Dynamics of Multiple Infection and Within-Host Competition in Genetically Diverse Malaria Infections

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abstract: Within-host competition between coinfecting parasite strains shapes the evolution of parasite phenotypes such as virulence and drug resistance. Although this evolution has a strong theoretical basis, within-host competition has rarely been studied experimentally, particularly in medically relevant pathogens with hosts that have pronounced specific and nonspecific immune responses against coinfecting strains. We investigated multiple infection in malaria, using two pairs of genetically distinct clones of the rodent malaria *Plasmodium chabaudi* in mice. Clones were inoculated into mice simultaneously or 3 or 11 days apart, and population sizes were tracked using immunofluorescence or quantitative polymerase chain reaction. In all experiments, at least one of the two clones suffered strong competitive suppression, probably through both resource- and immune-mediated (apparent) competition. Clones differed in intrinsic competitive ability, but prior residency was also an important determinant of competitive outcome. When clones infected mice first, they did not suffer from competition, but they did when infecting mice at the same time or after their competitor, more so the later they infected their host. Consequently, clones that are competitively inferior in head-to-head competition can be competitively superior if they infect hosts first. These results are discussed in the light of strain-specific immunity, drug resistance, and virulence evolution theory.

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Humans and animals often become infected with different strains of the same parasitic species, and so parasite strains have to compete for limited amounts of resources and interact through strain-transcending immunity (Read and Taylor 2001). Such competition—here defined as reductions in parasite density caused by the presence of another clone—can be intense and has important evolutionary and medical consequences. Within-host competition is a major determinant in the evolution and epidemiology of drug resistance (Hastings and D'Alessandro 2000; de Roode et al. 2004*a*) because the evolutionary success of drug-resistant mutants crucially depends on whether they are able to outcompete drug-sensitive wild-type strains. The same is true for vaccine-escape mutants (e.g., Kew et al. 2002): their evolution and spread through the population will depend on their competitiveness with attenuated pathogen forms in vaccinated hosts and wild-type strains in others (Bull 1994). Finally, it is frequently asserted that competition between parasite strains selects for increased virulence and thus more severe disease, because virulent genotypes are assumed to outcompete prudent genotypes, resulting in a selective advantage of high virulence (e.g., Levin and Pimentel 1981; Bremermann and Pickering 1983; Van Baalen and Sabelis 1995; Frank 1996).

Despite these consequences, an understanding of within-host competition and parasite evolution is still seriously hampered by a lack of experimental data. Whereas numerous authors have studied multiple infection mathematically (e.g., Levin and Pimentel 1981; Bremermann and Pickering 1983; Nowak and May 1994; May and Nowak 1995; Van Baalen and Sabelis 1995; Frank 1996; Mosquera and Adler 1998; Adler and Mosquera Losada 2002; Nowak and Sigmund 2002), our empirical understanding is currently patchy and inconclusive (Read and Taylor 2001). Thus, in many models it is assumed that parasites growing more rapidly and inflicting more damage to their host are competitively superior to less harmful parasites, yet experimental evidence to support this claim is prac-

Table 1: Numbers of mice that were analyzed from each treatment group

Experiment	Mice
Experiment 1:	
Coinfection experiment:	
AS alone	5
CB alone	3
AS+CB	19
Superinfection experiment:	
AS alone	6
CB alone	3
AS day 0, CB day 3	7
CB day 0, AS day 3	5
Experiment 2:	
AS(pyr1A) alone day 0	4
AS(pyr1A) alone day 3	5
AS(pyr1A) alone day 11	5
AJ alone day 0	3
AJ alone day 3	4
AJ alone day 11	5
AS(pyr1A)+AJ day 0	4
AS(pyr1A) day 0, AJ day 3	4
AS(pyr1A) day 0, AJ day 11	3
AJ day 0, AS(pyr1A) day 3	5
AJ day 0, AS(pyr1A) day 11	4

tically absent (but see de Roode et al. 2005). Experiments have shown that competitive success of competing clones cannot be easily predicted from their growth rates in single infections (Nakamura et al. 1992; Hodgson et al. 2004) and that host genotype (Wille et al. 2002; de Roode et al. 2004*b*) also plays an important role. Furthermore, vaccination with benign parasite strains can protect against challenge with more virulent strains, suggesting that residency could also be an important determinant of within-host competition (Orcutt and Schaedler 1973; Morrison et al. 1982; Duval-Iflah et al. 1983; Allaker et al. 1988; Barrow and Page 2000). Prior colonization of *Silene latifolia* flowers by one strain of the anther-smut pathogen also prevented colonization of competitors (Hood 2003), and competitive ability was enhanced for virus species infecting their larval insect hosts before their competitors (e.g., Ishii et al. 2002; Thomas et al. 2003).

Here we report the first detailed study on the role of residency on within-host competition in a mammalian disease: malaria. We used two pairs of clones of the rodent malaria *Plasmodium chabaudi* and infected mice with them simultaneously or 3 or 11 days apart. Thus, we asked how parasite genotype, residency, and time between first and second infection affected within-host competitive ability.

In human malaria, mixed infections are extremely common, with prevalence reaching more than 80% in high-transmission areas (e.g., Babiker et al. 1999; Konaté et al. 1999; Magesa et al. 2002). These mixed infections occur because hosts become infected with different strains simultaneously (i.e., coinfection) or sequentially (i.e., superinfection). Coinfection occurs because one mosquito bite can contain a range of genetically distinct genotypes (Conway et al. 1991; Druilhe et al. 1998). Superinfection is also common and occurs because people are bitten by numerous mosquitoes injecting different malaria strains (Ntoumi et al. 1995; Daubersies et al. 1996; Arnot 1998; Basco and Ringwald 2001; Arez et al. 2003).

Whether malaria strains infect their host simultaneously or sequentially will very likely affect the extent to which they suffer from competitive suppression. This is because malaria parasites replicate exponentially during an infection, causing severe anemia (Cox 1988; Jakeman et al. 1999; Menendez et al. 2000) and inducing strong antiparasitic immune responses (Taylor-Robinson 1995; Li et al. 2001; Artavanis-Tsakonas et al. 2003); thus, strains infecting an already infected host will have fewer resources and encounter a stronger immune response than strains infecting naive hosts (e.g., Hellriegel 1992).

Material and Methods

Parasites and Hosts

Two main experiments were conducted, each making use of a different pair of *Plasmodium chabaudi* clones. Experiment 1 used clones AS and CB, while experiment 2 used AS(pyr1A) and AJ. Clones AJ, AS, and CB were originally cloned from wild parasite isolates from thicket rats in the Central African Republic (Beale et al. 1978); AS(pyr1A) was then derived from AS by selection for resistance against the drug pyrimethamine (Walliker et al. 1975). So far as we know, clones AS, AJ, and CB are unrelated clones, because they originally came from different isolates (hosts) collected at different locations. They are genetically (and antigenically) distinct at multiple loci.

Hosts were 9-week-old C57Bl/6J male (experiment 1) or female (experiment 2) inbred mice (Harlan, United Kingdom) kept in a reversed (experiment 1) or normal (experiment 2) 12L : 12D cycle. They were kept and fed as described by de Roode et al. (2004*b*).

Experiment 1: AS and CB

The first experiment, studying competition between clones AS and CB, consisted of two subexperiments. The first subexperiment ("coinfection") tested whether parasite clones suffer from competition when infecting the same host simultaneously. Six mice were infected intravenously with 10^4 AS parasites, six mice with 10^4 CB parasites, and 24 mice with 10^4 AS + 10^4 CB parasites. The second subexperiment

("superinfection") then asked whether competition is stronger for clones that infect mice already infected with another clone. Two groups of seven mice were infected with either 10^4 AS or CB parasites alone; 11 mice were infected with 10^4 AS parasites on day 0 and then with 10^4 CB parasites on day 3, and 10 mice were infected with 10^4 CB parasites on day 0 and 10^4 AS parasites on day 3.

