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# WITHIN-HOST COMPETITION IN GENETICALLY DIVERSE MALARIA INFECTIONS: PARASITE VIRULENCE AND COMPETITIVE SUCCESS

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*Abstract.*—Humans and animals often become coinfected with pathogen strains that differ in virulence. The ensuing interaction between these strains can, in theory, be a major determinant of the direction of selection on virulence genes in pathogen populations. Many mathematical analyses of this assume that virulent pathogen lineages have a competitive advantage within coinfected hosts and thus predict that pathogens will evolve to become more virulent where genetically diverse infections are common. Although the implications of these studies are relevant to both fundamental biology and medical science, direct empirical tests for relationships between virulence and competitive ability are lacking. Here we use newly developed strain-specific real-time quantitative polymerase chain reaction protocols to determine the pairwise competitiveness of genetically divergent *Plasmodium chabaudi* clones that represent a wide range of innate virulences in their rodent host. We found that even against their background of widely varying genotypic and antigenic properties, virulent clones had a competitive advantage in the acute phase of mixed infections. The more virulent a clone was relative to its competitor, the less it suffered from competition. This result confirms our earlier work with parasite lines derived from a single clonal lineage by serial passage and supports the virulence-competitive ability assumption of many theoretical models. To the extent that our rodent model captures the essence of the natural history of malaria parasites, public health interventions which reduce the incidence of mixed malaria infections should have beneficial consequences by reducing the selection for high virulence.

*Key words.*—Competitive suppression, malaria, *Plasmodium chabaudi*, virulence, within-host competition.

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Infections often consist of more than one genetically distinct pathogen lineage (Read and Taylor 2001). In theory, in-host ecological interactions between these lineages can affect within-host selection and consequently the evolution and epidemiology of many important pathogen traits, including drug resistance (Hastings and D'Alessandro 2000; de Roode et al. 2004a) and vaccine escape (Kew et al. 2002). The importance of within-host selection has attracted particular theoretical attention in the context of the evolution of virulence. Most authors have assumed that virulent pathogen lineages have a competitive advantage within hosts, with the general conclusion that where genetically diverse infections are more common, pathogens will evolve to become on average more virulent (e.g., Levin and Pimentel 1981; Bremermann and Pickering 1983; Van Baalen and Sabelis 1995; Frank 1996; Gandon et al. 2001; Adler and Mosquera Losada 2002).

There is, however, no logical necessity for a link between competitive ability and intrinsic pathogen virulence (defined here as parasite-induced morbidity and mortality). Within-host competition is generally assumed to select for higher virulence when parasite competition is based on limited host resources; the rationale being that the most successful competitor would be the one that most effectively exploits the host, and as such does most damage to the host. However, pathogens could compete in different ways, for example, by attacking competitors: direct interference competition could reduce the efficiency of host exploitation, so that the best competitors do less damage (for this and other scenarios, see Chao et al. 2000; Brown et al. 2002; West and Buckling 2003).

Empirically, the situation is also confused. A substantial body of experiments actually demonstrates competitive suppression of virulent strains by avirulent strains (reviewed by Read and Taylor 2001), but much of this could be a reporting bias arising from the search for protective attenuated strains for use in animal and public health. Some indirect evidence points to a competitive advantage to virulence: serial passage of pathogens through new host environments typically increases virulence (e.g., Ebert 1998; Mackinnon and Read 1999a, 2004), and live attenuated vaccines occasionally revert to wild-type virulence (Bull 1994; Kew et al. 2002). Both phenomena are thought to be due to virulent variants increasing in frequency because they have a competitive advantage, although other explanations have been proposed (Read and Taylor 2001).

Recent advances in quantitative polymerase chain reaction (PCR; e.g., Cheesman et al. 2003) have made it possible to study within-host competition in great detail, and we recently provided the first direct in vivo evidence for a competitive advantage of virulent parasites in genetically diverse infections (de Roode et al. 2005a). That work involved a rodent model of malaria. In nature, malarial infections are often genetically diverse, with human hosts typically harboring more than one genotype of the same species (reviewed by Arnot 1999; Read and Taylor 2001). These mixed infections can be extremely common, constituting over 80% of infections in high-transmission areas (e.g., Day et al. 1992; Druilhe et al. 1998; Babiker et al. 1999; Konaté et al. 1999; Magesa et al. 2002; Jafari et al. 2004). Experimental evidence from rodent models demonstrates that genetically distinct malaria clones compete within hosts (e.g., Snounou et al. 1989; Taylor et al. 1997; Read et al. 2002; de Roode et al. 2003, 2004a,b, 2005a,b), and a variety of nonexperimental observations imply that competition also occurs in human malaria

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infections (e.g., Daubersies et al. 1996; Mercereau-Puijalon 1996; Arnot 1999; Smith et al. 1999; Bruce et al. 2000).

To determine whether such competition favors more virulent strains, we recently derived seven lines by serial passage from a single clonal lineage of the murine malaria *Plasmodium chabaudi* and then competed those lines against an unrelated tester strain in laboratory mice (de Roode et al. 2005a). Those experiments demonstrated that more virulent lines did indeed compete more successfully. Because relative in-host frequencies were positively related to clonal frequencies in mosquitoes, we concluded that in-host selection could promote the evolution of virulence in malaria populations.

Comparing the virulence and competitive ability of lines derived from a single clonal lineage mimics a scenario where variants with small genetic and phenotypic changes in virulence arise within a pool of wild-type parasites. Indeed, the fact that the strains we used in those earlier experiments had the same genetic background yet differed dramatically in their virulence and competitiveness shows that within-host selection can easily select for increased virulence (de Roode et al. 2005a). Here we expand our analyses on the relationship between virulence and competitive ability with pairwise competition experiments among a suite of clones that vary widely in their genetic and antigenic properties. These clones are a sample of *P. chabaudi* parasites circulating in the wild, so that this study mimics a scenario where parasite genotypes with divergent genetic and antigenic properties coinfect the same host. The majority of human malaria infections are of this sort (e.g., Day et al. 1992; Druilhe et al. 1998; Babiker et al. 1999; Konaté et al. 1999; Magesa et al. 2002; Jafari et al. 2004) and arise when individual hosts are infected with unrelated parasites from multiple mosquito bites or from single bites of multiply infected mosquitoes. Malaria parasites differ widely in their antigenic properties, and immune responses are at least partly strain specific (e.g., Jarra and Brown 1989; Snounou et al. 1989). Therefore, it is theoretically possible that in some clone combinations parasites can have a competitive disadvantage by attracting most immune attention toward themselves, thereby leaving less virulent parasites to grow unnoticed and transmit freely (e.g., Almogy et al. 2002). Indeed, transient competitive suppression giving way to competitive release has been seen in *P. chabaudi* (Taylor et al. 1997; Read and Taylor 2001; de Roode at al. 2004b). However, we show below that even when clones differ widely in their virulence as well as genetic and antigenic properties, virulence is again strongly related to competitiveness. This was most marked during the acute phase of infection. During the subsequent chronic phase of infection, when strain-specific immune responses become substantial, the virulence-competitive ability relationships were much more complex. Here, the presence of a competitor clone could sometimes enhance parasite densities, suggesting a role for clone-specific immune responses and antigenic variation.

## Materials and Methods

### *Parasites and Hosts*

Two experiments were performed, each of which used four genetically distinct *P. chabaudi chabaudi* clones. The first experiment used clones denoted AS, CB, AJ, and AT. The panel of clones selected was chosen to represent parasites that differ in their virulence, with AS and CB expected to be less virulent than AJ and AT (Mackinnon and Read 1999b; de Roode et al. 2005a,b). Unexpectedly, CB proved to be quite virulent, so in the second experiment the less virulent CW clone was used instead. All clones were originally isolated from different thicket rats collected at different locations (Beale et al. 1978) and are genetically (and antigenically) distinct at multiple loci (McLean et al. 1991; Mackinnon and Read 2004; S. J. Cheesman and R. Carter, unpubl. data). These clones are maintained as frozen stabilates; we use subscript codes to denote the precise point in the clonal histories from which they come. Below, we refer to them only using their letter codes for simplicity, but the lines were as follows. In the first experiment: $AS_{11843}$ (derived from AS by selection for resistance against pyrimethamine [Walliker et al. 1975] and subsequently passaged several times through mice for maintenance purposes), $AJ_{4787}$, $AT_{53}$, and $CB_{914}$. This last clone was more virulent in these experiments than previously reported (e.g., Read et al. 2002; de Roode et al. 2005b) and, upon sequencing of the *msp-1* gene used for quantitative PCR (see below), was found to differ from the CB used by de Roode et al. (2005b). This is probably because the two CB lineages represent different clones in the original rodent from which the CB isolate was taken. In the second experiment, clone $CB_{914}$ was replaced with $CW_{175}$, $AS_{11843}$ with $AS_{11986}$ ($AS_{11843}$ with two additional passages through mice), $AJ_{4787}$ with $AJ_{4815}$ ($AJ_{4787}$ with two additional passages through mice), and the original $AT_{57}$ retained.

