher virulence (15).

We used the rodent malaria *Plasmodium chabaudi* in laboratory mice to determine whether virulent parasites have a competitive advantage in genetically diverse infections, and whether the genetic composition of transmitted parasite populations are a reflection of the outcome of this competition.

To address the competition-virulence question, we used seven strains that were genetically closely related but differed in their virulence (here denoted AS1, AS3a, AS3m, AS3v, AS4, AS5, and AS6) and competed these against an unrelated, and more virulent strain, denoted AJ. We infected groups of mice with either of the strains on their own or with two-strain mixtures of AJ and one of the AS strains. Thus, we used AJ as a reference strain, against which we competed the different AS strains. Densities of individual strains in mixed infections were tracked by using strain-specific quantitative real-time PCR (22, 23) and virulence measured as anemia (24). Our conclusions were unaltered if mouse weight loss had been used instead (data not shown).

To test whether the outcome of within-host competition was a determinant of transmission success, we carried out two experiments in which malaria mosquitoes were fed to mice infected with either single or mixed infections of AS and AJ strains. The infection status of mosquitoes and the number of AS and AJ parasites were assessed by using real-time PCR and related to the AS and AJ parasite densities in the mice on which the mosquitoes fed. These transmission experiments were important, because it is theoretically possible that parasites that are competitively suppressed could put more resources into transmission-stage production, so that competitive suppression could actually lead to higher relative transmission (13).

This paper was submitted directly (Track II) to the PNAS office.

Abbreviation: RBC, red blood cell.

*To whom correspondence should be addressed. E-mail: jderood@emory.edu.

†Present address: Department of Environmental Studies, Emory University, 400 Dowman Drive, Atlanta, GA 30322.

‡Present address: Evolutionary Anthropology Research Group, Department of Anthropology, University of Durham, 43 Old Elvet, Durham DH1 3HN, United Kingdom.

§Present address: Entomology Unit, Agriculture and Biotechnology Laboratory, International Atomic Energy Agency (IAEA), A-2444 Seibersdorf, Austria.

www.pnas.org/cgi/doi/10.1073/pnas.0500078102

Methods

Parasites and Hosts. Parasite strains AS3m and AJ were originally clonally derived from different thicket rats of the species *Thamnomys rutilans* (25); AS3a was then obtained from AS3m through drug selection (26), and AS3v, AS1, AS4, AS5, and AS6 were subsequently derived from AS3a by passage through mice, thicket rats, and mosquitoes. Mice were age-matched 6- to 8-week-old female C57BL/6J mice (Harlan Olac, Bichester, U.K.; Ann Walker, University of Edinburgh), fed, and kept as described (22).

Virulence and Competitiveness. Five experiments were carried out, each making use of different AS strains. In all experiments, closely related strains of AJ were used as the tester strains against which we competed the different AS strains. In the first experiment, five mice were infected with AS1, four with AJ, and four with AS1+AJ. In the second, groups of five mice were infected with AS3a, AS3m, AS3v, or AJ alone or with mixtures of each AS strain and AJ. In the third, five mice were infected with AS4, three with AJ, and four with AS4+AJ. In the fourth, seven mice were infected with AS5, six with AJ, and six with AS5+AJ. In the final experiment, nine mice were infected with AS6, 15 with AJ, and 15 with AS6+AJ.

Mice infected with just AS or AJ received 10^6 parasite-infected red blood cells, whereas mice infected with both strains received 2×10^6 parasites, made up of 10^6 AS and 10^6 AJ parasites. The latter was done because, in our analysis, we wanted to compare the performance of a strain on its own with its performance in a mixed infection, requiring equal numbers of each parasite strain at inoculation. A 2-fold difference in parasite numbers has a negligible effect on parasite dynamics and virulence (27). In the final experiment, infections were initiated with $1 \times 10^5/2 \times 10^5$ or $1 \times 10^7/2 \times 10^7$ parasites. Parasite densities were tracked for 14–50 days by using strain-specific quantitative PCR (22, 23) and mouse red blood cell densities and body weights monitored (22). All procedures were regulated and carried out under the U.K. Home Office Animals (Scientific Procedures) Act, 1986.