Mouse red blood cell densities were determined using flow cytometry (Beckman Coulter) on 5 μL of mouse blood obtained from the tail. Thin blood smears were taken to determine parasitemias (here defined as the percentage of red blood cells infected with parasites) using 1,000 × microscopy; parasite densities were then calculated as the product of red blood cell densities and parasitemias taken on the same day. For mixed infections, numbers of AS and CB parasites were determined using immunofluorescent antibody assays (IFAs). The monoclonal antibodies used were H3, reacting specifically to CB, and B4 and B15, reacting specifically to AS (Boyle et al. 1982; McLean et al. 1991); IFA protocols were as described elsewhere (Taylor et al. 1997). These assays give ratios of the two infecting clones, which—multiplied by the overall parasite density—give the clonal parasite densities on a given day. As IFAs can give biased estimates of ratios (Taylor et al. 1997), comparisons of observed and expected ratios in artificial mixtures of AS and CB were used to calculate appropriate correction factors (as described by Taylor et al. [1997]). All measurements were taken three times a week until day 35 (coinfection) or day 57 (superinfection).

Experiment 2: AS(pyr1A) and AJ

Using a different pair of *P. chabaudi* clones, the second experiment asked whether clones suffered more from competition the later they infected a host already infected with another clone. On days 0, 3 and 11, groups of five mice were infected intraperitoneally with either 10^6 AS(pyr1A) or 10^6 AJ parasites alone, while another group of mice were infected with both 10^6 AS(pyr1A) and 10^6 AJ parasites simultaneously. Mice in two further treatment groups were infected with 10^6 AS(pyr1A) parasites on day 0 and then with 10^6 AJ parasites on days 3 or 11, while the final two groups of mice were infected with 10^6 AJ parasites on day 0 and then with 10^6 AS(pyr1A) parasites on days 3 or 11.

Mouse red blood cell densities were determined using flow cytometry (Beckman Coulter) of 2 μL of tail blood. Thin blood smears were taken, and parasitemias were calculated using microscopy (de Roode et al. 2004*b*); parasite densities were then calculated as the product of red blood cell densities and parasitemias taken on the same day. In mixed infections, AS(pyr1A) and AJ were distinguished and quantified using strain-specific real-time quantitative polymerase chain reaction (Cheesman et al. 2003; de Roode et al. 2004*b*). All measures were taken daily for 12 days, after which they were taken every 2 days until day 35 postinfection (for mice infected with one clone or with a mixture simultaneously) or 35 days after infection with the second clone (for the other mixed infections).

Statistical Analysis

All analyses were carried out using ANOVA (Minitab, ver. 13.30); data were log-transformed when necessary to meet normality and homogeneity-of-variance assumptions. Total numbers of parasites of specific clones produced over the whole infection were calculated. *Plasmodium chabaudi* parasites have a 24-h replication cycle, so the sum of the daily parasite numbers gives the number produced over the entire infection. For all comparisons, analyses were based on the same number of days of sampling. For example, to compare coinfection and superinfection in experiment 1, we calculated the overall densities produced over the first 35 days of the infection, even though mice in the superinfection experiment were sampled for 57 days. In experiments where mice were not sampled daily, densities were interpolated from the densities 1 day before and 1 day after each data-absent day. Finally, we measured anemia as a measure of virulence by averaging mice's red blood cell densities over the entirety of the infection.

Results

Several mice died, while a few others had substantially delayed and reduced parasite densities, suggesting that they received lower parasite densities than anticipated (Timms et al. 2001). All these mice were excluded from the analysis; numbers of mice on which the analysis was based are shown in table 1.

Experiment 1: AS and CB

In mice simultaneously infected with AS and CB, both parasite clones produced about half of the total parasite population; AS had a slightly higher peak than CB around day 10, while CB had a higher peak than AS around day 16 (fig. 1*A*). Compared to single infections, both AS and CB suffered from competition (fig. 1*B*, 1*C*), producing about 3 million fewer parasites in mixtures than they did alone (fig. 2).

Compared to infections initiated with AS and CB simultaneously, parasite dynamics were markedly different in mice infected sequentially. In mice infected with AS on day 0 and CB 3 days later, AS dominated until day 16 (fig. 1*D*) and competitively suppressed CB (fig. 1*F*) during this time. Parasite numbers for AS then plummeted and were suppressed (fig. 1*E*), while CB densities rose sharply, and