Hosts were eight-week-old C57B1/6J inbred female mice (Harlan, Blackthorn, U.K.). Mice were fed on 41B maintenance diet (Harlan) and their drinking water supplemented with 0.05% para-amino benzoic acid to enhance parasite growth (Jacobs 1964). They were maintained at 21°C with a 12L:12D photoperiod.

### *Experimental Design and Inoculation of Mice with Parasites*

Both experiments consisted of 10 treatments, infections with each of the four clones singularly and all pairwise mixed infections: AS + CB, AS + AT, AS + AJ, CB + AT, CB + AJ, and AT + AJ in the first experiment and AS + CW, AS + AT, AS + AJ, CW + AT, CW + AJ, and AT + AJ in the second experiment. Each treatment group comprised five mice, giving a total of 50 mice per experiment. Mice infected with a single clone received $10^6$ parasites, whereas those challenged with two clones were injected with $10^6$ of each (total $2 \times 10^6$ parasites). The number of parasites inoculated in mixed infections allowed a direct comparison between the performance of a particular clone in a single infection with its performance in a mixed infection. Although this means that mice infected with a mixture received a double dose, it has previously been shown that a two-fold dose of parasites has a negligible effect on parasite dynamics and virulence (Timms et al. 2001; B. H. K. Chan, J. C. de Roode, and A. F. Read, unpubl. data).

Inoculations were prepared from donor mice by diluting blood in 0.1 ml of calf serum solution (50% heat-inactivated

TABLE 1. Real-time quantitative polymerase chain reaction assays: primer and probe sequences and details of assay specificities.

| Primer identification (target gene) | Primer sequence 5′-3′ | Amplicon size (bp) |
|---|---|---|
| AS(*ama*)F | GGA AAA GGT ATA ACT ATT CAA AAT TCT AAG GT | 129 |
| AS(*ama*)R | AAT TGT TAT AGG AGA AAT GTT TAC ATC TGT TTG | |
| AJ(*ama*)F | GGA AAA GGT ATA ACT AAT CAA AAA TCT ACT AAA | 127 |
| AJ(*ama*)R | GTG TTA TAG GAG AAA TGT GTA CAT CTG TTT T | |
| AS/AJ(*msp-1*)F | CCG GAA GAA CTA CAG AAT ACA CCA T | 105 |
| AS/AJ(*msp-1*)R | AGA AGT AGA AAA TGC AGA TAG GGA AAA | |
| AT(*msp-1*)F | GGA AGA ACT ACA GAA TAC ACC AGC ATA | 111 |
| AT(*msp-1*)longR | GAA TGT AGA GAA GTA GAA AAT ACA GAT ACA ACT AA | |
| CB(*msp-1*)F | As AS/AJ(*msp-1*)F | 107 |
| CB(*msp-1*)R | GAA GAA GTA GAA AAT GCA GAT AGT GCT AA | |
| CW(*msp-1*)F | As AS/AJ(*msp-1*)F | 196 |
| CW(*msp-1*)upR | AAG ATG AAG ATG GTA ATA GAT GTG TAG CA | |

calf serum, 50% Ringer's solution [27 mM KCl, 27 mM $CaCl_2$, and 150 mM NaCl] and 20 units of heparin per millilitre) and were introduced by intraperitoneal injection.

### *Monitoring of Virulence and Infection Dynamics*

Virulence was monitored by recording red blood cell (RBC) densities (using flow cytometry; Beckman Coulter, High Wycombe, U.K.) and body weights. RBC counts and body weight have been shown to decrease dramatically during infection and the decrease to correlate with host mortality and thus virulence (Mackinnon et al. 2002). Thin blood smears from tail blood were also taken, enabling confirmation and standardization of real-time quantification of parasite densities.

Parasites in the blood of experimental hosts were counted using real-time quantitative PCR. Capillaries were used to collect 5-μl samples of tail blood from each mouse before 1200 h on each sampling day (i.e., when most parasites in the peripheral blood were in the ring or early trophozoite stages, containing only a single copy of the parasite genome). The blood was immediately expressed into a 0.5-ml microcentrifuge tube containing citrate saline and was kept on ice. Blood samples were subsequently centrifuged at maximum speed to ensure precipitation of blood cells (13,000 rpm) for 1 min, the supernatant removed and the blood pellet stored at −80°C until DNA extraction. All samples were collected daily from days 1 to 21 postinoculation and then every second day until day 35.

DNA extractions were performed using the BloodPrep kit (Applied Biosystems, Foster City, CA) on the ABI Prism 6100 Nucleic Acid PrepStation according to manufacturer's instructions. DNA was eluted in a total volume of 200 μl and stored at −80°C until quantification.

Clone-specific PCR primers and minor grove-binder (MGB) probes, targeting either the *P. chabaudi msp-1* gene or *ama* gene, were designed using Primer Express (Applied Biosystems) software. Real-time quantitative PCRs were performed on an ABI Prism 7000 machine with an initial denaturation of 95°C for 10 min followed by 45 cycles of denaturation at 95°C for 15 sec and annealing/extension at 60°C for 1 min. Two microliters of DNA was included in a 25-μl volume PCR reaction with the following components: 1.5 μl each of forward and reverse primer, both at a final concentration of 300 nM; 12.5 μl of 2× TaqMan Universal PCR Master Mix (hot start; Applied Biosystems); 1 μl of MGB probe at a final concentration of 200 nM; and 6.5 μl of sterile water.

Absolute quantification of experimental samples was determined by comparing threshold cycle numbers against a standard curve. DNA standards were generated for each target clone by extracting DNA from duplicate blood samples bearing known parasite numbers as described previously (Bell and Ranford-Cartwright 2004). Standards were extracted from the same volume of blood and using the same methodology as for experimental samples. To ensure that DNA standards for each clone were totally comparable, each set was standardized against those for clone AS using a *P. chabaudi* generic real-time assay (details not given). Three replicates of each DNA standard (covering six orders of magnitude) were included in each quantitative PCR run.

Assay specificities and quantitative sensitivities were tested against DNA of the clones used. Primer and probe sequences and details of assay specificities are provided in Table 1. All assays were sensitive to <10 parasites per PCR reaction (data not shown), which is equivalent to <200 parasites per microliter of mouse blood (× 20: 2 μl of DNA used from a total eluted volume of 200 μl that was extracted from 5 μl of mouse blood). Quantification of target parasites was not affected by the presence of large excesses of nontarget parasite DNA (10 target parasite genomes reliably quantified among 100,000 nontarget genomes, data not shown), and mouse DNA was never amplified. Quantification of a particular clone in different mixed infections necessitated, in certain circumstances, the use of different assays, for example, AS in AS + CW required the AS-*ama* assay (AS/AJ-*msp1* assay amplifies CW-identical sequences) and AS in AS + AT required the AS/AJ-*msp1* assay (AS-*ama* assay amplifies AT-identical sequences; see Table 1). Although both assays were found to give highly comparable results when tested against AS- or AJ-only infections, all real-time quantifications were adjusted to smear-derived parasite densities to ensure no quantification errors arising from the use of different PCR assays (data not shown). Regression analyses showed quantitative PCR counts provided by the *msp1* and *ama* assays to correlate highly with parasite densities (parasitaemia × rbc density) determined by thin blood smears ($R^2 > 84.3\%$, $F_{1,37} > 205$, $P < 0.001$; data not shown). The repeatability (Lessells and Boag 1987) of the quantitative

TABLE 1. Extended.

| MGB probe sequence | Nontarget clones amplified | Primer usage (target clone in bold) |
|---|---|---|
| *ama* probe: 6-FAM-ATC CTC CTT CTC TTA CTT TC-MGB | AT | **AS** + AJ, **AS** + CW |
| | none | AS + **AJ**, **AJ** + CW |
| *msp1* probe: 6-FAM-ACA AGT ACA TAC AAT TTT T-MGB | CW | **AS**, **AJ**, **AS** + AT, **AJ** + AT, **AS** + CB, **AJ** + CB |
| | none | **AT**, AS + **AT**, AJ + **AT**, **AT** + CB, **AT** + CW |
| | none | **CB**, AS + **CB**, AJ + **CB**, AT + **CB** |
| | none | **CW**, AS + **CW**, AJ + **CW**, AT + **CW** |

PCR assays of duplicate blood samples run on separate extraction plates and quantified in separate quantitative PCR runs was found to be 0.98 (data not shown).

