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

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ABSTRACT

Humans and animals often become co-infected 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 co-infected hosts and, because of this, 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 any relationship between virulence and competitive ability

are lacking. Here we use newly developed strain-specific real-time quantitative PCR protocols to determine the pair-wise 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, human interventions aimed at reducing 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

Infections often consist of more than one genetically distinct pathogen lineage (e.g. 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 & 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. Here, 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 & Pimentel 1981; Bremermann & Pickering 1983; Van Baalen & Sabelis 1995; Frank 1996; Gandon et al. 2001; Adler & 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 that which 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 e.g. Chao et al. 2000; Brown et al. 2002; West & Buckling 2003).

Empirically, the situation is also confused. There is a substantial body of experiments demonstrating 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: the serial passage of pathogens through new host environments typically increases virulence (e.g. Ebert 1998; Mackinnon & 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 & Taylor 2001).

Recent advances in quantitative 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 harbouring more than one genotype of the same species (reviewed by Arnot 1999; Read & Taylor 2001). These mixed infections can be extremely common, constituting over 80% of infections in high-transmission areas (e.g. Day et al. 1992; Babiker et al. 1999; Konaté et al. 1999; Druilhe et al. 1998; 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, 2004 a,b, 2005a,b), and a variety of non-experimental observations imply that competition also occurs in human malaria 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 favours 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). These experiments demonstrated that more virulent lines did indeed compete more successfully. Since 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 pair-wise 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 co-infect the same host. The majority of human malaria infections are of this sort (e.g. Day et al. 1992; Babiker et al. 1999; Konaté et al. 1999; Druilhe et al. 1998; 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 with multiply-infected mosquitoes. Malaria parasites differ widely in their antigenic properties, and immune responses are at least partly strain-specific (e.g. Jarra & 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 4 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 & 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 & Read 2004; Cheesman et al. unpublished results). 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 latter 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, UK). Mice were fed on 41B maintenance diet (Harlan, UK) and their drinking water supplemented with 0.05% para-amino benzoic acid to enhance parasite growth (Jacobs 1964). They were maintained at 21°C in a 12L:12D photoperiod.

Experimental design and inoculation of mice with parasites

Both experiments consisted of ten treatments: infections with each of the four clones singularly and all pair-wise mixed infections, i.e. 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 whilst those challenged with two clones were injected with 10^6 of each (total 2 x 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, unpublished results).

Inoculations were prepared from donor mice by diluting blood in 0.1ml of calf serum solution (50% heat-inactivated calf serum, 50% Ringer's solution [27mM KCl, 27mM $CaCl_2$ and 150mM NaCl] and 20 units of heparin per ml, and were introduced by intra-peritoneal injection.

Monitoring of virulence and infection dynamics

Virulence was monitored by recording red blood cell (RBC) densities (using flow cytometry; Beckman Coulter) and body weights with respect to day post-infection (p.i.). 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 standardisation of real-time quantification of parasite densities.

Parasite numbers present in the blood of experimental hosts were determined using real-time quantitative PCR (QPCR). Capillaries were used to collect 5µl samples of tail blood from each mouse

before 12.00 pm 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 0.5ml micro-centrifuge 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 r.p.m) for 1 min, the supernatant removed and the blood pellet stored at -80ºC until DNA extraction. All samples were collected daily from day 1 to day 21 post-inoculation and then every second day until day 35.

DNA extractions were performed using the BloodPrep® kit (Applied Biosystems) on the ABI Prism® 6100 Nucleic Acid PrepStation according to manufacturer's instructions. DNA was eluted in a total volume of 200µl and stored at -80^0C until quantification.

Clone-specific PCR primers and minor grove-binder (MGB) probes, targeting either the *Plasmodium chabaudi chabaudi msp-1* gene or *ama* gene, were designed using Primer Express® (Applied Biosystems) software. Real-time quantitative PCRs were performed on an Applied Biosystems Prism 7000 machine with an initial denaturation of 95ºC for 10min followed by 45 cycles of denaturation at 95ºC for 15s and annealing/extension at 60ºC for 1min. 2µl of DNA was included in a 25µl volume PCR reaction with the following components: 1.5µl each of forward and reverse primer, both at a final concentration of 300nM; 12.5µl of 2x TaqMan® Universal PCR Master Mix (hot start); 1µl of MGB probe at a final concentration of 200nM and 6.5µl 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 & 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 6 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 microlitre of mouse blood (x20: 2µl of DNA used from a total eluted volume of 200µl that was extracted from 5µl of mouse blood). Quantification of target parasites was not affected by the presence of large excesses of non-target parasite DNA (10 target parasite genomes reliably quantified among 100,000 non-target 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, e.g. 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. Whilst 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 QPCR counts provided by the *msp1* and *ama* assays to correlate highly with parasite densities (parasitaemia x rbc density) determined by thin blood smears: R^2>84.3%, $F_{1,37}$>205, p<0.001 (data not shown). The repeatability (Lessells & Boag 1987) of the QPCR assays of duplicate blood samples run on separate extraction plates and quantified in separate QPCR 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. Consequently 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 the "maximum RBC loss" or "maximum weight loss" attained by each mouse (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.