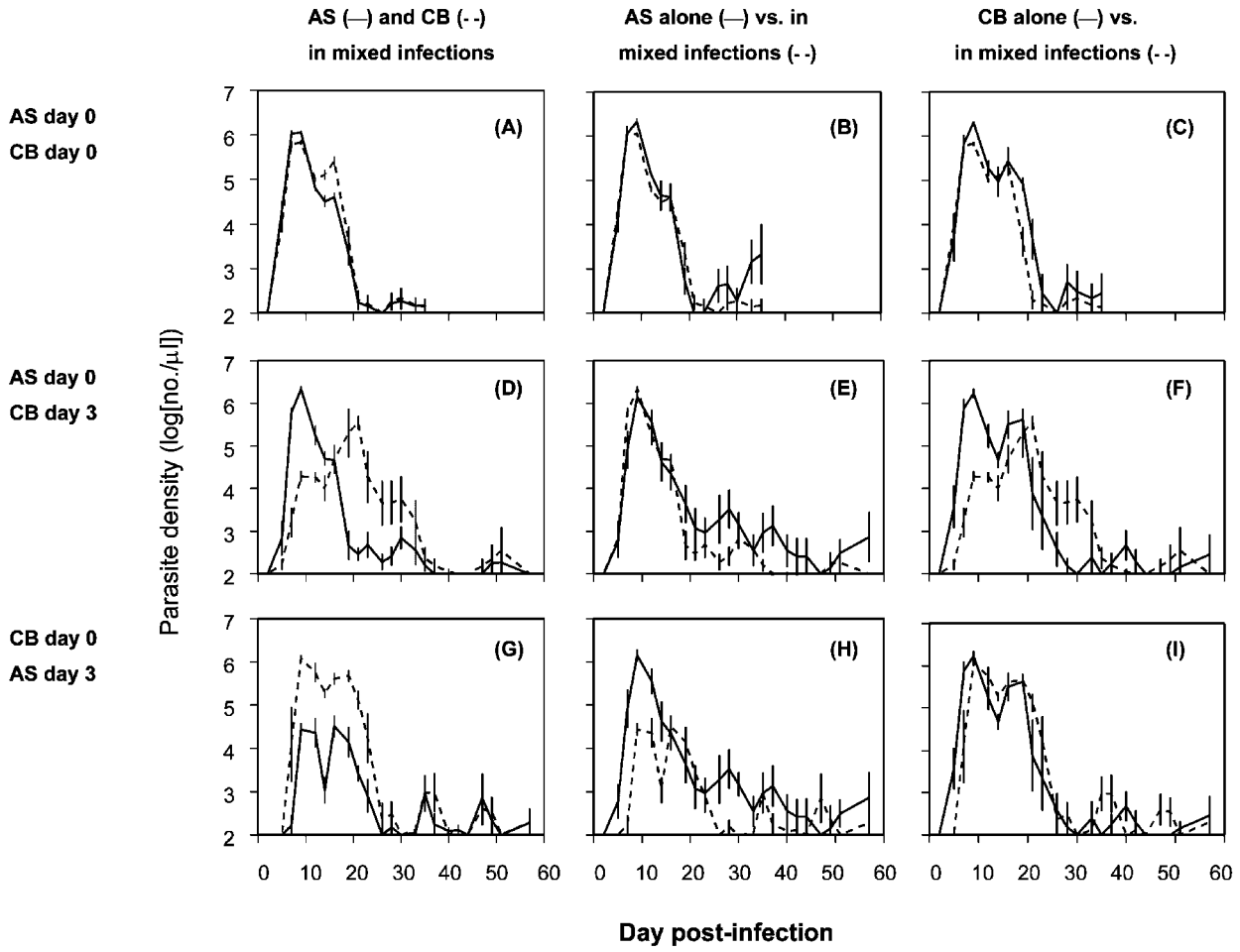

Figure 1: Log AS and CB parasite densities over time (mean ± 1 SEM) in experiment 1. The left-hand column shows AS and CB densities in mixed infections, the middle column compares AS densities in single and mixed infections, and the right-hand column compares CB densities in single and mixed infections. *A–C*, Mice were infected with AS and CB simultaneously; *D–F*, mice were infected with CB 3 days after AS infection; *G–I*, mice were infected with AS 3 days after CB infection. The threshold density at which parasites could be detected was around 100/μL mouse blood, so the *Y*-axes start at 2. See table 1 for the numbers of mice on which data points are based.

so CB started to dominate the infection; it remained the dominant clone for the rest of the infection (fig. 1*D*).

In mice infected with CB first (fig. 1*G*), AS was competitively suppressed for almost the entirety of the infection (fig. 1*H*), whereas CB was not (fig. 1*I*). In contrast with CB, which managed to overgrow AS when inoculated second, AS never managed to overgrow CB. In fact, AS parasite numbers followed the same up- and downward dynamics as CB in these infections; whenever CB numbers decreased, AS numbers decreased with them (fig. 1*G*).

Thus, when inoculated into mice 3 days before the other clone, neither AS nor CB suffered a reduction in parasite numbers, but they did when infecting the same host simultaneously or 3 days after their competitor (fig. 2). Both clones were competitively suppressed when simultaneously inoculated (AS: $F = 10$, df $= 1, 22$, $P = .004$; CB: $F = 7.9$, df $= 1, 20$, $P = .011$); AS, but not CB, was even further suppressed when inoculated 3 days later (fig. 2; single/mixed × day interaction to test whether competitive suppression varied by day—AS: $F = 68$, df $= 1, 31$, $P < .001$; CB: $F = 1.3$, df $= 1, 28$, $P = .26$).

Experiment 2: AS(pyr1A) and AJ

Using a different set of clones and different techniques for distinguishing them, we again found competitive suppression by the resident clone. Moreover, this second experiment showed that the longer the period between inoculations of the clones, the greater was the competitive suppression of the second clone.

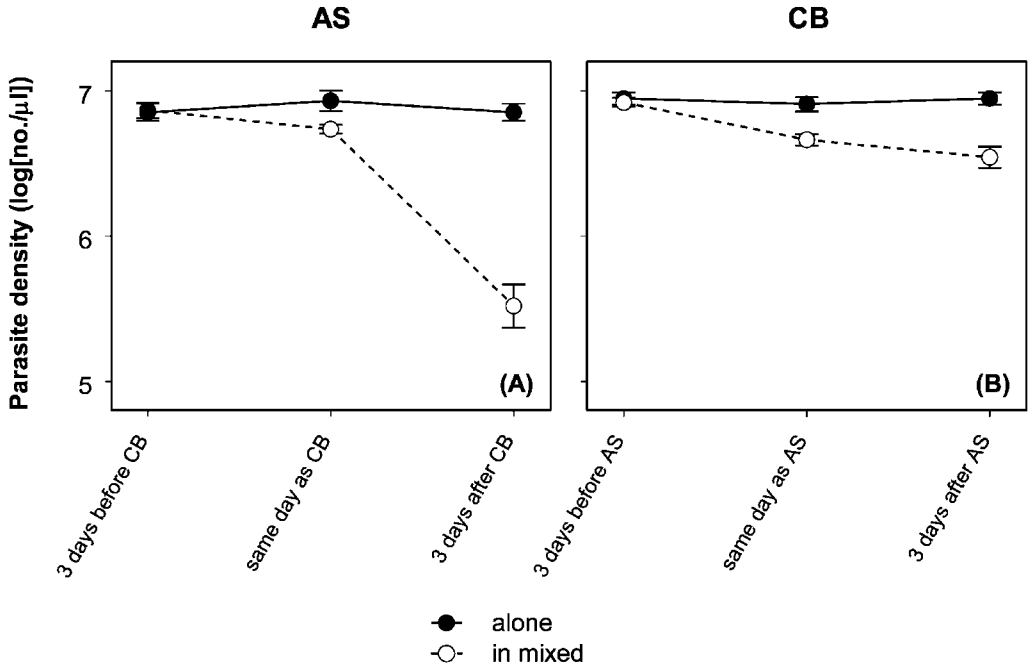

Figure 2: Total numbers of AS (*A*) and CB (*B*) parasites produced over the whole infection in single and mixed (AS and CB simultaneously or 3 days apart) infections in experiment 1 (mean ± 1 SEM). See table 1 for the numbers of mice on which data points are based.

In mice infected simultaneously, AJ was competitively dominant for the entirety of the infection, with AS(pyr1A) disappearing below detectable levels within 11 days (fig. 3*A*), as in our previous experiments (de Roode et al. 2004*b*). Consequently, AS(pyr1A) produced far fewer parasites in these mixed infections than in single infections (figs. 3*B*, 4*A*), whereas AJ did not (figs. 3*C*, 4*B*).

Clone AJ suffered from competition only when it infected mice 3 or 11 days after AS(pyr1A) (fig. 3*F*, 3*I*; fig. 4*B*). Even then, it always became the dominant clone later on in infections, overgrowing AS(pyr1A) around day 10 or day 25, respectively (fig. 3*D*, 3*G*). Because AJ was always able to overgrow AS(pyr1A) in these infections, AS(pyr1A) was competitively suppressed by AJ, even when it was inoculated 3 days before AJ (figs. 3*E*, 4*A*). Clone AS(pyr1A) also suffered greatly from competitive suppression when it was inoculated after AJ, never going on to become the dominant clone (fig. 3*J*, 3*K*, 3*M*, 3*N*; fig. 4*A*). Competitive suppression was so strong that AS(pyr1A) was only detected in three of the five mice that it infected 11 days after AJ.

Thus, overall, timing importantly affected how much clones suffered from competition. Clone AS(pyr1A) produced as many parasites in mixed infections when inoculated 11 days before AJ as it did alone, but it was competitively suppressed in all other treatments, with suppression becoming stronger the later it was inoculated (fig. 4*A*; single/mixed × day interaction to test whether competitive suppression varied by day: $F = 4.8$, df $= 4, 32$, $P = .004$). In contrast, AJ suffered from competition only when infecting mice after AS(pyr1A) (fig. 4*B*), the more so when infecting 11 days rather than 3 days later (single/mixed × day interaction: $F = 11.5$, df $= 4, 28$, $P < .001$). Thus, both clones suffered more from competition the later they infected mice already infected with the other clone, but over the whole range of infections, AS(pyr1A) suffered more than AJ.

Experiments 1 and 2: Overall Parasite Densities and Virulence

Among the single-clone infections in experiment 2, mice infected with AJ lost more red blood cells than those infected with AS(pyr1A) (clone: $F = 33$, df $= 1, 23$, $P < .001$), confirming that AJ was more virulent than AS(pyr1A), as found previously (de Roode et al. 2004*b*). This was in contrast with experiment 1, in which mice infected with AS or CB became similarly anemic (coinfection: $F = 0.42$, df $= 1, 6$, $P > .05$; superinfection: $F = 5.5$, df $= 1, 7$, $P > .05$).