The real-time quantitative PCR assays used here were not able to discriminate between asexual parasites and gametocytes, the transmissible parasite stages. Thus, parasite numbers obtained also include gametocytes when present, although gametocyte densities constitute at most 1% of parasites (see also Taylor and Read 1997) and hence contributed minimally to overall quantifications.

### *Trait Definition*

Prior to statistical analysis we defined and constructed the following traits that described the infection in part or as a whole. Virulence was defined as ''maximum RBC loss'' or ''maximum weight loss'' (Mackinnon et al. 2002), as the RBC density or weight on day of inoculation minus the minimum density or weight subsequently recorded. Maximum RBC loss and maximum weight loss are highly correlated, so for simplicity we report results for RBC loss only. The conclusions are unaltered if maximum weight loss is used.

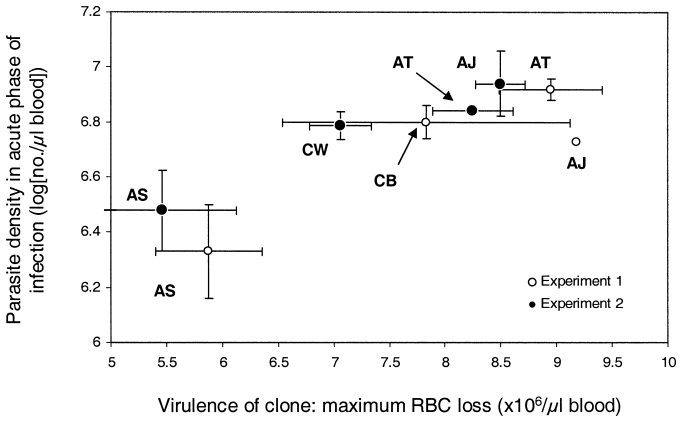


FIG. 1. Clone differences in virulence and parasite densities in single-clone infections. Parasite density in the acute phase of infection is the summed total of all parasites present during this period. Plotted points are the means of the number of mice surviving through to the end of the infection period (day 35). Error bars are ±1 SEM. Open circles represent data from experiment 1 and closed circles data from experiment 2. Error bars are missing for AJ in experiment 1 because only a single mouse survived the entire acute phase of infection in this treatment group. Vertical error bars for AT in experiment 2 are smaller than the symbol; horizontal error bars for CB in experiment 1 are based on just two animals.

Analyses of parasite numbers were performed on summed counts. *Plasmodium chabaudi* parasites invade new red cells synchronously every 24 h, so that summed daily counts give the total number of parasites present during a particular period. Parasite dynamics are radically different during the acute and chronic phases of infection. We defined the distinction between acute and chronic phases as the day parasite numbers first began to recover at the end of the first wave of infection (first recrudescence), which is after about two weeks, although this is variable between mice. In experiment 1 there was considerable mortality (see below), which typically occurred after peak parasite densities were reached but before the end of the acute phase. This disproportionately affected some treatment groups, so that total acute phase data were only available for one or two mice in some treatment groups. To maximize statistical power, we included these mice in the analyses by analyzing peak parasite densities, rather than total parasite numbers for the acute phase of experiment 1. Total parasite numbers and peak densities were highly correlated (e.g., across-mouse correlation in experiment 2: $F_{1,57} = 454$, $P < 0.001$, $R^2_{adj} = 0.887$). All other analyses were based on total parasite numbers. In experiment 2, substantially fewer mice died; these mice were excluded from analyses.

Two measures of competitive outcome were used. Competitive suppression, the proportional reduction in the number of parasites of a focal clone due to the presence of a competitor, was defined as 1 − [(parasite density of target clone in competition)/(parasite density of target clone on its own)]. Note that this measure has a maximum of one (when the focal clone is competitively excluded), is zero when a coinfecting clone has no impact, and becomes negative if the performance of the focal clone is improved by the presence of another clone (facilitation). Competitiveness, the frequency of the focal clone in a mixed infection, was defined as the parasite density of the target clone divided by the total parasite density in a mouse (sum of focal and competitor clones). Importantly, intrinsic differences between clones could result in divergence from the 1:1 inoculum frequencies, even in the absence of competition. To estimate this null expectation, we divided

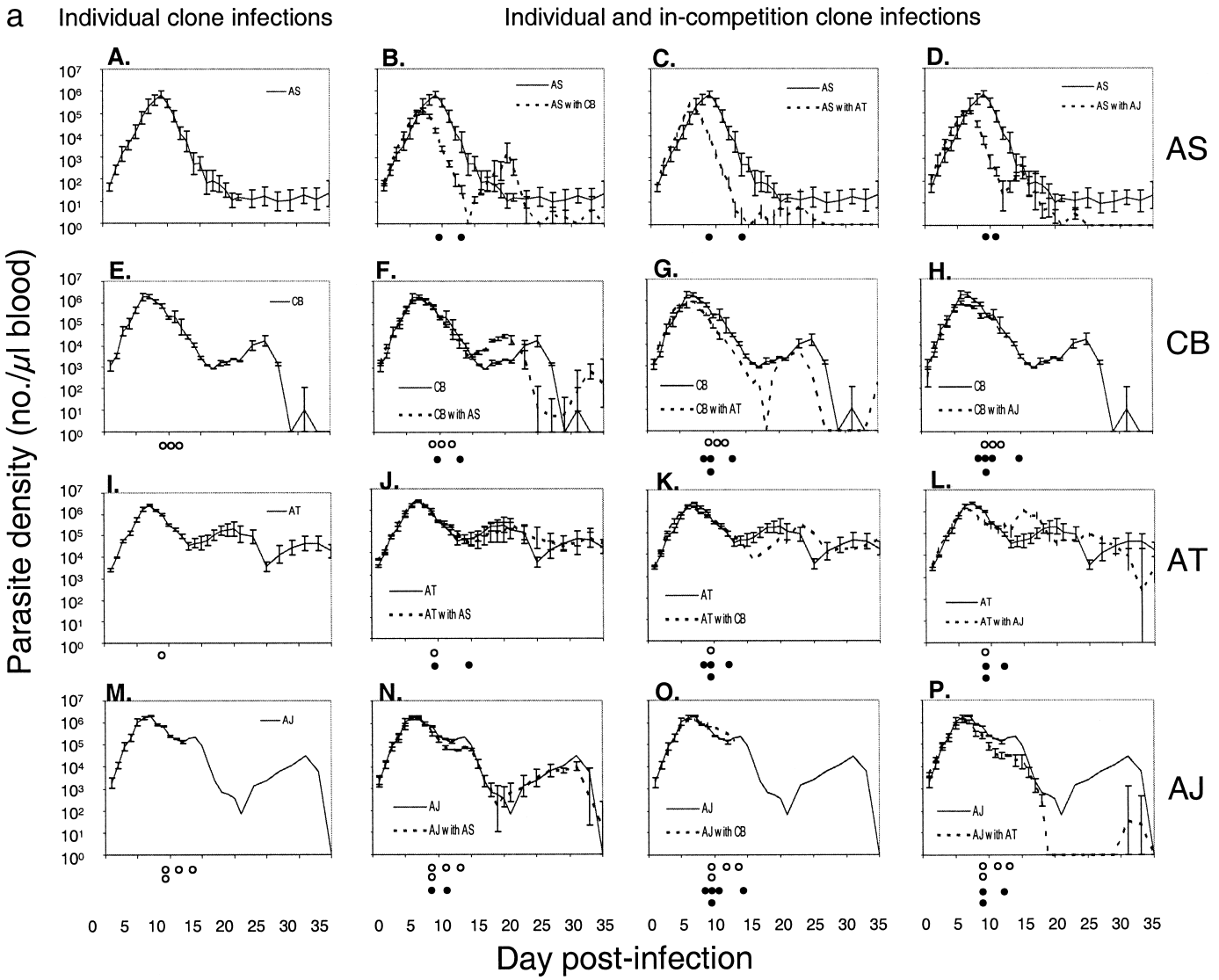


FIG. 2. Densities of individual parasite clones through time (mean ± 1 SEM), showing the four clones in single-clone infections (left columns), and then comparing their performance alone and in competition with each of the other clones in experiment 1 (a, above) and experiment 2 (b, next page). Means are based on the number of mice alive at that point (maximum $n = 5$). The days postinfection of mouse deaths are indicated as open circles (individual infections) or closed circles (in competition).

the average parasite density of the focal clone in single infections by the sum of the average densities of the focal and competitor clones in their respective single infections.