Analyses of parasite numbers, with respect to time post-infection, were performed on summed counts. *P. chabaudi* parasites invade new red cells synchronously every 24 hours, 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 these 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 had occurred but before the end of the acute phase. This disproportionately affected some treatment groups, so that total acute phase data were only available for 1 or 2 mice in some treatment groups. To maximize statistical power, we included these mice in the analyses by analysing 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: $F_{1,57}=454$, $p<0.001$, $R^2_{adj}=88.7\%$). 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. (1) *Competitive suppression*, the proportional reduction in the number of parasites of a focal clone due to the presence of a competitor, 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 1 (when the focal clone is competitively excluded), is zero when a co-infecting clone has no impact, and becomes negative if the performance of the focal clone is improved by the presence of another clone (facilitation). (2) *Competitiveness*, the frequency of the focal clone in a mixed infection, 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 the parasite density of the focal clone in a single infection by the sum 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 pair-wise clone comparison, this was calculated as the maximum red blood cell loss of the lesser virulent clone in a single infection divided by the maximum red blood cell loss of the more virulent clone in a single infection. This value had a maximum of 1 (with both clones being equally virulent); and a value of 0.5 would imply that the lesser virulent clone caused only half as much red blood cell 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, in prep.).

The clonal dynamics in the two experiments were analysed separately because the substitution of clone CB for CW in the second experiment 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 analysed 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 estimated for each mixed infection the deviation from this reference point, giving up to 15 data points for each clone in each experiment. These scores were then used to test whether (i) the performance of a clone was affected by presence of a competitor, irrespective of the identity of the competing clone (ie. whether the average performance of a clone was less than zero, using a one-sample t-test), and (ii) whether performance depended on the clonal identity of the competitor (one way ANOVA). Relationships between virulence and competitive suppression/competitiveness and competitive suppression/competitiveness were analysed using ANCOVA, with experiment, virulence and interactions between these terms as the explanatory variables; models were minimised by removing non-significant ($p>0.05$) terms from the model, starting with interactions. Maximum RBC losses and parasite densities were all log-transformed prior to analysis in order 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 the first experiment, 26 of the 50 mice were euthanised 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 analysed using peak parasite densities (which occurred before death), so that all mice were included in the analysis (n=50; see methods for further details). In the second experiment, fewer mice (13/50) 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 competitive outcome, with dead mice being excluded (n=37).

In both experiments, and as expected, individual clone infections varied in virulence (Experiment1: $F_{3,8}$=9.1, p=0.006; Experiment 2: $F_{3,11}$=6.8, p=0.007), with maximum red blood cell losses greatest for clones AJ and AT, and lowest for AS (Figure 1). Infections with more virulent clones contained more parasites during the acute phase (Figs 1, 2a,b, [left hand panels], 3a [panels A and B]): in experiment 1, CB, AT and AJ had higher densities than AS (Fig 3a, panel A), and in experiment 2, AJ and AT had higher densities than AS, with CW having intermediate densities (Fig 3a, panel 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, panels E, I, M; Fig 3b, panel A) remained at higher densities for longer than AS (Fig 2a, panel A; Fig 3b, panel A), and in experiment 2, clones AT and AJ (Fig 2b, panels I and M; Fig 3b, panel B) had higher densities for longer than AS and CW (Fig 2b, panels A and E; Fig 3b, panel 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 & 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, panels B, C, D; Fig 3a, panel C), but CB suffered less competitive suppression by the avirulent clone AS (Fig 2a, panel F; Fig 3a, panel E) than by the virulent clones AT and AJ (Fig 2a, panels G and H; Fig 3a, panel E). Furthermore, in experiment 2, clone AS was competitively suppressed more by the virulent clones AT and AJ (Fig 2b, panels C and D; Fig 3a, panel D) than by the moderately virulent clone CW (Fig 2b, panel B; Fig 3a, panel D). Also, clone CW was not suppressed by the less virulent AS (Fig 2b, panel F; Fig 3a, panel F), but it was by the virulent clones AT and AJ (Fig 2b, panels G and H; Fig 3a, panel F). Finally, clone AJ was not suppressed by the less virulent clones AS and CW (Fig 2b, panels N and O; Fig 3a, panel J), but it was by the equally virulent AT (Fig 2b, panel P; Fig 3a, panel J).