In both experiments, mixed-clone infections never contained significantly more parasites than found in infections of one or both clones on their own (fig. 5), implying that

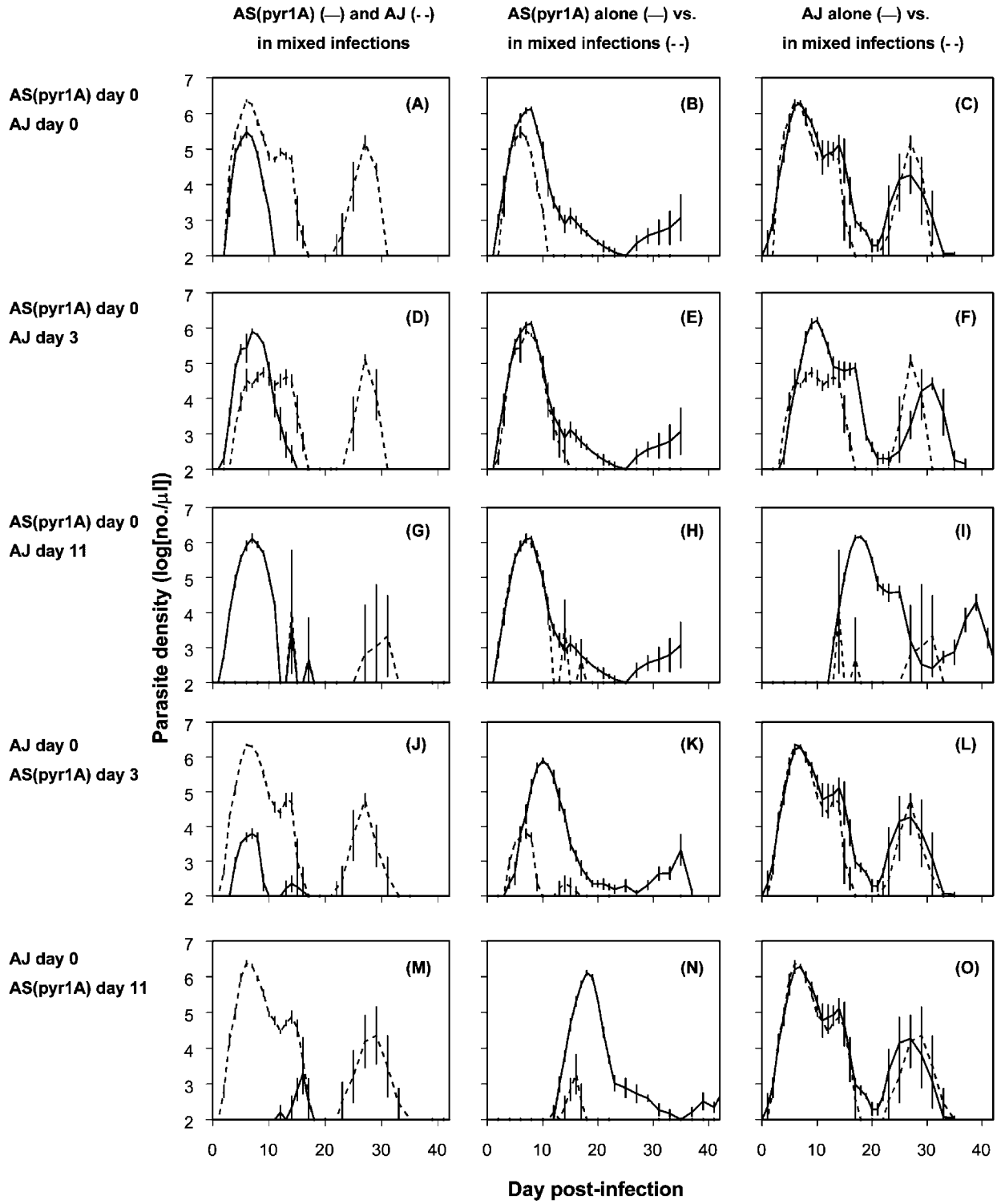

Figure 3: Log parasite densities over time for infections in experiment 2 (mean ± 1 SEM). The left-hand column shows AS(pyr1A) and AJ densities in mixed infections, the middle column compares AS(pyr1A) densities in single and mixed infections, and the right-hand column compares AJ densities in single and mixed infections. *A–C*, Mice were infected with AS(pyr1A) and AJ simultaneously; *D–F*, mice were infected with AJ 3 days after AS(pyr1A) infection; *G–I*, mice were infected with AJ 11 days after AS(pyr1A) infection; *J–L*, mice were infected with AS(pyr1A) 3 days after AJ infection; *M–O*, mice were infected with AS(pyr1A) 11 days after AJ infection. The threshold density at which parasites could be detected was around 100/μL mouse blood, so the *Y*-axes start at 2. See table 1 for the numbers of mice on which data points are based.

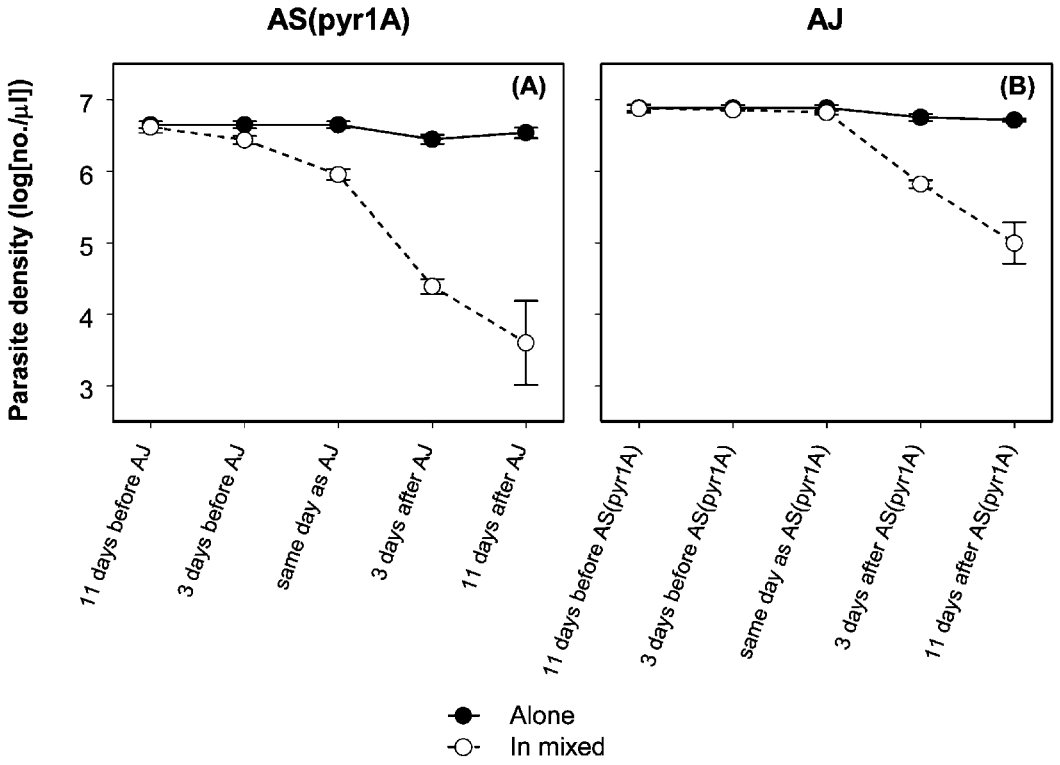

Figure 4: Total numbers of AS(pyr1A) (*A*) and AJ (*B*) parasites produced over the whole infection in single and mixed infections in experiment 2 (mean ± 1 SEM). See table 1 for the numbers of mice on which data points are based.

competition was a direct effect of these clones having to share the available niche space between them. In experiment 1, mice infected with AS first and then CB experienced more parasites than those infected with AS alone (fig. 5*C*); as a result, these mice became more anemic than those infected with AS alone ($F = 10.9$, df $= 1, 11$, $P = .007$). In experiment 2, however, prior infection with AS(pyr1A) protected mice from high AJ densities, and so these mice experienced fewer parasites than those infected with AJ alone (fig. 5*D*). Mice infected with AJ 11 days after AS were therefore indeed protected from disease: they lost fewer red blood cells than did mice infected with AJ alone (single/mixed: $F = 57$, df $= 1, 5$, $P = .001$).