To relate competitive suppression and competitiveness to virulence, we calculated relative virulence: for each pairwise clone comparison, this was calculated as the maximum RBC loss of the lesser virulent clone in a single infection divided by the maximum RBC loss of the more virulent clone in a single infection. This value had a maximum of one (with both clones being equally virulent), and a value of 0.5 would imply that the lesser virulent clone caused only half as much RBC loss as the more virulent clone.

### *Statistical Analyses*

The analysis was restricted to assess the effect of competitors on individual clones' dynamics. Thus, we did not analyse overall parasite densities and the overall virulence that mice experienced from mixed versus single infections; these analyses will be presented in a companion paper (A. S. Bell, J. C. de Roode, D. Sim, and A. F. Read, unpubl. ms.).

The clonal dynamics in the two experiments were analyzed separately because the substitution of clone CB for CW in experiment 2 means that clone identity was not fully cross-factored with experiment. Moreover, the high levels of mortality in experiment 1 necessitated a different measure of acute-phase parasite densities (see above).

Parasite densities of clones in single and mixed infections were analyzed as follows. To estimate the performance of a clone in mixed infections, we used the mean density of each clone in single infections as a reference point. As an estimate of a focal clone's performance in mixed infections, we then

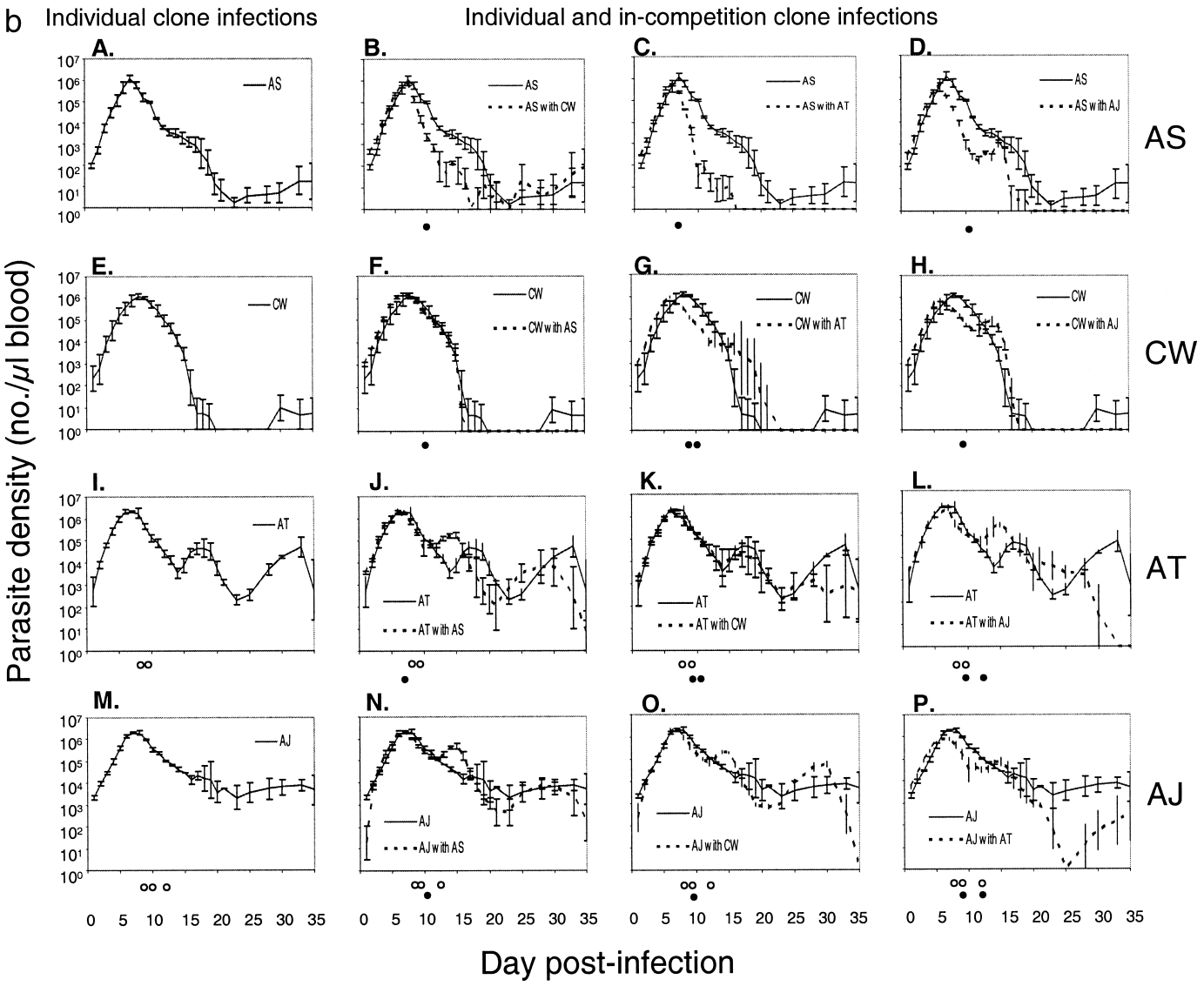


FIG. 2. Continued.

estimated for each mixed infection the deviation from this reference point, giving up to 15 datapoints for each clone in each experiment. These scores were then used to test whether the performance of a clone was affected by presence of a competitor, irrespective of the identity of the competing clone (i.e., whether the average performance of a clone was less than zero, using a one-sample *t*-test), and whether performance depended on the clonal identity of the competitor (one-way ANOVA). Relationships between relative virulence and competitive suppression/competitiveness were analyzed using ANCOVA, with experiment, virulence, and interactions between these terms as the explanatory variables; models were minimized by removing nonsignificant ($P > 0.05$) terms from the model, starting with interactions. Maximum RBC losses and parasite densities were all log-transformed prior to analysis to meet the necessary normality and homogeneity-of-variance assumptions of the models used. Occasionally log-transformed data failed to satisfy these requirements and these data were arcsine-square-root-transformed (for proportions) or square-rooted.

## RESULTS

In experiment 1, 26 of the 50 mice were euthanized before the end of the acute phase of infection because of signs of extreme morbidity. This disproportionately affected treatment groups containing virulent clones, so that there was only a single surviving mouse infected with AJ, one infected with CB + AT, and none infected with CB + AJ. Acute-phase competitive outcomes were thus analyzed using peak parasite densities (which occurred before death), so that all mice were included in the analysis ($n = 50$; see Materials and Methods for further details). In experiment 2, fewer mice (13 of 50) required euthanasia and all treatment groups contained at least two mice throughout the monitoring period, so that total parasite numbers could be used in analyses of