Whereas clones were generally suppressed by competitors during the acute phase of infection, in the chronic phase, competitive suppression was a lot rarer, and more common was that clone densities were unaffected by the presence of another clone (Table 2), or indeed 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, panels G and H; Fig 3a, panel F), it obtained higher densities subsequently than it did on its own (Fig 2b, panels G and H; Fig 3b, panel 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 co-infecting clone was AS or CW rather than AT

(Fig 2b, panels N-P; Fig 3b, panel J). Facilitation was also observed in the second experiment for clone AT in combination with AJ (Fig 2b, panel L; Fig 3b, panel H). Finally, in experiment 1, where clone AS was strongly suppressed by CB during the acute phase (Fig 2a, panel B; Fig 3a, panel C), it actually had higher densities in the presence of CB around day 20 than it did on its own (Fig 2a, panel B). More generally, however, clone AS was the one clone that did not obtain facilitation during the chronic phase, and was indeed suppressed by clone AT in experiment 1 (Fig 3b, panel C) and AT and AJ in experiment 2 (Fig 3b, panel D), with its densities eventually suppressed below detectible levels in both experiments (Fig 2a, panel C-D; Fig 2b, panels 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}=84.5%). 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, panel 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 co-infecting clones, the less virulent clone became disproportionately rarer (Fig 4c; $F_{1,9}$=30.1, p<0.001, r^2_{adj}=74.4%; 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 n.s.).

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 *Plasmodium chabaudi*, more virulent strains have a competitive advantage within their rodent host (Fig. 4a, c). During 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 which is relevant both when novel virulence variants arise within an infection of wild type parasites, and when unrelated parasites differing in virulence co-infect 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 *Plasmodium 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 & Read 1998; de Roode et al. 2005a). There is thus no evidence that suppressed clones increase their relative investment in transmission-stage production, a possibility suggested by several authors (e.g. Van Baalen & Sabelis 1995; Paul et al. 2003).

In other experiments with just two clones (AS and AJ), we have found a number of factors which *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 & A.F. Read, unpublished results). 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 messy-world realities 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 & Pimentel 1981; Bremermann & Pickering 1983; Van Baalen & Sabelis 1995; Frank 1996; Mosquera & 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. in press).

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 and 3; see also e.g. 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, companion paper in prep.), presumably due to a combination of resource limitation (Yap & Stevenson 1994; Li et al. 2001) and strain-transcending immunity (immune-mediated apparent competition; Read & Taylor 2000, 2001; de Roode et al. 2003; Haydon et al. 2003; Stevenson & Riley 2004; Råberg et al. in press). 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 red blood cells – considered a factor in severe human *P. falciparum* malaria (Chotivanich et al. 2000; reviewed in Mackinnon & Read 2004) – or by better avoidance of initial host immune responses, possibly by differences in cytoadherence or other antigenic variation abilities (reviewed in Mackinnon & Read 2004). In principle it is possible that there is direct interference between competing clones, analogous for example to bacteriocin-mediated warfare which 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. in press). Most notably, the acute phase was characterised by competitive suppression of clones, whereas the chronic phase often showed facilitation (Tab. 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 & Brown 1989; Snounou et al. 1989; Buckling & Read 2001, de Roode et al. 2005b).

Importantly, 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 pair-wise clone combination. For instance, in experiment 2, clone AS was suppressed during the chronic phase, but

only by AT and AJ (Fig 3b, panel D), whereas AJ benefited from the presence of any of the three clones it competed with (Fig 3b, panel 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 typically recrudesced earlier when in competition (Fig. 2 panels 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 panel F], whereas clone AS was suppressed in the presence of CW (Fig 2b panel B]) and produced no parasites during the chronic phase (Fig 3b, panel F). In contrast, in co-infections 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, panels G&H and Fig. 3b panel F). This suggests that where CW was dominant, the immune system focused on CW and controlled it before it could recrudesce; where it 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 & A.F. Read, unpublished 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 generalise to the field situation, it might be hypothesised that between-season selection on virulence is