Discussion

We found strong competitive interactions between genetically different clones of *Plasmodium chabaudi*. The extent to which a particular clone suffered from competition depended strongly on its genotype, whether it was resident before a second clone infected the host, and the duration of prior residency.

The importance of genotype was most apparent in experiment 2, where clone AJ was always able to overgrow AS(pyr1A), even when the latter was already established, but AS(pyr1A) never became dominant in mice already infected with AJ. Moreover, AS(pyr1A) suffered from competition by AJ even when it infected mice before AJ, whereas AJ suffered from competition only when infecting mice after AS. Such genotype-specific competitive suppression has been reported before with different clones (Snounou et al. 1992; Taylor and Read 1998) and appears to be a general finding in *P. chabaudi*.

Residency and the amount of time between first and second infections also affected competition. In general, clones did not suffer from competition when they infected mice first, but they did when they infected mice simultaneously with or after their competitor (figs. 2, 4). Moreover, the later a clone was inoculated after another, the more it suffered from competition. Relative timing of infection could thus turn around competitive hierarchies, and so clones that were competitively inferior in simultaneous coinfections became competitively dominant when inoculated first.

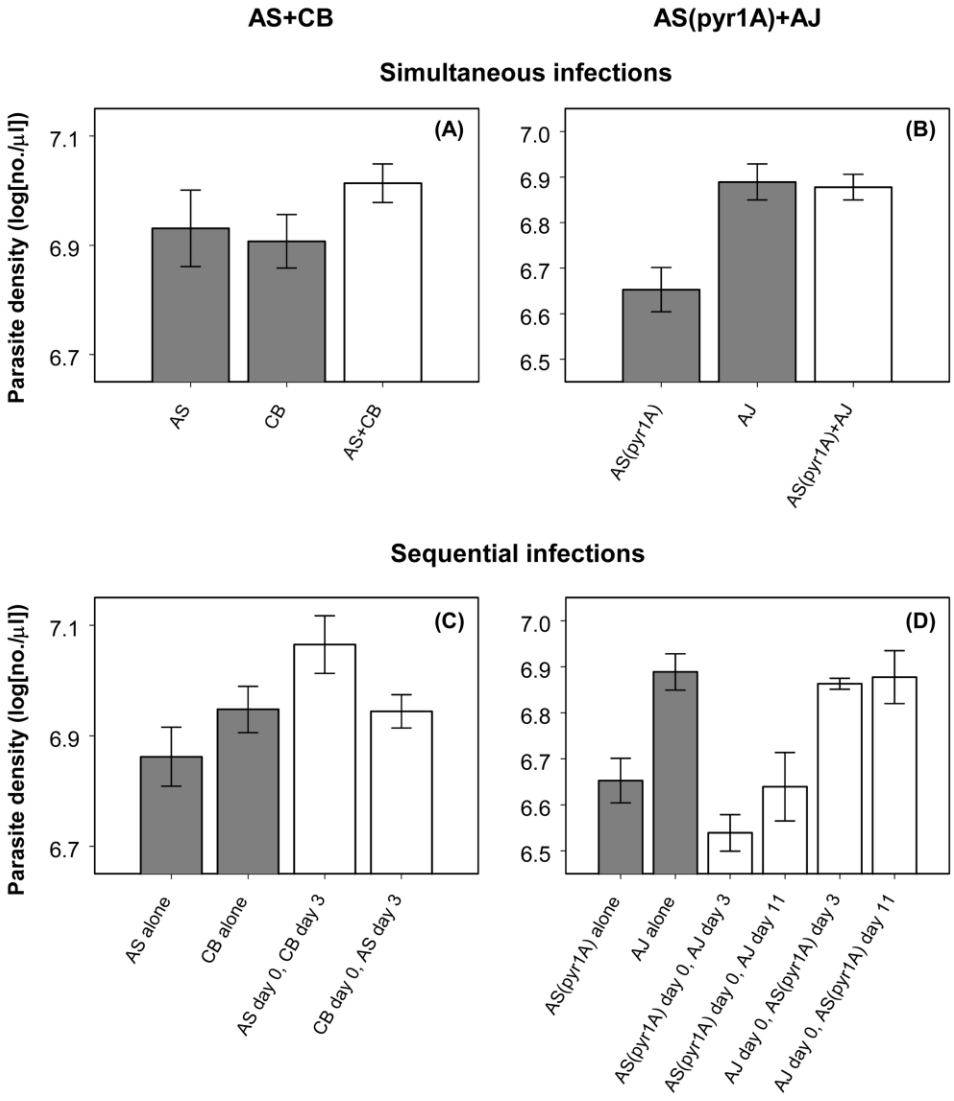

Figure 5: Overall parasite numbers over the whole infection (mean ± 1 SEM) for infections in experiments 1 and 2. In experiment 1, mice infected with AS and CB simultaneously had as many parasites as those infected with AS or CB alone (*A*; $P > .05$); mice infected with CB 3 days after AS infection had higher parasite densities than those with AS alone (*C*; $P < .05$) but as many as those with CB alone (*C*; $P > .05$). In experiment 2, mice infected with AS(pyr1A) and AJ simultaneously had higher parasite densities than mice infected with AS(pyr1A) alone (*B*; $P \leq .05$) but as many as those with AJ alone (*B*; $P > .05$); mice infected with AJ 3 or 11 days after AS(pyr1A) infection had lower parasite densities than mice infected with AJ alone (*D*; $P < .05$) and as many as those infected with AS(pyr1A) alone (*D*; $P > .05$). See table 1 for the numbers of mice on which data points are based.

What ecological factors caused the within-host competition that we observed? In malaria, within-host competition is most likely caused by competition for resources, such as red blood cells, and strain-transcending immunity (e.g., Richie 1988; Hellriegel 1992; Snounou et al. 1992; Read and Taylor 2000, 2001; de Roode et al. 2003; Haydon et al. 2003). During an infection, malaria parasites replicate exponentially, reaching parasite densities exceeding millions of parasites per microliter of blood (billions of parasites per mouse) between 8 and 10 days into the infection (figs. 1, 3). This exponential growth is followed by an exponential collapse of parasite numbers, which is the result of both parasite destruction of red blood cells and immune clearance of infected and uninfected red blood cells (e.g., Cox 1988; Hellriegel 1992; Yap and Stevenson 1994; Taylor-Robinson 1995; Jakeman et al. 1999; Menendez et al. 2000; Li et al. 2001). Thus, parasite clones infecting mice simultaneously must divide the available

red blood cells and many other resources, such as blood glucose, between them and will be affected by cross-reactive immune responses induced against them. Such processes presumably explain why parasites inoculated after their competitor suffered more competitive suppression than when they infected mice at the same time or after their competitor, because they should have less time to grow up to high numbers before conditions become unfavorable. This should be especially so for clones inoculated 11 days after their competitor, as they infect a host severely depleted of resources and with strong immune responses in place (see also Hellriegel 1992).