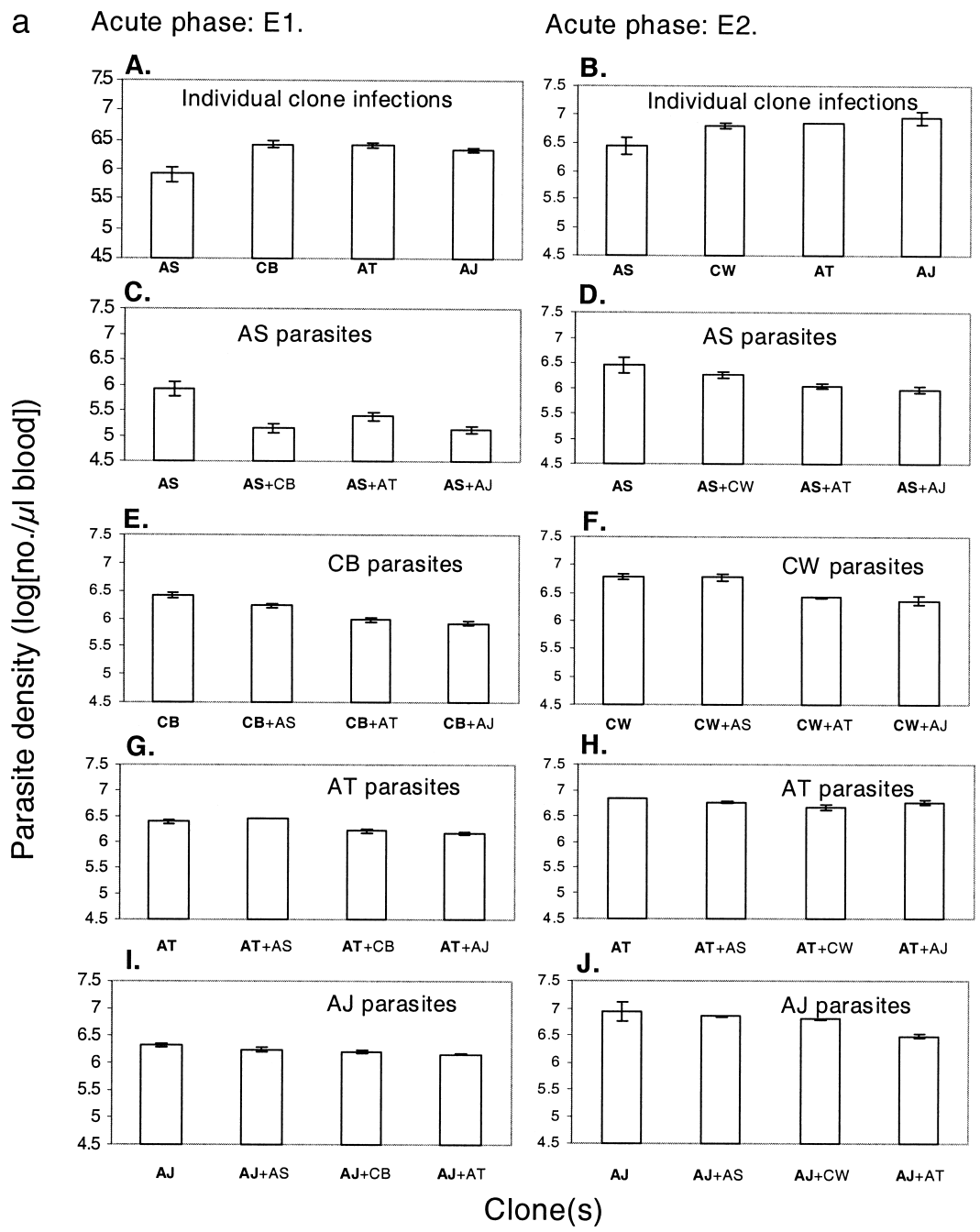


FIG. 3. Parasite density of the four clones individually and in pairwise competition during the acute (a, above) and chronic (b, next page) phases of infection in each experiment. For the acute phase of infection (a), experiment 1 panels (E1: panels A, C, E, G, I) show peak parasite densities, whereas experiment 2 panels (E2: B, D, F, H, J) show total parasite densities. Bars represent mean (±1 SEM) of the number of mice surviving up to the end of each phase (maximum $n = 5$). A single asterisk indicates a single mouse in a treatment group and two asterisks a treatment group in which all mice died.

competitive outcome, with dead mice being excluded (leaving $n = 37$).

In both experiments, and as expected, individual clone infections varied in virulence (experiment 1: $F_{3,8} = 9.1$, $P = 0.006$; experiment 2: $F_{3,11} = 6.8$, $P = 0.007$), with maximum RBC losses greatest for clones AJ and AT and lowest for AS (Fig. 1). Infections with more virulent clones contained more parasites during the acute phase (Figs. 1; 2a,b, left panels; 3a, panels A and B). In experiment 1, CB, AT, and AJ had higher densities than AS (Fig. 3a, A), and in experiment 2, AJ and AT had higher densities than AS, with CW having intermediate densities (Fig 3a, B). More virulent clones also persisted for longer and at higher densities during the chronic phase. Thus, in experiment 1, clones CB, AT, and AJ (Fig. 2a, E, I, M; Fig. 3b, A) remained at higher densities for longer than AS (Fig. 2a, A; Fig. 3b, A), and in experiment 2, clones

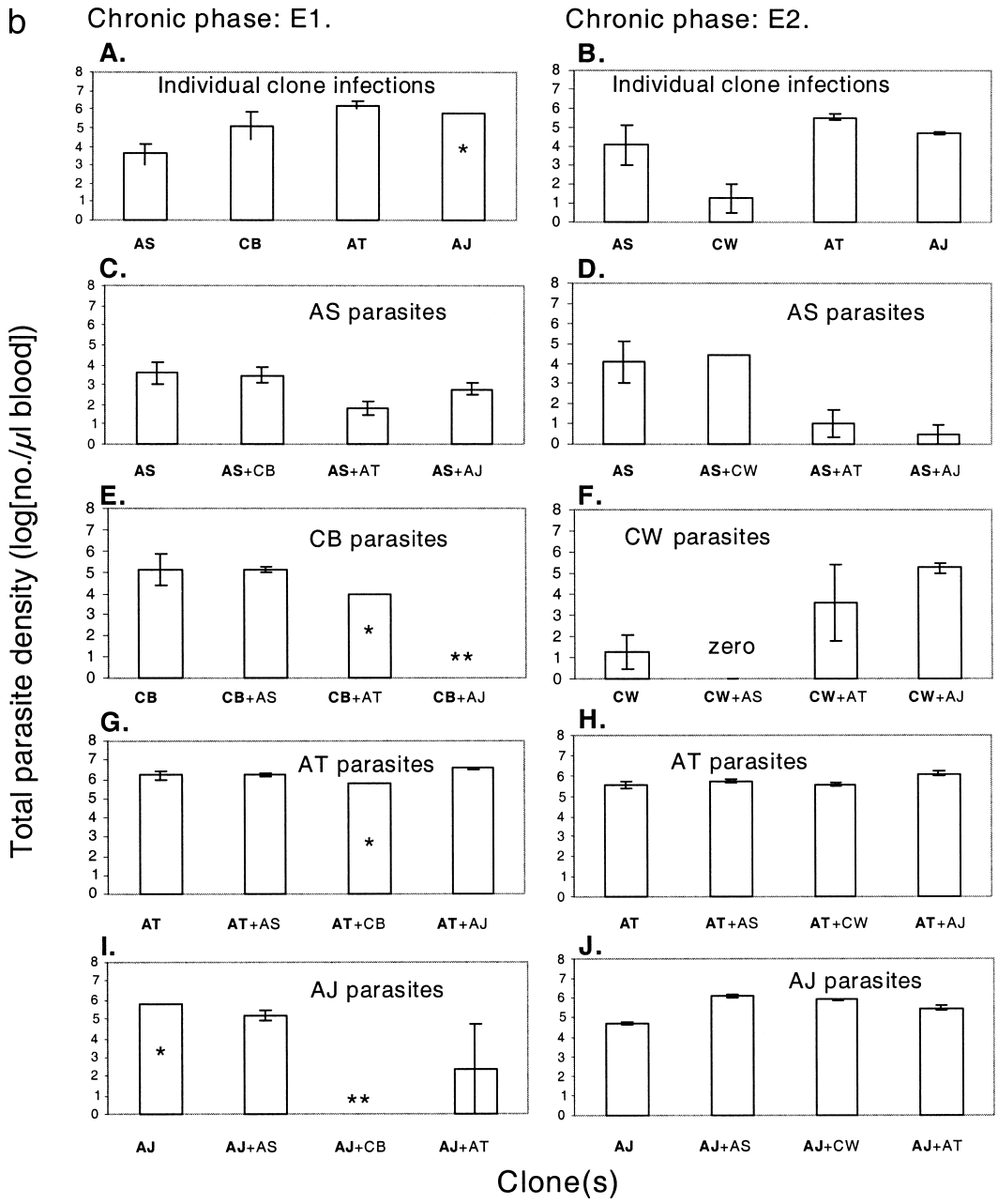


FIG. 3. Continued.

AT and AJ (Fig. 2b, I and M; Fig. 3b, B) had higher densities for longer than AS and CW (Fig. 2b, A and E; Fig. 3b, B). These clone differences in parasite densities and morbidity were reflected in mortality rates and time to death (Fig. 2) and are in line with those found previously (e.g., Mackinnon and Read 1999a; de Roode et al. 2005a,b).