Mosquito Transmission. Two experiments were carried out to study the relationship between within-host competition and transmission. In the first, we infected groups of nine mice with either AS5, AJ, or a mixture of AS5 and AJ, as described above. We fed groups of 30 2- to 5-day-postemergence female *Anopheles stephensi* mosquitoes on individual mice on days 7, 14, and 21 postinfection (protocols as in ref. 28). Mice were killed after mosquito feeds, so that they were fed once only. Eight days postfeed, mosquito midguts were dissected and DNA extracted from them. We then used real-time PCR to assess whether mosquitoes were infected and how many AS and AJ parasites they harbored. The experiment was repeated by using groups of six mice, feeding 3- to 7-day-postemergence mosquitoes on individual mice on days 6 and 7 postinfection and dissecting mosquito midguts 10 days postfeed.

Trait Definition. For all experiments, we calculated the total numbers of AS and AJ parasites produced over the first 14 days of infection. Because *P. chabaudi* undergoes a 24-hour replica-

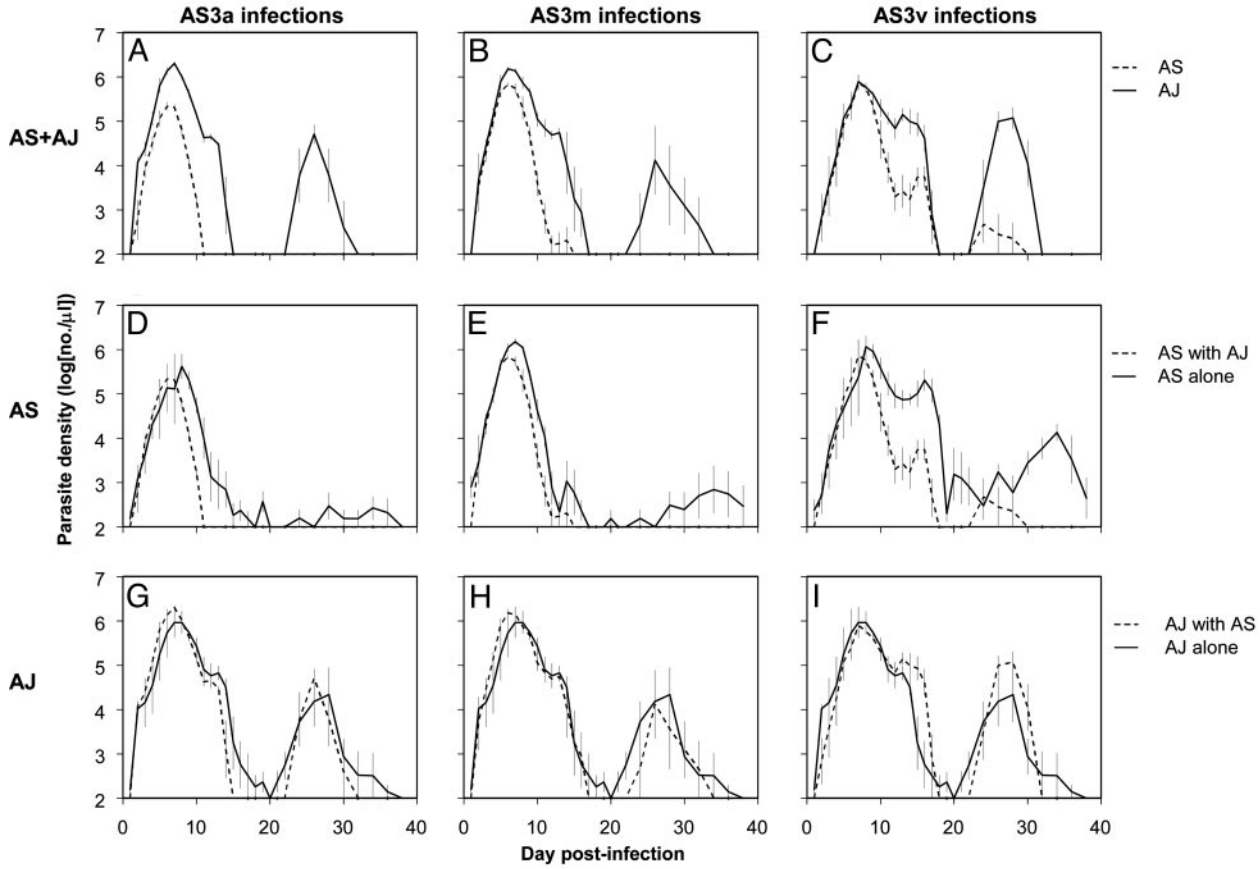

Fig. 1. Parasite densities over time (mean ± 1 SEM) for AS3a, AS3m, and AS3v. Graphs compare AS with AJ strains in mixed infections (*A–C*), AS strains in mixed and single infections (*D–F*), and AJ strains in mixed and single infections (*G–I*). AS strains persisted for at least 30 days when infecting mice alone but for a much shorter time period in competition with AJ [$F_{(1,24)} = 42$, $P < 0.001$ for AS3a, AS3m, and AS3v; $F_{(1,61)} = 36$, $P < 0.001$ for all AS strains]. The limit of detection was 100 parasites per microliter of blood, so that *y* axes start at 2.

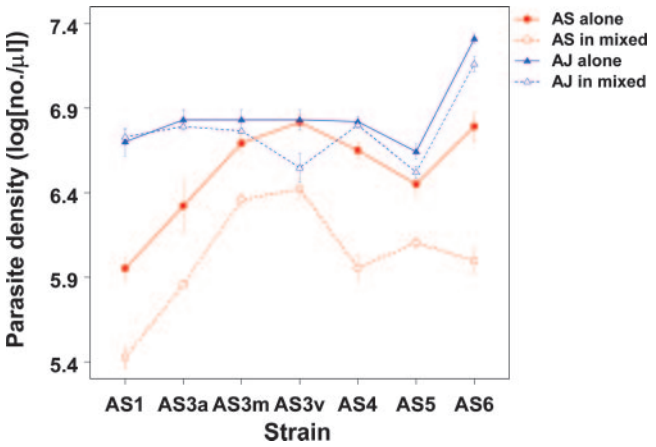

Fig. 2. AS and AJ parasite densities in single and mixed infections (mean ± 1 SEM). Strains of both lineages were suppressed by the presence of a competitor.

tion cycle, summing the daily parasite densities gives the total number of parasites produced. "Relative AS density" was defined as the proportion of the total number of parasites produced over the first 14 days of an infection that were AS. Relative AS density was determined for each mouse separately; means were then calculated per experiment and used for analysis. "Competitive