not affected by interactions among co-infecting strains. In contrast, during transmission seasons, virulence would be favoured 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 & 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 anti-multiplicity effects, such as bed nets, transmission blocking vaccines or partially effective drugs. However, some vaccines (those designed to reduce parasite growth rate and/or toxicity) can also diminish selection *against* virulent pathogens (Gandon et al. 2001; Mackinnon & Read 2003, 2004; Porco et al. 2005) thus favouring higher levels of intrinsic virulence and resulting in more severe disease in unvaccinated individuals. We are currently experimentally investigating how immunisation and semi-immunity affect in-host competition; semi-immunity promotes the evolution of virulence in serially passaged malaria lines (Mackinnon & 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 & Mosquera Losada 2002; Galvani 2003).

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Table 1. Real-time quantitative PCR (QPCR) assays: primer and probe sequences and details of assay specificities

Primer identification (target gene)	Primer sequence 5'-3'	Amplicon size (bp)	MGB probe sequence	Non-target clones amplified	Primer usage (target clone in bold)
AS(*ama*)F AS(*ama*)R	GGA AAA GGT ATA ACT ATT CAA AAT TCT AAG GT AAT TGT TAT AGG AGA AAT GTT TAC ATC TGT TTG	129	*ama* probe: 6-FAM-ATC CTC CTT CTC TTA CTT TC-MGB	AT	**AS**+AJ, **AS**+CW
AJ(*ama*)F AJ(*ama*)R	GGA AAA GGT ATA ACT AAT CAA AAA TCT ACT AAA GTG TTA TAG GAG AAA TGT GTA CAT CTG TTT T	127		None	AS+**AJ**, **AJ**+CW
AS/AJ(*msp-1*)F AS/AJ(*msp-1*)R	CCG GAA GAA CTA CAG AAT ACA CCA T AGA AGT AGA AAA TGC AGA TAG GGA AAA	105	*msp1* probe: 6-FAM-ACA AGT ACA TAC AAT TTT T-MGB	CW	**AS**, **AJ**, **AS**+AT, **AJ**+AT, **AS**+CB, **AJ**+CB
AT(*msp-1*)F AT(*msp-1*)longR	GGA AGA ACT ACA GAA TAC ACC AGC ATA GAA TGT AGA GAA GTA GAA AAT ACA GAT ACA ACT AA	111		None	**AT**, AS+**AT**, AJ+**AT**, **AT**+CB, **AT**+CW
CB(*msp-1*)F CB(*msp-1*)R	As AS/AJ(*msp-1*)F GAA GAA GTA GAA AAT GCA GAT AGT GCT AA	107		None	**CB**, AS+**CB**, AJ+**CB**, AT+**CB**
CW(*msp-1*)F CW(*msp-1*)upR	As AS/AJ(*msp-1*)F AAG ATG AAG ATG GTA ATA GAT GTG TAG CA	196		None	**CW**, AS+**CW**, AJ+**CW**, AT+**CW**

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). Significant results indicate suppression of the focal clone, with the exception of an asterisk, which shows 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	
		Competiton[a]	Competitor[b]	Competition[a]	Competitor[b]
Acute	AS	$t_{14} = 12.9$, $p<0.001$	$F_{2,12}=3.2$, $p=0.08$	$t_{11} = 7.9$, $p<0.001$	$F_{2,9}=7.71$, $p=0.01$
	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	$t_{14} = 5.5$, $p<0.001$	$F_{2,12}=31.7$, $p<0.001$	$t_{9} = 5.0$, $p=0.001$	$F_{2,7}=1.88$, $p=0.22$
	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	$t_{8} = 2.7$, $p=0.030$	$F_{2,6}=6.07$, $p=0.04$	$t_{11} = 3.4$, $p=0.004$	$F_{2,9}=18.93$, $p=0.001$
	CW	-	-	$t_{10} = 2.0$, $p=0.08$	$F_{2,8}=5.46$, $p=0.03$*
	CB	NP	NP	-	-
	AT	$t_{4} = 1.2$, $p=0.31$	$F_{2,3}=7.0$, $p=0.07$	$t_{9} = 2.9$, $p=0.02$*	$F_{2,7}=6.21$, $p=0.03$*
	AJ	NP	$F_{1,3}=3.51$, $p=0.16$	$t_{10} = 12.4$, $p<0.001$*	$F_{2,8}=9.93$, $p=0.007$*

[a]'*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).