Although it is easy to explain why parasites that infected their host after their competitor suffered more from competition, it is harder to disentangle the two processes by which this happened. One important reason for this is that resource and apparent competition are much intertwined in this system; thus, the sharp decline in red blood cell numbers is partly due to parasite destruction of red blood cells and partly due to red blood cell destruction by the immune system. Nevertheless, our results do imply that the immune system plays a crucial role. First, the initial growth of parasite clones in simultaneous coinfections was seemingly unaffected by the presence of a competitor clone, and so the initial growth rates for individual clones in single and mixed infections were surprisingly parallel (fig. 1*B*, 1*C*; fig. 3*B*, 3*C*), suggesting that during this initial phase, when immunity is not yet in place, clones did not affect each other. Second, parasite numbers of both clones CB and AJ declined more rapidly and earlier in simultaneous mixed infections than in single infections (figs. 1*C*, 3*C*); as these mixed infections had the same overall numbers of red blood cells as the respective single infections, it seems likely that this rapid decline was due to immunity rather than resource limitation. Finally, competitive suppression was generally more striking during the chronic phase of infection (fig. 1*B*, 1*E*; fig. 3*B*, 3*E*, 3*H*), where immune responses are in place and red blood cell densities have recovered, again suggesting that immunity rather than resources limited parasite numbers during that phase.

Further support for a role for immunity comes from the fact that clones apparently affected each other asymmetrically (figs. 1, 3). Thus, clone CB was able to invade a host already infected with AS and indeed overgrow AS later on during the infection (fig. 1*D*); AS, on the other hand, never became dominant in mice already infected with CB and followed the exact up- and downward patterns as CB (fig. 1*G*). One potential reason for this is that immunity directed at AS did not have a strong effect on CB, whereas immunity against CB severely affected AS. Support for this hypothesis comes from experiments in which mice were infected or immunized with AS or CB parasites and then challenged with the same (homologous) or the alternate (heterologous) clone (Jarra et al. 1986; Jarra and Brown 1989; Snounou et al. 1989). These experiments showed that, as suggested here, immunity induced by CB is able to control AS parasites better than AS-induced immunity is able to control CB parasites. The result is that CB could invade and become dominant in mice whose immune systems are entirely focused on AS. Interestingly, however, despite CB's ability to invade mice already infected with AS, it still obtained lower densities than it would have done on its own, suggesting that the strain-transcending component of the immune response was also strong. Generally and ecologically speaking, these results imply that competition between coinfecting pathogens should be stronger the more similar their ecological niches are. Indeed, a lack of competition between strains of the trypanosome *Crithidia bombi* in bumblebees was ascribed to those strains inhabiting different niches inside their host (Schmid-Hempel et al. 1999).

Although our experiments suggest that immunity plays an important role in causing competition between coinfecting clones, further experiments are necessary to elucidate the relative importance of resource limitation and strain-transcending immunity. Hellriegel (1992), for example, used theoretical models of malaria infections to show that even in the absence of immunity, parasite growth in mixed infections was limited by reduced numbers of red blood cells. Possible experiments to study the relative roles of resource limitation and immunity could include studies in which mice are blood-transfused to keep red blood cell densities high even when strong immune responses are in place (e.g., Yap and Stevenson 1994) and studies on within-host competition in immunocompetent and immunocompromised mice. We have recently extended the competition studies we report here to include hosts of varying immune status. These reveal that thymic-dependent immunity is a determinant of competitive outcome and also innate responses and/or resource limitation (e.g., L. Råberg, J. C. de Roode, A.S. Bell, P. Stamou, D. Gray, and A. F. Read, unpublished results).

Regardless of the underlying mechanism, our results show unequivocally that parasite strains that inhabit very similar niches inside their host compete with each other, suffering large reductions in their densities. Such competition will affect the evolution of a number of parasite traits of theoretical and biomedical interest. For instance, in the absence of drug treatment, drug-resistant mutants are likely to be less competitive than wild-type parasites (or they would have spread to fixation in the predrug era), but in drug-treated hosts they will have two advantages. First, they are not killed by drugs, and second, their competitors will be removed. This removal of competitors—that is, competitive release—has the potential to dramatically increase the spread of drug resistance in a population

(Hastings and D'Alessandro 2000). We have previously shown that prophylactic drug use indeed leads to competitive release (de Roode et al. 2004*a*); the implication of the results we report here is that this competitive release would occur for treatment administered at least 11 days after infection and be particularly pronounced in infections where drug-sensitive clones were initially resident (e.g., fig. 3*K*, 3*N*). Thus, there may be situations where the evolution of drug resistance could be slowed by minimizing the use of drugs so as to maximize the competitive suppression of drug-resistant mutants.

A large number of theoretical studies have assumed that intense competition occurs within infections and have asked how such competition imposes selection on virulence phenotypes. Although these models differ in their details, they all predict virulence to be elevated with respect to equilibrium levels that would evolve in a population where only single-clone infections occurred (reviewed in de Roode et al. 2005). Our data provide support for many of the assumptions made in these models. Thus, in AS + CB mixed infections, we observed coexistence of different clones with competitive suppression of at least one of the clones, as envisaged in several studies (e.g., Sasaki and Iwasa 1991; Van Baalen and Sabelis 1995; Frank 1996), while the infections in which AS(pyr1A) infected mice after AJ mostly resemble a situation in which infected hosts cannot be reinfected at all (e.g., Bremermann and Thieme 1989). We found the least support for the assumption that virulent clones take over hosts already infected with less virulent clones, outcompeting these clones instantly (e.g., Levin and Pimentel 1981; Nowak and May 1994). In experiment 2, where AJ was more virulent than AS(pyr1A), causing more anemia in the mice it infected, it suffered greatly from competitive suppression when infecting mice after AS(pyr1A). Interestingly, however, it did overgrow AS(pyr1A) in the end, at least partly supporting the claim that virulent clones can take over hosts already infected with less virulent clones.

An important question would then be how much transmission the more virulent clone actually obtained. Clone AJ parasite densities were between two and three orders of magnitude lower than they were in single AJ infections (fig. 3*F*, 3*I*). Previous studies have shown that densities of gametocytes (the malaria life stages that are transmitted to the mosquito vector) are correlated with densities of asexual parasites (Taylor and Read 1998; Mackinnon and Read 2004) and that within-host clonal density in mixed infections is correlated with transmission success (Taylor and Read 1998; de Roode et al. 2005); thus, the initial competitive suppression of AJ undoubtedly resulted in substantial transmission losses for the virulent clone.

Our results demonstrate that the real-life complexities of a disease like malaria may be hard to capture in a single evolutionary model. Not only do our results suggest that competitive suppression is genotype-specific and that levels of strain-transcending and strain-specific immunity depend on the exact clones infecting the host, we have even demonstrated that competitive hierarchies can be completely reversed depending on which clone infects the host first. Interestingly, such a mosaic of competitive interactions and reversal of competitive dominance could explain why in many diseases (e.g., Chotivanich et al. 2000; Nevils et al. 2000; Taylor et al. 2003) virulence polymorphisms are maintained (Mackinnon and Read 2004). They indeed suggest that, contrary to theoretical expectations, avirulent parasites may play a larger role in nature than we have up till now granted them.

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Literature Cited

Adler, F. R., and J. Mosquera Losada. 2002. Super- and coinfection: filling the range. Pages 139–149 *in* U. Dieckmann, J. A. J. Metz, M. A. Sabelis, and K. Sigmund, eds. Adaptive dynamics of infectious diseases: in pursuit of virulence management. Cambridge University Press, Cambridge.