### *Competition*

During the acute phase, all clones suffered from competitive suppression in at least one of the experiments and, in most cases, the densities achieved by individual clones in mixed infections depended on which clone was the competitor (Table 2). For example, in experiment 1, the avirulent clone AS suffered equivalent competitive suppression by the virulent clones CB, AT, and AJ (Fig. 2a, B, C, D; Fig. 3a, C), but CB suffered less competitive suppression by the avirulent clone AS (Fig. 2a, F; Fig. 3a, E) than by the virulent clones AT and AJ (Fig. 2a, G and H; Fig. 3a, E). Furthermore, in experiment 2, clone AS was competitively suppressed more by the virulent clones AT and AJ (Fig. 2b, C and D; Fig. 3a, D) than by the moderately virulent clone CW (Fig. 2b, B; Fig. 3a, D). Also, clone CW was not suppressed by the less virulent AS (Fig. 2b, F; Fig. 3a, F), but it was by the virulent clones AT and AJ (Fig. 2b, G and H; Fig. 3a,

TABLE 2. Parasite densities during the acute and chronic phases of infection. Competition during the acute phase in experiment 1 is based on peak parasite densities rather than total parasite densities (see trait definitions in Materials and Methods). ''Competition'' is the effect of the presence of a competitor, irrespective of the identity of the clone (i.e., a comparison of the focal clone's performance in mixed infections relative to that achieved alone). ''Competitor'' is whether the performance of the focal clone in mixed infections varied depending on the identity of the competitor (i.e., a comparison of the performance of the focal clone against each of the three other clones). Significant results indicate suppression of the focal clone, with the exception of an asterisk, which indicates increased numbers (facilitation) of the focal clone in at least some mixed infections. A dash indicates that the clone was absent from the experiment; NP, not possible, where too few mice survived to the chronic phase to enable statistical testing.

| Infection phase | Clone | Experiment 1 | | Experiment 2 | |
|---|---|---|---|---|---|
| | | Competition | Competitor | Competition | Competitor |
| Acute | AS | $t_{14} = 12.9$, $P < 0.001$ | $F_{2,12} = 3.2$, $P = 0.08$ | $t_{11} = 7.9$, $P < 0.001$ | $F_{2,9} = 7.7$, $P = 0.01$ |
| | CW | — | — | $t_{10} = 3.8$, $P = 0.004$ | $F_{2,8} = 11.6$, $P = 0.004$ |
| | CB | $t_{14} = 9.0$, $P < 0.001$ | $F_{2,12} = 21.8$, $P < 0.001$ | — | — |
| | AT | $t_{14} = 5.5$, $P < 0.001$ | $F_{2,12} = 31.7$, $P < 0.001$ | $t_{9} = 5.0$, $P = 0.001$ | $F_{2,7} = 1.9$, $P = 0.22$ |
| | AJ | $t_{14} = 5.9$, $P < 0.001$ | $F_{2,12} = 1.4$, $P = 0.28$ | $t_{10} = 4.2$, $P = 0.002$ | $F_{2,8} = 66.2$, $P < 0.001$ |
| Chronic | AS | $t_{8} = 2.7$, $P = 0.03$ | $F_{2,6} = 6.1$, $P = 0.04$ | $t_{11} = 3.4$, $P = 0.004$ | $F_{2,9} = 18.9$, $P = 0.001$ |
| | CW | — | — | $t_{10} = 2.0$, $P = 0.08$ | $F_{2,8} = 5.5$, $P = 0.03$* |
| | CB | NP | NP | — | — |
| | AT | $t_{4} = 1.2$, $P = 0.31$ | $F_{2,3} = 7.0$, $P = 0.07$ | $t_{9} = 2.9$, $P = 0.02$* | $F_{2,7} = 6.2$, $P = 0.03$* |
| | AJ | NP | $F_{1,3} = 3.5$, $P = 0.16$ | $t_{10} = 12.4$, $P < 0.001$* | $F_{2,8} = 9.9$, $P = 0.007$* |

F). Finally, clone AJ was not suppressed by the less virulent clones AS and CW (Fig. 2b, N and O; Fig. 3a, J), but it was by the equally virulent AT (Fig. 2b, P; Fig. 3a, J).

Whereas clones were generally suppressed by competitors during the acute phase of infection, in the chronic phase competitive suppression was rarer. More common was that clone densities were unaffected by the presence of another clone (Table 2) or densities were elevated (i.e., facilitation rather than competition). For example, in experiment 2, after clone CW was suppressed by clones AT and AJ in the acute phase (Fig. 2b, G and H; Fig. 3a, F), it obtained higher densities subsequently than it did on its own (Fig. 2b, G and H; Fig. 3b, F). Furthermore, in experiment 2, when another clone was present, AJ always did better than it did on its own. This facilitation of AJ was even greater if the coinfecting clone was AS or CW rather than AT (Fig. 2b, N-P; Fig. 3b, J). Facilitation was also observed in experiment 2 for clone AT in combination with AJ (Fig. 2b, L; Fig. 3b, H). Finally, in experiment 1, where clone AS was strongly suppressed by CB during the acute phase (Fig. 2a, B; Fig. 3a, C), it actually had higher densities in the presence of CB around day 20 than it did on its own (Fig. 2a, B). More generally, however, clone AS was the one clone that did not obtain facilitation during the chronic phase; AS was suppressed by clone AT in experiment 1 (Fig. 3b, C) and AT and AJ in experiment 2 (Fig. 3b, D), with its densities eventually suppressed below detectible levels in both experiments (Fig. 2a, C, D; Fig. 2b, C, D).

### *Virulence and Competitive Ability in Acute Phase*

In the acute phase of infection, the extent of competitive suppression was related to the difference in the intrinsic virulence of the competing clones, with competitive suppression being greater the larger the disparity between the intrinsic virulence of the competing clones (Fig. 4a; $F_{1,9} = 55.6$, $P < 0.001$, $R^2_{adj} = 0.845$). The slope of this relationship did not differ between experiments (experiment × virulence interaction, $F_{1,7} = 0.04$, $P = 0.84$), but the intercepts differed slightly ($F_{1,8} = 11.1$, $P = 0.01$). The negative relationship between virulence and competitive suppression came about as described above: clone AS densities were barely reduced by the moderately virulent clone CW, but strongly by the virulent clones AJ and AT. Also, clone CW, of intermediate virulence, was unaffected by the presence of the less virulent AS but suppressed by the more virulent clones AJ and AT. One notable anomaly to the strong general trend was the greater than expected competitive suppression of the virulent clone AT by the moderately virulent clone CW (Fig. 3a, H).

The competitive suppression of clones by more virulent clones affected the frequency of the clones in mixed infections (Fig. 5). Clones of similar intrinsic virulence (i.e., relative virulence = 1) were equally represented in infections. With increasing disparity in the intrinsic virulence of coinfecting clones, the less virulent clone became disproportionately rarer (Fig. 4c; $F_{1,9} = 30.1$, $P < 0.001$, $R^2_{adj} = 0.744$; trend line significantly steeper than the null expectation: interaction between null expectation and observed trend lines: $F_{1,18} = 11.2$, $P = 0.004$). The virulence-competitiveness relationship did not differ between experiments (experiment main effect and experiment × virulence interactions both $P > 0.05$).

### *Virulence and Competitiveness in Chronic Phase*

During the chronic phase, when so little competitive suppression was detected and some facilitation was apparent, there was no relationship between virulence and the extent of competitive suppression (Fig. 4b; $F_{1,9} = 0.04$, $P = 0.84$). Nevertheless, there was a suggestion that frequency in the mixed infections was related to intrinsic virulence differences, with clones achieving higher frequencies when competed with more similarly virulent clones (Fig. 4d, $F_{1,9} = 5.06$, $P = 0.051$).

## DISCUSSION

This study shows that, in a range of mixed-strain infections of the malaria *P. chabaudi*, more virulent strains have a competitive advantage within their rodent host (Fig. 4a, c). During