[b]'*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).

Figure Legends

Figure 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 s.e.m. Open circles represent data from experiment 1 and closed circles data from experiment 2. Error bars are missing for AJ in experiment 1 as only a single mouse survived the entire acute phase of infection in this treatment group. Vertical error bars for AT in the second experiment are smaller than the symbol; horizontal error bars for CB in experiment 1 are based on just two animals.

Figure 2. Densities of individual parasite clones through time (mean ± 1 s.e.m.), showing the 4 clones in single-clone infections (left hand columns), and then comparing their performance alone and in competition with each of the other clones in experiment 1 (a) and 2 (b). Means are based on the number of mice alive at that point (max n=5). The days p.i. of mouse deaths are indicated as open circles (individual infections) or closed circles (in competition).

Figure 3. Parasite density of the four clones individually and in pair-wise competition during the acute (a) and chronic (b) 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 s.e.m.) of the number of mice surviving up to the end of each phase (maximum of five). A single asterisk indicates a single mouse in a treatment group and two asterisks an treatment group in which all mice died.

Figure 4. Relationship between the relative virulence of clones and the extent of competitive suppression (top panels) and competitiveness (lower panels) in competition. Data are pooled across both experiments during the acute (left hand panels) and chronic (right hand panels) phases of infectionsRelative virulence is the anaemia induced by the two competing clones when on their own, expressed as the anaemia induced by least virulent competitor as a fraction of that induced by the more virulent. Thus a value of 1 means the competing clones induce equal levels of anaemia; a value of 0.5 that the less virulent clone induces half the anaemia 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 panel A, open circles indicate data points from experiment 1 and closed circles data points from experiment 2. Solid lines in panels C & D indicate the null expectation if there was no competition present between the two co-infecting clones (see methods). There are a total of 11, not 12, pair-wise competitions because there were no surviving mice in the CB+AJ co-infection group in experiment 1.

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

Figure 1.

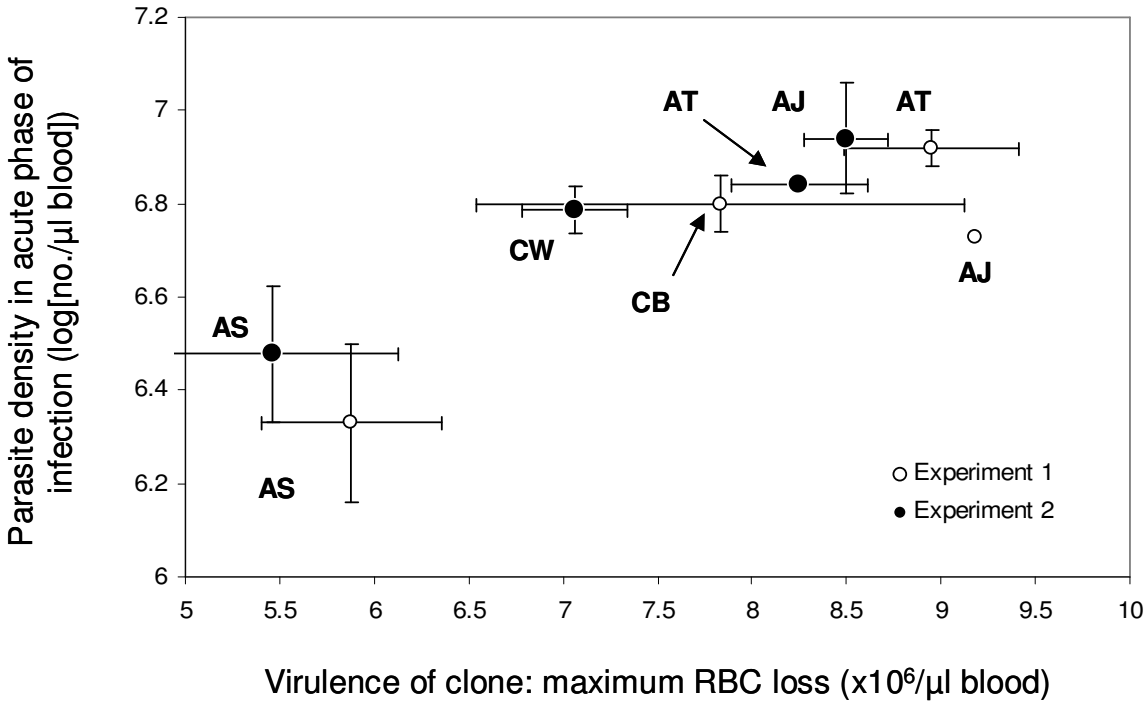
Parasite density in acute phase of infection (log[no./µl blood])
7.2
7
6.8
6.6
6.4
6.2
6
5
5.5
6
6.5
7
7.5
8
8.5
9
9.5
10
AT
AJ
AT
CW
CB
AJ
AS
AS
Experiment 1
Experiment 2
Virulence of clone: maximum RBC loss (x10⁶/µl blood)