suppression" was calculated by dividing the mean density that AS obtained in competition by the mean density it obtained alone in an experiment and subtracting this from 1. "Relative anemia" was calculated as follows: for each mouse, the minimum red blood cell (RBC) density was determined and subtracted from the initial RBC density to give RBC loss; this was then divided by the initial RBC density to give proportional RBC loss. Mean proportional RBC losses were calculated for AS- and AJ-infected mice within an experiment, and relative RBC loss was calculated by dividing the mean of AS by the mean of AJ. In this way, the virulence of an AS strain could be expressed relative to the AJ strain against which it was competed.

To estimate transmission success, we analyzed the proportions of mosquitoes infected with AS or AJ and the numbers of AS and AJ parasites per PCR-positive mosquito. We also determined the proportions of AS in the mosquito population and compared these with the proportions of AS in the parasite population in the mouse. Proportions were calculated in two ways. The first summed the AS parasites over all of the mosquitoes fed on a single mouse and then divided this number by the overall AS+AJ parasites summed over those mosquitoes. The second determined the proportion of AS in each PCR-positive mosquito and then averaged these per mouse. This analysis was aimed at assessing whether within-host frequency was a good predictor of relative transmission to the mosquito vector. Because *P. chabaudi* gametocytes (the transmissible malaria stages that develop from asexual blood parasites) take ≈36–54 h to mature (29), and because mice were fed to mosquitoes around 1600 h in the afternoon on feed days, we analyzed the corresponding asexual densities as monitored around 0900 h the day before feeding.

Statistical Analysis. All analyses were performed in statistical packages MINITAB 13.30 (Minitab, State College, PA) or R 1.6.2 (www.stats.uwaterloo.ca/Stats_Dept/StatSoftware/R) by using general linear modeling. Absolute parasite densities were log-transformed and relative AS densities and AS proportions arcsine-square-root-transformed before analysis to meet the homogeneity-of-variance and normality-of-error assumptions of the models used.