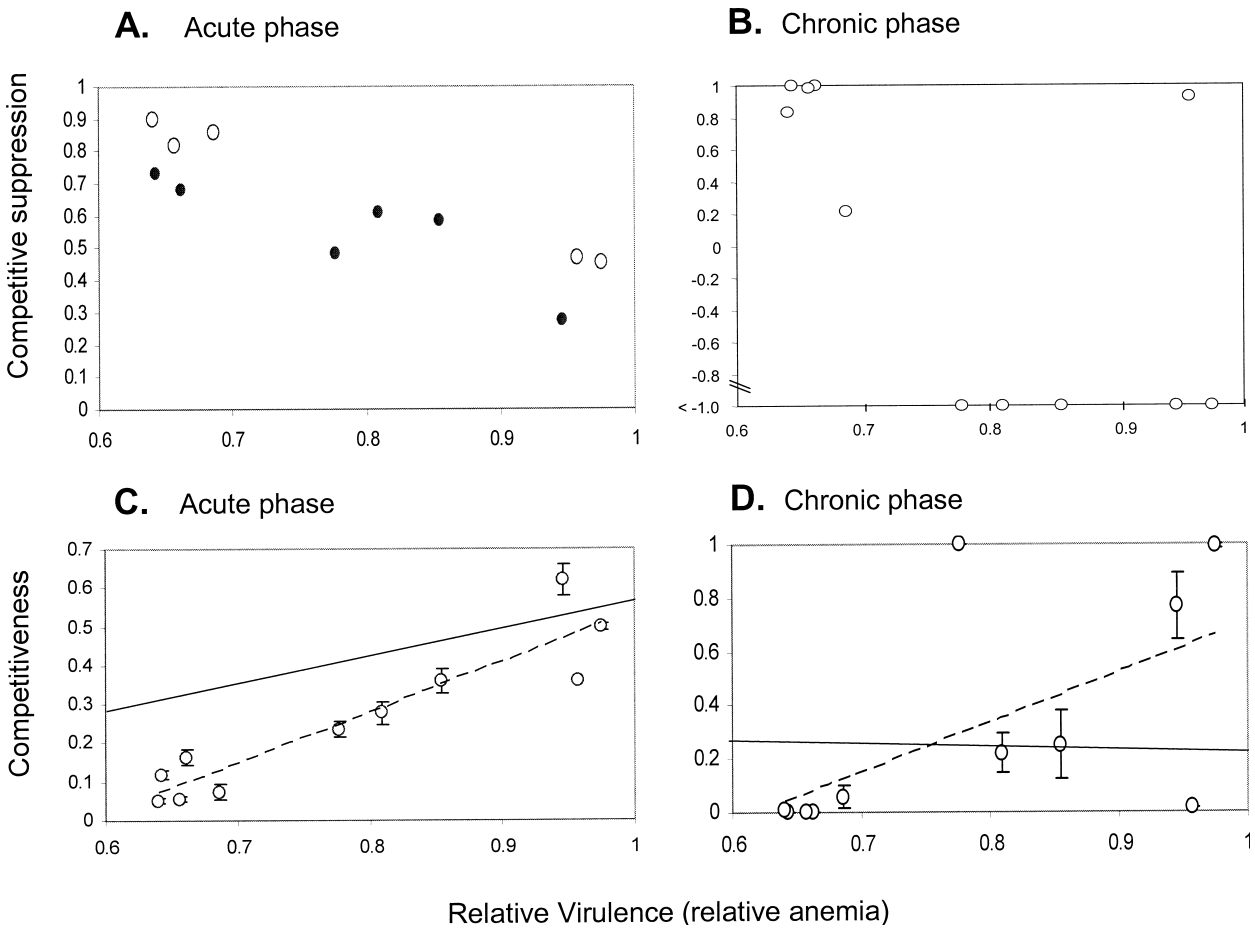


FIG. 4. Relationship between the relative virulence of clones and the extent of competitive suppression (A, B) and competitiveness (C, D) in competition. Data are pooled across both experiments during the acute (A, C) and chronic (B, D) phases of infections. Relative virulence is the anemia induced by the two competing clones when on their own, expressed as the anemia induced by least virulent competitor as a fraction of that induced by the more virulent. Thus, a value of 1.0 means the competing clones induce equal levels of anemia; a value of 0.5 that the less virulent clone induces half the anemia of the more virulent. Competitive suppression is the proportional reduction in clone density due to the presence of a competitor (1, competitive exclusion; 0, no suppression; <0, facilitation); competitiveness is clone frequency in mixed infections (see text). Dotted lines are least squares regression lines for plotted data. In (A) open circles indicate datapoints from experiment 1 and closed circles datapoints from experiment 2. Solid lines in panels (C) and (D) indicate the null expectation if there was no competition present between the two coinfecting clones (see Materials and Methods). There are a total of 11, not 12, pairwise competitions because there were no surviving mice in the CB + AJ coinfection group in experiment 1.

the acute phase of infection, less virulent clones were competitively suppressed (Fig. 4a) and attained progressively lower parasite numbers as the disparity between the virulence of the clones increased (Fig. 4c). This relationship between virulence and competitive ability bears striking resemblance with the relationship we found in our earlier analysis of closely related lines derived from a single clonal lineage (de Roode et al. 2005a). Between that study and the results we report here, we have tested for competition with 18 different clone combinations, each of which is based on at least five replicate mixed infections and five of each of the relevant single clone infections. Thus, the competitive advantage of virulent clones in mixed infections appears to be a general phenomenon in this host-parasite system, and one that is relevant both when novel virulence variants arise within an infection of wild-type parasites and when unrelated parasites differing in virulence coinfect the same host.

Most human malaria infections are genetically diverse (e.g., Day et al. 1992; Arnot 1999; Babiker et al. 1999), and such infections are also very common in *P. chabaudi* in its natural host (e.g., Beale et al. 1978). Our experiments show that more virulent clones are the better competitors in mixed infections, so that mixed infections should select for higher virulence. Indeed, over 70% of the variance in competitive ability was explained by virulence differences. Although we did not test here whether competitive superiority led to greater transmission success, previous experiments have shown that competitive suppression results in reduced transmission to the mosquito vector (de Roode et al. 2005a) and that clone frequency in mixed infections is positively related to relative transmission success (Taylor and Read 1998; de Roode et al. 2005a). Thus, there is no evidence that suppressed clones increase their relative investment in transmission-stage production, a possibility suggested by several authors (e.g., Van Baalen and Sabelis 1995; Paul et al. 2003).

In other experiments with just two clones (AS and AJ), we

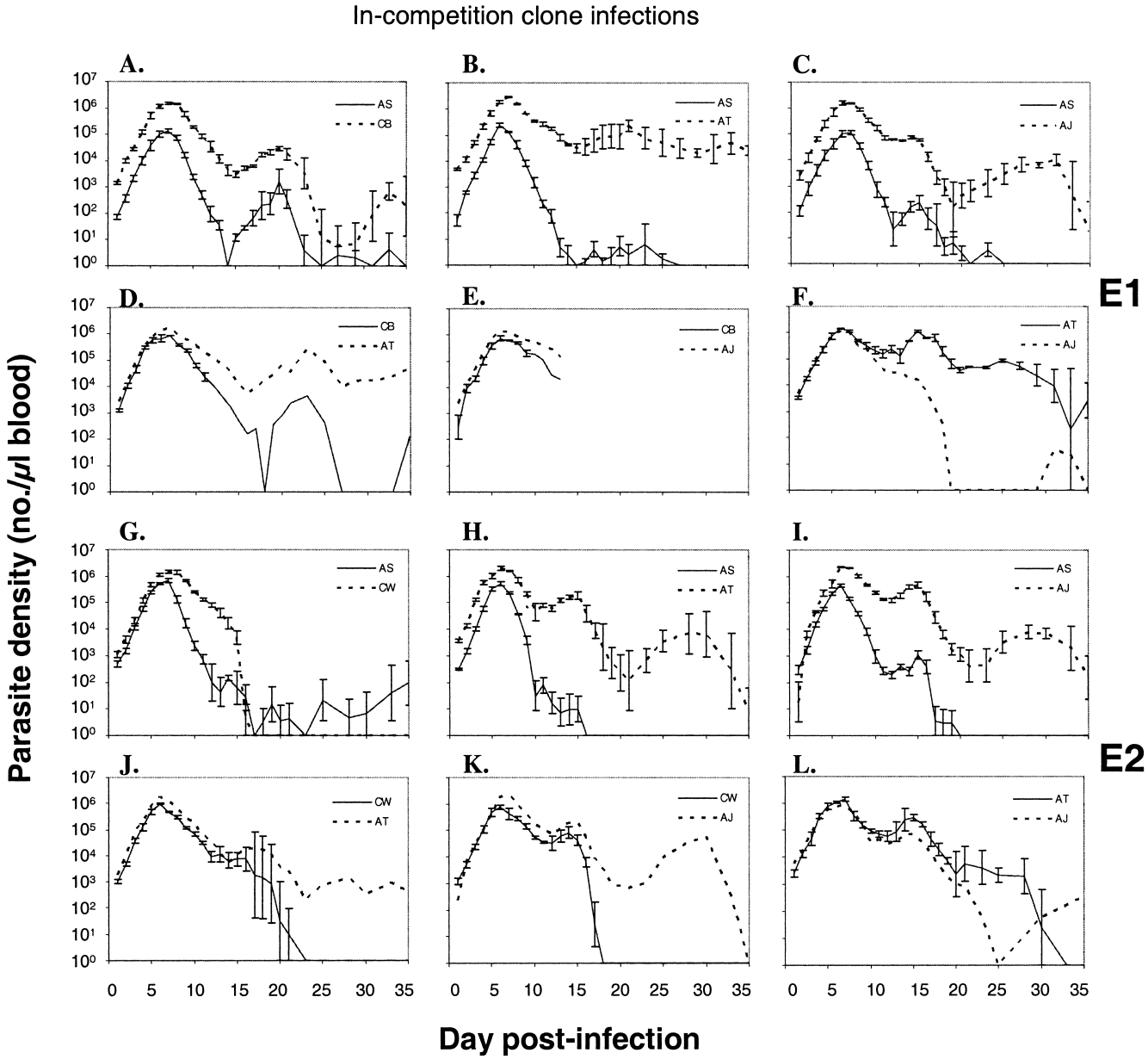


FIG. 5. Densities of individual parasite clones through time in mixed intections (mean ± 1 SEM). Panels (A–F) experiment 1 (E1), panels (G–L) experiment 2 (E2). Means are based on the number of mice alive at that point (maximum $n = 5$). Traces terminate at the time point of the last mouse death if all mice in a treatment group died.