Allaker, R. P., D. H. Lloyd, and I. M. Smith. 1988. Prevention of exudative epidermitis in gnotobiotic piglets by bacterial interference. Veterinary Record 123:597–598.

Arez, A. P., J. Pinto, K. Pålsson, G. Snounou, T. G. T. Jaenson, and V. E. Do Rosário. 2003. Transmission of mixed *Plasmodium* species and *Plasmodium falciparum* genotypes. American Journal of Tropical Medicine and Hygiene 68:161–168.

Arnot, D. 1998. Clone multiplicity of *Plasmodium falciparum* infections in individuals exposed to variable levels of disease transmission. Transactions of the Royal Society of Tropical Medicine and Hygiene 92:580–585.

Artavanis-Tsakonas, K., J. E. Tongren, and E. M. Riley. 2003. The war between the malaria parasite and the immune system: immunity, immunoregulation and immunopathology. Clinical and Experimental Immunology 133:145–152.

Babiker, H. A., L. C. Ranford-Cartwright, and D. Walliker. 1999. Genetic structure and dynamics of *Plasmodium falciparum* infections in the Kilombero region of Tanzania. Transactions of the Royal Society of Tropical Medicine and Hygiene 93(suppl. 1):11–14.

Barrow, P. A., and K. Page. 2000. Inhibition of colonisation of the alimentary tract in young chickens with *Campylobacter jejuni* by precolonisation with strains of *C. jejuni*. FEMS (Federation of European Microbiological Societies) Microbiology Letters 182:87–91.

Basco, L. K., and P. Ringwald. 2001. Molecular epidemiology of malaria in Yaounde, Cameroon. VIII. Multiple *Plasmodium falciparum* infections in symptomatic patients. American Journal of Tropical Medicine and Hygiene 65:798–803.

Beale, G. H., R. Carter, and D. Walliker. 1978. Genetics. Pages 213–245 *in* R. Killick-Kendrick and W. Peters, eds. Rodent malaria. Academic Press, London.

Boyle, D. B., C. I. Newbold, C. C. Smith, and K. N. Brown. 1982. Monoclonal antibodies that protect in vivo against *Plasmodium chabaudi* recognize a 250,000-dalton parasite polypeptide. Infection and Immunity 38:94–102.

Bremermann, H. J., and J. Pickering. 1983. A game-theoretical model of parasite virulence. Journal of Theoretical Biology 100:411–426.

Bremermann, H. J., and H. R. Thieme. 1989. A competitive exclusion principle for pathogen virulence. Journal of Mathematical Biology 27:179–190.

Bull, J. J. 1994. Virulence. Evolution 48:1423–1437.

Cheesman, S. J., J. C. de Roode, A. F. Read, and R. Carter. 2003. Real-time quantitative PCR for analysis of genetically mixed infections of malaria parasites: technique validation and applications. Molecular and Biochemical Parasitology 131:83–91.

Chotivanich, K., R. Udomsangpetch, J. A. Simpson, P. Newton, S. Pukrittayakamee, S. Looareesuwan, and N. J. White. 2000. Parasite multiplication potential and the severity of falciparum malaria. Journal of Infectious Diseases 181:1206–1209.

Conway, D. J., B. M. Greenwood, and J. S. McBride. 1991. The epidemiology of multiple-clone *Plasmodium falciparum* infections in Gambian patients. Parasitology 103:1–6.

Cox, F. E. G. 1988. Major models in malaria research: rodent. Pages 1503–1543 *in* W. H. Wernsdorfer and I. McGregor, eds. Malaria: principles and practice of malariology. Churchill Livingstone, Edinburgh.

Daubersies, P., S. Sallenave-Sales, S. Magne, J.-F. Trape, H. Contamin, T. Fandeur, C. Rogier, O. Mercereau-Puijalon, and P. Druilhe. 1996. Rapid turnover of *Plasmodium falciparum* populations in asymptomatic individuals living in a high transmission area. American Journal of Tropical Medicine and Hygiene 54:18–26.

de Roode, J. C., A. F. Read, B. H. K. Chan, and M. J. Mackinnon. 2003. Rodent malaria parasites suffer from the presence of conspecific clones in three-clone *Plasmodium chabaudi* infections. Parasitology 127:411–418.

de Roode, J. C., R. Culleton, A. S. Bell, and A. F. Read. 2004*a*. Competitive release of drug resistance following drug treatment of genetically diverse *Plasmodium chabaudi* infections. Malaria Journal 3:33.

de Roode, J. C., R. Culleton, S. J. Cheesman, R. Carter, and A. F. Read. 2004*b*. Host heterogeneity is a determinant of competitive exclusion or coexistence in genetically diverse malaria infections. Proceedings of the Royal Society of London B 271:1073–1080.

de Roode, J. C., R. Pansini, S. J. Cheesman, M. E. H. Helinski, S. Huijben, A. R. Wargo, A. S. Bell, B. H. K. Chan, D. Walliker, and A. F. Read. 2005. Virulence and competitive ability in genetically diverse malaria infections. Proceedings of the National Academy of Sciences of the USA 102:7624–7628.

Druilhe, P., P. Daubersies, J. Patarapotikul, C. Gentil, L. Chene, T. Chongsuphajaisiddhi, S. Mellouk, and G. Langsley. 1998. A primary malarial infection is composed of a very wide range of genetically diverse but related parasites. Journal of Clinical Investigation 101:2008–2016.

Duval-Iflah, Y., J. P. Chappuis, R. Ducluzeau, and P. Raibaud. 1983. Intraspecific interactions between *Escherichia coli* strains in human newborns and in gnotobiotic mice and piglets. Progress in Food and Nutrition Science 7:107–116.

Frank, S. A. 1996. Models of parasite virulence. Quarterly Review of Biology 71:37–78.

Hastings, I. M., and U. D'Alessandro. 2000. Modelling a predictable disaster: the rise and spread of drug-resistant malaria. Parasitology Today 16:340–347.

Haydon, D. T., L. Matthews, R. Timms, and N. Colegrave. 2003. Top-down or bottom-up regulation of intra-host blood-stage malaria: do malaria parasites most resemble the dynamics of prey or predator? Proceedings of the Royal Society of London B 270:289–298.

Hellriegel, B. 1992. Modelling the immune response to malaria with ecological concepts: short-term behaviour against long-term equilibrium. Proceedings of the Royal Society of London B 250:249–256.

Hodgson, D. J., R. B. Hitchman, A. J. Vanbergen, R. S. Hails, R. D. Possee, and J. S. Cory. 2004. Host ecology determines the relative fitness of virus genotypes in mixed-genotype nucleopolyhedrovirus infections. Journal of Evolutionary Biology 17:1018–1025.

Hood, M. E. 2003. Dynamics of multiple infection and within-host competition by the anther-smut pathogen. American Naturalist 162:122–133.

Ishii, T., J. Takatsuka, M. Nakai, and Y. Kunimi. 2002. Growth characteristics and competitive abilities of a nucleopolyhedrovirus and an entomopoxvirus in larvae of the smaller tea tortrix, *Adoxophyes honmai* (Lepidoptera: Tortricidae). Biological Control 23:96–105.

Jakeman, G. N., A. Saul, W. L. Hogarth, and W. E. Collins. 1999. Anaemia of acute malaria infections in non-immune patients primarily results from destruction of uninfected erythrocytes. Parasitology 119:127–133.

Jarra, W., and K. N. Brown. 1989. Protective immunity to malaria: studies with cloned lines of rodent malaria in CBA/Ca mice. IV. The specificity of mechanisms resulting in crisis and resolution of the primary acute phase parasitaemia of *Plasmodium chabaudi chabaudi* and *P. yoelii yoelii*. Parasite Immunology 11:1–13.