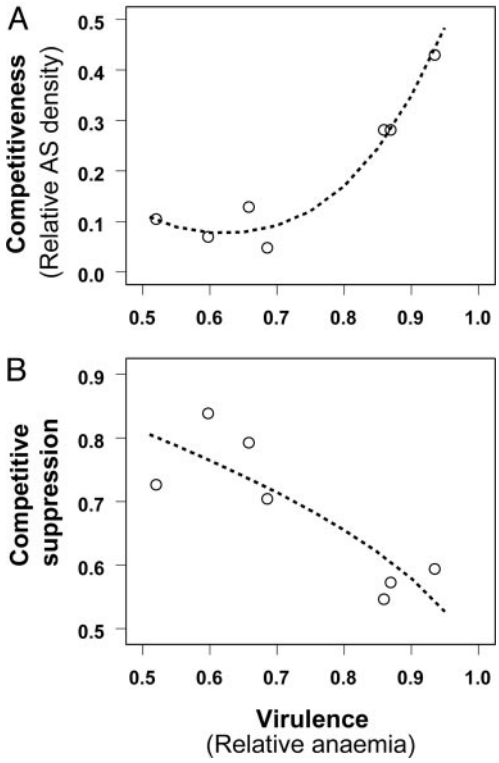

Fig. 3. The relationships between virulence and competitiveness (*A*) and virulence and competitive suppression (*B*). Virulence of the AS strains was measured as the anemia induced in single-strain infections and expressed as the fraction of the anemia contemporaneously induced by single-strain infections of AJ. (*A*) Competitiveness was defined as the proportion that AS obtained of the total parasite population in the mixed-strain infections. Regression analysis was done by using arcsine-square-root-transformed data; the plotted regression line is back-transformed. Because the minimal model included a quadratic virulence term ($P = 0.035$, $R^2 = 0.90$), the relationship between virulence and competitiveness is curvilinear. (*B*) Relative competitive suppression was defined as the proportional reduction of parasite numbers of AS strains in competition from what they achieved in single-strain infections. Regression analysis was done by using arcsine-square-root-transformed data ($P = 0.021$, $R^2 = 0.63$); the plotted regression line is back-transformed.

Proportions of infected mosquitoes and numbers of parasites per mosquito were analyzed by using general models with quasibinomial and Poisson error distributions, respectively, to correct for overdispersion (30). Because mouse, and not mosquito, was the proper replication unit in these experiments, numbers of parasites per mosquito were averaged over the total number of infected mosquitoes that fed on a single mouse to get rid of pseudoreplication (30, 31). Significance of model terms was based on deletion of such terms followed by model comparison as described (30).

Results

Virulence and Competitiveness. In all experiments, strain AJ competitively suppressed AS, prematurely truncating infections (see Fig. 1 for a subset of infections) and reducing parasite densities [Fig. 2; single/mixed: $F_{(1,67)} = 125$, $P < 0.001$]. The tester strain AJ, which was the dominant strain in all mixed infections, also suffered competitive suppression, although less so than the AS strains [Fig. 2; single/mixed: $F_{(1,76)} = 16$, $P < 0.001$]. It is likely that competition occurred because the total number of parasites of both strains that could be present in an infection was constrained: overall parasite numbers (AS+AJ) in mixed infections were never different from those produced by AJ alone [$F_{(1,68)} = 1.23$, $P = 0.27$].