found a number of factors that quantitatively affect the outcome of competition, such as infection order and relative frequency in inocula (de Roode et al. 2005b), host genotype (de Roode et al. 2004b), and dose (B. H. K. Chan, J. C. de Roode, and A. F. Read, unpubl. data). However, in each case, the qualitative result is as reported here: AJ, the more virulent clone, was always competitively superior. Thus, to date we have no experimental data to suggest that realities of disease ecology will affect the direction of selection on virulence imposed by within-host competition. Taken together, the acute-phase data support the virulence-competitive ability assumption of numerous mathematical models that show that within-host competition will promote the evolution of more virulent parasites in a population when mixed infections are common (e.g., Levin and Pimentel 1981; Bremermann and Pickering 1983; Van Baalen and Sabelis 1995; Frank 1996; Mosquera and Adler 1998).

Although it is clear that virulent clones numerically dominate mixed infections, the mechanism by which they do this is less clear. Many models of virulence evolution assume that intrinsic virulence of a parasite genotype is associated with its intrinsic replication rate. The clones we used in this experiment did differ in intrinsic replication rate during the initial phase of infections, but these clone differences were not associated with the differences in clone virulence (data not shown). Mackinnon et al. (2002, 2005) and Mackinnon

and Read (2004) also reported that there was little evidence to suggest that increased *P. chabaudi* virulence was due to a higher intrinsic asexual multiplication rate. In human malaria, there are some data to show that virulence is associated with intrinsic replication rate at least in vitro (Chotivanich et al. 2000), but other studies have not found such an effect (Simpson et al. 1999; Deans et al. 2006).

In our experiments, what does appear key is the ability of a virulent clone to continue to multiply at high parasite densities (e.g., Figs. 2, 3; see also Mackinnon et al. 2002). In particular, the overall number of parasites that can be attained in an infection is constrained (results from this experiment: A. S. Bell, J. C. de Roode, D. Sim, A. F. Read, unpubl. ms.), presumably due to a combination of resource limitation (Yap and Stevenson 1994; Li et al. 2001) and strain-transcending immunity (immune-mediated apparent competition; Read and Taylor 2000, 2001; de Roode et al. 2003; Haydon et al. 2003; Stevenson and Riley 2004; Råberg et al. 2006). It is notable, for example, that during the acute phase of infection even the most virulent of our clones (AJ) was competitively suppressed in the presence of a similarly virulent clone (AT). In the face of such resource limitation and immune attack, it is likely that the most successful parasites are those that are able to maintain high numbers, perhaps by being less selective in their invasion of RBC, which is considered a factor in severe human *P. falciparum* malaria (Chotivanich et al. 2000; reviewed in Mackinnon and Read 2004), or by better avoidance of initial host immune responses, possibly by differences in cytoadherence or other antigenic variation abilities (reviewed in Mackinnon and Read 2004). In principle, it is possible that there is direct interference between competing clones, analogous, for example, to bacteriocin-mediated warfare that occurs in some bacterial infections (Riley and Gordon 1999), but we know of no direct evidence for the secretion of alleopathic substances by malaria parasites (or indeed any protozoa).

Several observations suggest that the immune system does indeed play an important role in the interaction between malaria clones (see also de Roode et al. 2005b; Råberg et al. 2006). Most notably, the acute phase was characterized by competitive suppression of clones, whereas the chronic phase often showed facilitation (Table 2, Fig. 4). This is in line with findings that immunity during the acute phase of infection is predominantly strain transcending (e.g., Taylor-Robinson 1995; Phillips et al. 1997; Li et al. 2001), so that the immune response invoked by the presence of one clone also affects a competitor clone. During the chronic phase, however, immunity is predominated by immune responses that are largely strain specific and more effective against the clone that invoked them than against other clones (e.g., Jarra et al. 1986; Jarra and Brown 1989; Snounou et al. 1989; Buckling and Read 2001; de Roode et al. 2005b).

Many of these studies have also shown that strain-specific responses can be highly asymmetric (e.g., Jarra and Brown 1989; Buckling and Read 2001; de Roode et al. 2005b): for example, the response invoked by clone AS has been shown to be relatively ineffectual against CB, but the response against CB appears also to be highly effective against AS. Such asymmetries could explain why interactions between clones in the chronic phase depended on the specific pairwise clone combination. For instance, in experiment 2, clone AS was suppressed during the chronic phase, but only by AT and AJ (Fig. 3b, D), whereas AJ benefited from the presence of any of the three clones (Fig. 3b, J). Furthermore, it is possible that immune avoidance could be more readily achieved when the competitor was also present in sufficient numbers to be a focus of the immune response. For example, the more virulent clones often recrudesced earlier when in competition (Fig. 2, J–L, N–P). In addition, in competition with AS, clone CW was the dominant clone during the acute phase (its performance unaffected by the presence of clone AS [Fig. 2b, F], whereas clone AS was suppressed in the presence of CW (Fig. 2b, B]) and produced no parasites during the chronic phase (Fig. 3b, F). In contrast, in coinfections with AT and AJ, CW was dominated during the acute phase (Fig. 5), but produced much higher numbers during the chronic phase than it would have done alone (Fig. 2b, G, H; Fig. 3b, F). This suggests that where CW was dominant, the immune system focused on CW and controlled it before it could recrudesce; where CW was dominated by AT and AJ, the immune system may have left CW free to recrudesce strongly during the chronic phase.

Without direct transmission data, it is difficult to evaluate the evolutionary significance of these dynamics in the chronic phase. The majority of transmission in this model system, however, typically occurs during or shortly after the acute phase (H. M. Ferguson, K. Grech, J. C. de Roode, K. Watt, B. H. K. Chan, and A. F. Read, unpubl. data), with infectivity declining through time, possibly as a consequence of reduced densities of transmission stages during the chronic phase (e.g., Buckling et al. 1997) and possibly because transmission blocking immunity develops (e.g., Naotunne et al. 1990). Clearly persistence in chronic infections must be important in epidemiological situations where malaria frequently survives very long dry seasons (e.g., Abdel-Wahab et al. 2002). To the extent that our results generalize to the field situation, it might be hypothesized that between-season selection on virulence is not affected by interactions among coinfecting strains. In contrast, during transmission seasons, virulence would be favored because of the competitive advantage.

If our experimental results capture the relevant natural history of malaria infections in the field, the relationship between virulence and competitive ability we have demonstrated implies that any public health interventions that actively reduce strain multiplicity could have beneficial evolutionary consequences (e.g., Adler and Mosquera Losada 2002; Galvani 2003): by reducing the number of within-host competing clones, the evolutionary selection for increased virulence could be reduced, thus selecting for less virulent parasites. A range of different interventions could have antimultiplicity effects, such as bed nets, transmission blocking vaccines, or partially effective drugs. However, interventions designed to reduce parasite growth rate and/or toxicity can diminish selection against virulent pathogens (Gandon et al. 2001; Mackinnon and Read 2003, 2004; Porco et al. 2005), thus favoring higher levels of intrinsic virulence and resulting in more severe disease in unvaccinated individuals. We are currently investigating how immunization and semi-immunity affect in-host competition; semi-immunity promotes the evolution of virulence in serially passaged malaria lines (Mackinnon

and Read 2004), as would be expected if immunity enhances the advantages of virulence by intensifying competition. Strain-specific vaccination could also change the outcome of competition. Of equal concern is competitive release following drug treatment (see de Roode et al. 2004a), where less virulent but drug-resistant strains survive better than sensitive strains and are able to exploit the opportunities presented by the removal of their competitors, thereby increasing their relative transmission. These findings point to an urgent need to better understand the selective consequences for virulence of chemotherapy and various classes of vaccination. One tentative conclusion can perhaps be made at this stage: use of bed nets and infection-blocking vaccines that reduce the number of clones per host would reduce in-host competition and select for reduced virulence (Gandon et al. 2001; Adler and Mosquera Losada 2002; Galvani 2003).

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