Jarra, W., L. A. Hills, J. C. March, and K. N. Brown. 1986. Protective immunity to malaria. Studies with cloned lines of *Plasmodium chabaudi chabaudi* and *P. berghei* in CBA/Ca mice. II. The effectiveness and inter- or intra-species specificity of the passive transfer of immunity with serum. Parasite Immunology 8:239–254.

Kew, O., V. Morris-Glasgow, M. Landaverde, C. Burns, J. Shaw, Z. Garib, J. André, et al. 2002. Outbreak of poliomyelitis in Hispaniola associated with circulating type 1 vaccine-derived poliovirus. Science 296:356–359.

Konaté, L., J. Zwetyenga, C. Rogier, E. Bischoff, D. Fontenille, A. Tall, A. Spiegel, J. F. Trape, and O. Mercereau-Puijalon. 1999. Variation of *Plasmodium falciparum msp1* block 2 and *msp2* allele

prevalence and of infection complexity in two neighbouring Senegalese villages with different transmission conditions. Transactions of the Royal Society of Tropical Medicine and Hygiene 93(suppl. 1):21–28.

Levin, S., and D. Pimentel. 1981. Selection of intermediate rates of increase in parasite-host systems. American Naturalist 117:308–315.

Li, C., E. Seixas, and J. Langhorne. 2001. Rodent malarias: the mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite. Medical Microbiology and Immunology 189:115–126.

Mackinnon, M. J., and A. F. Read. 2004. Virulence in malaria: an evolutionary viewpoint. Philosophical Transactions of the Royal Society of London B 359:965–986.

Magesa, S. M., K. Y. Mdira, H. A. Babiker, M. Alifrangis, A. Färnert, P. E. Simonsen, I. C. Bygbjerg, D. Walliker, and P. H. Jakobsen. 2002. Diversity of *Plasmodium falciparum* clones infecting children living in a holoendemic area in north-eastern Tanzania. Acta Tropica 84:83–92.

May, R. M., and M. A. Nowak. 1995. Coinfection and the evolution of parasite virulence. Proceedings of the Royal Society of London B 261:209–215.

McLean, A. P., F. A. Lainson, A. M. Sharkey, and D. Walliker. 1991. Genetic studies on a major merozoite surface antigen of the malaria parasite of rodents, *Plasmodium chabaudi*. Parasite Immunology 13:369–378.

Menendez, C., A. F. Fleming, and P. L. Alonso. 2000. Malaria-related anaemia. Parasitology Today 16:469–476.

Morrison, W. I., P. W. Wells, S. K. Moloo, J. Paris, and M. Murray. 1982. Interference in the establishment of superinfections with *Trypanosoma congolense* in cattle. Journal of Parasitology 68:755–764.

Mosquera, J., and F. R. Adler. 1998. Evolution of virulence: a unified framework for coinfection and superinfection. Journal of Theoretical Biology 195:293–313.

Nakamura, T., T. Konishi, H. Kawaguchi, and J. Imose. 1992. Estimation of relative fecundity in *Eimeria tenella* strains by a mixed infection method. Parasitology 104:11–17.

Nevils, M. A., J. V. Figueroa, J. R. Turk, G. J. Canto, V. Le, M. R. Ellersieck, and C. A. Carson. 2000. Cloned lines of *Babesia bovis* differ in their ability to induce cerebral babesiosis in cattle. Parasitology Research 86:437–443.

Nowak, M. A., and R. M. May. 1994. Superinfection and the evolution of parasite virulence. Proceedings of the Royal Society of London B 255:81–89.

Nowak, M. A., and K. Sigmund. 2002. Super- and coinfection: the two extremes. Pages 125–137 *in* U. Dieckmann, J. A. J. Metz, M. A. Sabelis, and K. Sigmund, eds. Adaptive dynamics of infectious diseases: in pursuit of virulence management. Cambridge University Press, Cambridge.

Ntoumi, F., H. Contamin, C. Rogier, S. Bonnefoy, J. F. Trape, and O. Mercereau-Puijalon. 1995. Age-dependent carriage of multiple *Plasmodium falciparum* merozoite surface antigen-2 alleles in asymptomatic malaria infections. American Journal of Tropical Medicine and Hygiene 52:81–88.

Orcutt, R., and R. W. Schaedler. 1973. Control of staphylococci in the gut of mice. Pages 435–440 *in* J. B. Heneghan, ed. Germfree research: biological effect of gnotobiotic environments. Academic Press, New York.

Read, A. F., and L. H. Taylor. 2000. Within-host ecology of infectious diseases: patterns and consequences. Pages 59–75 *in* R. C. A. Thompson, ed. Molecular epidemiology of infectious diseases. Arnold, London.

———. 2001. The ecology of genetically diverse infections. Science 292:1099–1102.

Richie, T. L. 1988. Interactions between malaria parasites infecting the same vertebrate host. Parasitology 96:607–639.

Sasaki, A., and Y. Iwasa. 1991. Optimal growth schedule of pathogens within a host: switching between lytic and latent cycles. Theoretical Population Biology 39:201–239.

Schmid-Hempel, P., K. Puhr, N. Kruger, C. Reber, and R. Schmid-Hempel. 1999. Dynamic and genetic consequences of variation in horizontal transmission for a microparasitic infection. Evolution 53:426–434.

Snounou, G., W. Jarra, S. Viriyakosol, J. C. Wood, and K. N. Brown. 1989. Use of a DNA probe to analyse the dynamics of infection with rodent malaria parasites confirms that parasite clearance during crisis is predominantly strain- and species-specific. Molecular and Biochemical Parasitology 37:37–46.

Snounou, G., T. Bourne, W. Jarra, S. Viriyakosol, J. C. Wood, and K. N. Brown. 1992. Assessment of parasite population dynamics in mixed infections of rodent plasmodia. Parasitology 105:363–374.

Taylor, L. H., and A. F. Read. 1998. Determinants of transmission success of individual clones from mixed-clone infections of the rodent malaria parasite, *Plasmodium chabaudi*. International Journal for Parasitology 28:719–725.

Taylor, L. H., D. Walliker, and A. F. Read. 1997. Mixed-genotype infections of malaria parasites: within-host dynamics and transmission success of competing clones. Proceedings of the Royal Society of London B 264:927–935.

Taylor, L. H., F. Katzer, B. R. Shiels, and S. C. Welburn. 2003. Genetic and phenotypic analysis of Tunisian *Theileria annulata* clones. Parasitology 126:241–252.

Taylor-Robinson, A. W. 1995. Regulation of immunity to malaria: valuable lessons learned from murine models. Parasitology Today 11:334–342.

Thomas, M. B., E. L. Watson, and P. Valverde-Garcia. 2003. Mixed infections and insect-pathogen interactions. Ecology Letters 6:183–188.

Timms, R., N. Colegrave, B. H. K. Chan, and A. F. Read. 2001. The effect of parasite dose on disease severity in the rodent malaria *Plasmodium chabaudi*. Parasitology 123:1–11.

Van Baalen, M., and M. W. Sabelis. 1995. The dynamics of multiple infection and the evolution of virulence. American Naturalist 146: 881–910.

Walliker, D., R. Carter, and A. Sanderson. 1975. Genetic studies on *Plasmodium chabaudi*: recombination between enzyme markers. Parasitology 70:19–24.

Wille, P., T. Boller, and O. Kaltz. 2002. Mixed inoculation alters infection success of strains of the endophyte *Epichloë bromicola* on its grass host *Bromus erectus*. Proceedings of the Royal Society of London B 269:397–402.

Yap, G. S., and M. M. Stevenson. 1994. Blood transfusion alters the course and outcome of *Plasmodium chabaudi* AS infection in mice. Infection and Immunity 62:3761–3765.

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