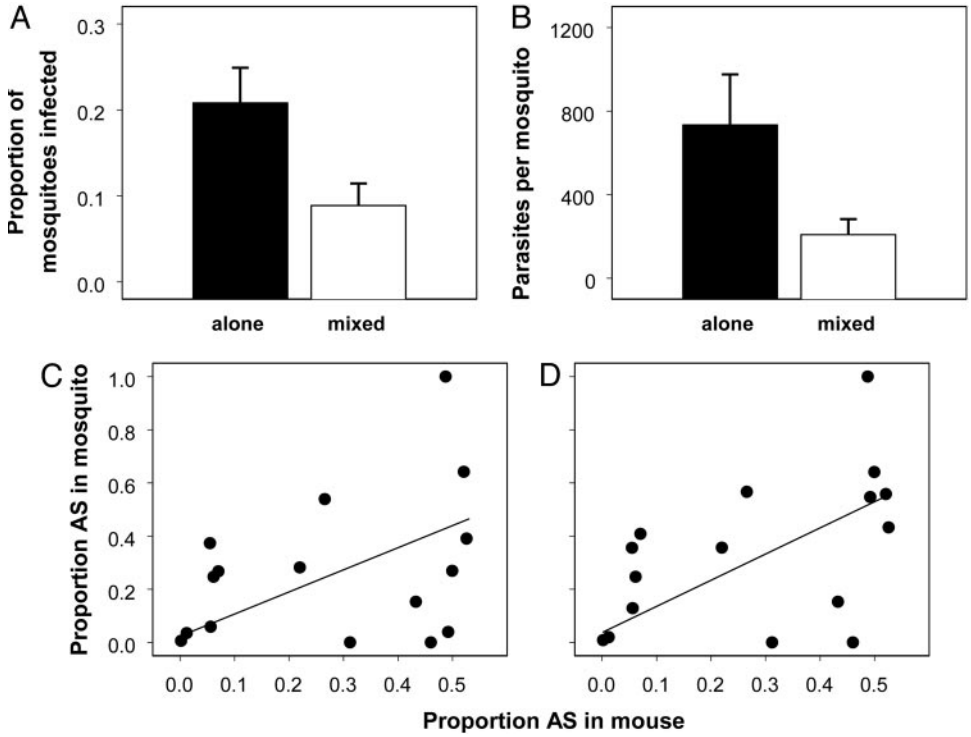

Fig. 4. Transmission of AS to mosquitoes. (*A*) Proportions of mosquitoes infected with AS parasites from single and mixed infections. (*B*) Numbers of AS parasites per infected mosquito from single and mixed infections. For *A* and *B*, the same patterns were found for both experiments (single/mixed × experiment interaction; $P > 0.05$). (*C*) Proportion of AS (calculated by summing the number of AS parasites over all mosquitoes fed on a single mouse divided by the overall AS+AJ parasites in those mosquitoes) vs. its asexual proportion in the mouse [$F_{(1,15)} = 7.04$, $P = 0.018$, $R^2 = 0.27$]. (*D*) Average proportion of AS (calculated by determining the proportion of AS in each PCR-positive mosquito and then averaging these per mouse) vs. its asexual proportion in the mouse [$F_{(1,15)} = 9.54$, $P = 0.007$, $R^2 = 0.35$]. For *C* and *D*, the same patterns were found in both experiments (experiment and blood-stage proportion AS × experiment interaction: $P > 0.05$).

Although all AS strains suffered from competition, the extent of competitive suppression was related to intrinsic virulence, so that more virulent AS strains achieved higher densities in competition with AJ (Fig. 3*A*). AS strains that were more virulent in single-strain infections not only obtained greater parasite densities; they also suffered relatively less competitive suppression than did less virulent AS strains (Fig. 3*B*). The curvilinear shape of the virulence-competitiveness relationship (Fig. 3*A*) suggests that relatively small changes in virulence can have large effects on competitiveness. Thus, only AS strains as virulent as AJ would obtain large population sizes in mixed infections (Fig. 3*A*).

Mosquito Transmission. In both experiments, the transmission of strain AS to mosquitoes was drastically reduced by competition within the vertebrate host (Fig. 4), in terms of both the numbers of mosquitoes infected [Fig. 4*A*; single/mixed: $F_{(1,67)} = 9.3$, $P = 0.003$] and the parasite burdens in mosquitoes it did infect [Fig. 4*B*; single/mixed: $F_{(1,39)} = 4.5$, $P = 0.040$]. AJ also produced fewer parasites in mosquitoes that it infected from mixed than from single infections [single/mixed: $F_{(1,32)} = 7.2$, $P = 0.012$], but it did not infect significantly fewer mosquitoes [single/mixed: $F_{(1,66)} = 3.7$, $P = 0.06$]. In both experiments, and for both strains, transmission success increased with the density of blood-stage parasites (slopes significantly greater than zero in all cases), whether transmission was measured as the proportion of mosquitoes infected [AS, $F_{(1,67)} = 26$, $P < 0.001$; AJ, $F_{(1,66)} = 10.8$, $P = 0.002$] or the parasite numbers in mosquitoes they did infect [AS, $F_{(1,39)} = 4.7$, $P = 0.036$; AJ, $F_{(1,32)} = 10.3$, $P = 0.003$]. Thus, competitive suppression of blood-stage densities decreased mosquito transmission.

Importantly, in both experiments, we found a significant positive relationship between the frequency of a strain within a mouse and its frequency in the parasite population transmitted to mosquitoes (Fig. 4 *C* and *D*). The frequency of AS within the mouse was the same as that in the transmitted population (intercepts and slopes of regression lines not different from 0 and 1, respectively; $P > 0.05$ in all cases). Thus, the outcome of within-host competition directly determines between-host transmission success.

Discussion

This study shows that in mixed-strain infections of rodent malaria parasites, more virulent strains have a competitive advantage within their rodent host (Fig. 3). Importantly, our transmission experiments showed that competitive suppression of a strain within hosts also suppresses that strain's transmission to mosquitoes. Moreover, strain frequency in mosquitoes was directly related to strain frequency in the mouse (Fig. 4). It is theoretically possible that parasites that are competitively suppressed increase their relative investment in transmission stage production (13). Malaria parasites can do this in response to subcurative drug treatment (32), but we found no evidence that they do this in response to competitive suppression (Fig. 4; see also ref. 33). Thus, high within-host competitive ability increases between-host fitness.

The relationship between virulence and competitiveness explains several biological phenomena. First, in serial passage experiments, malaria (19) and other parasites (5) often become more virulent and in live attenuated vaccines, mutants with wild-type virulence can arise (20, 21). In both cases, the virulence increases could be a direct result of new virulent mutants outcompeting the variants that predominate in the current population within the host.

EVOLUTION

Second, the virulence-competitive ability relationship we have demonstrated also explains why parasite species can be highly virulent to their hosts, despite the fact that they depend on them for their survival. Many theoretical studies have suggested that virulence is a consequence of a parasite's efforts to maximize its fitness: parasites require extensive within-host replication to achieve transmission to the next host, but at the same time, such replication damages host tissues, increasing the chances of killing the host (2, 3, 7–9). A general conclusion from these studies is that parasites evolve some intermediate level of virulence. Higher levels of virulence, however, could arise due to within-host competition (9–14), if more virulent parasites outcompete less virulent parasites and transmit at a relatively higher rate to the next round of hosts. Within-host competition can thus increase the average virulence of a population of parasites above the equilibrium level expected when there is no competition. Without within-host competition, parasites will maximize transmission with risk of host death as the sole factor selecting against excessively virulent strains. When within-host competition occurs, risk of competitive suppression enters the evolutionary equation, favoring parasites that have a higher chance of killing their host because they are less likely to be competitively suppressed.

That we found a strong relationship between virulence and within-host competitiveness suggests this is indeed the case; selection for more competitive strains will also select for strains with higher virulence, resulting in higher average levels of virulence in a population (9–13). Of course, the exact average level of virulence in a population will also strongly depend on how common mixed infections are. Thus, even though a highly virulent parasite strain may be competitively superior and transmit at a higher rate from a mixed infection than a prudent parasite, it could transmit from single infections at a lower rate than prudent parasites, because it more frequently kills its hosts. Moreover, if a mutant arose that was so virulent that it killed the multiply infected host before any transmission could take place, it would soon be selected against at the between-host level.

It is becoming increasingly clear that parasite infections commonly consist of several, if not many, distinct genotypes (reviewed in ref. 15). In human malaria, which is perhaps the most widely genotyped parasitic disease in the field, the majority of infections are mixed, and the number of coinfecting strains, in extreme cases, can exceed 10 (e.g., refs. 34–36). Our data strongly suggest that such mixed infections importantly shape parasite virulence. Interventions aimed at reducing the frequency of mixed-strain infections could then indeed lead to reduced levels of virulence by taking away the evolutionary pressure on the maintenance of competitiveness (17, 18).

We thank N. Colegrave, M. Guinnee, T. Little, M. Mackinnon, S. Nee, L. Råberg, and S. West for discussions; R. Mooney, D. Sim, and K. Watt for technical assistance; and the March animal house personnel for excellent husbandry. C. Lively and N. Moran, as well as an anonymous reviewer, provided valuable comments that improved the manuscript. This work was supported by the Wellcome Trust and the Biotechnology and Biological Sciences Research Council. J.C.d.R. was supported by the Darwin Trust of Edinburgh.

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