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Figure 2a

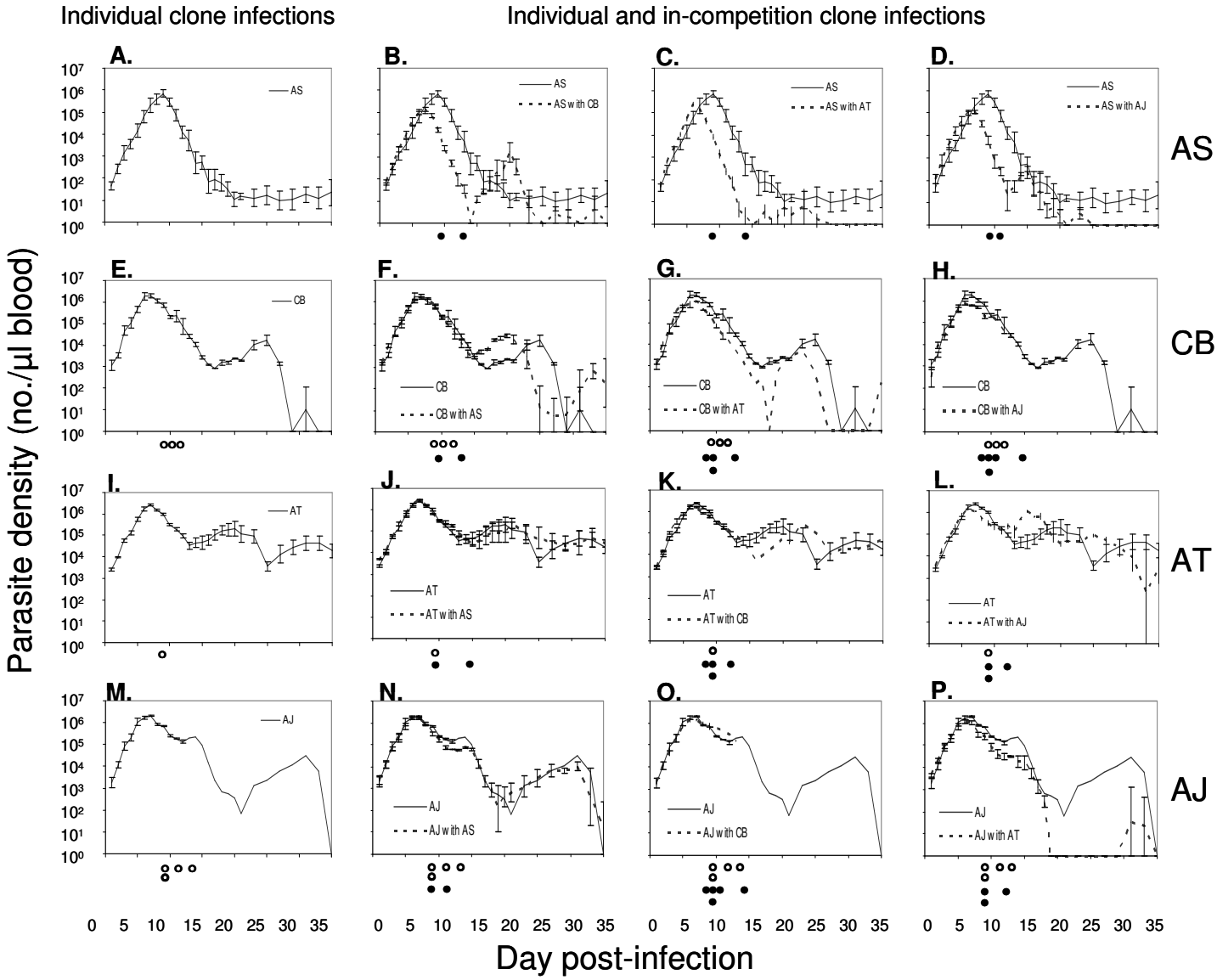

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Fig 2b

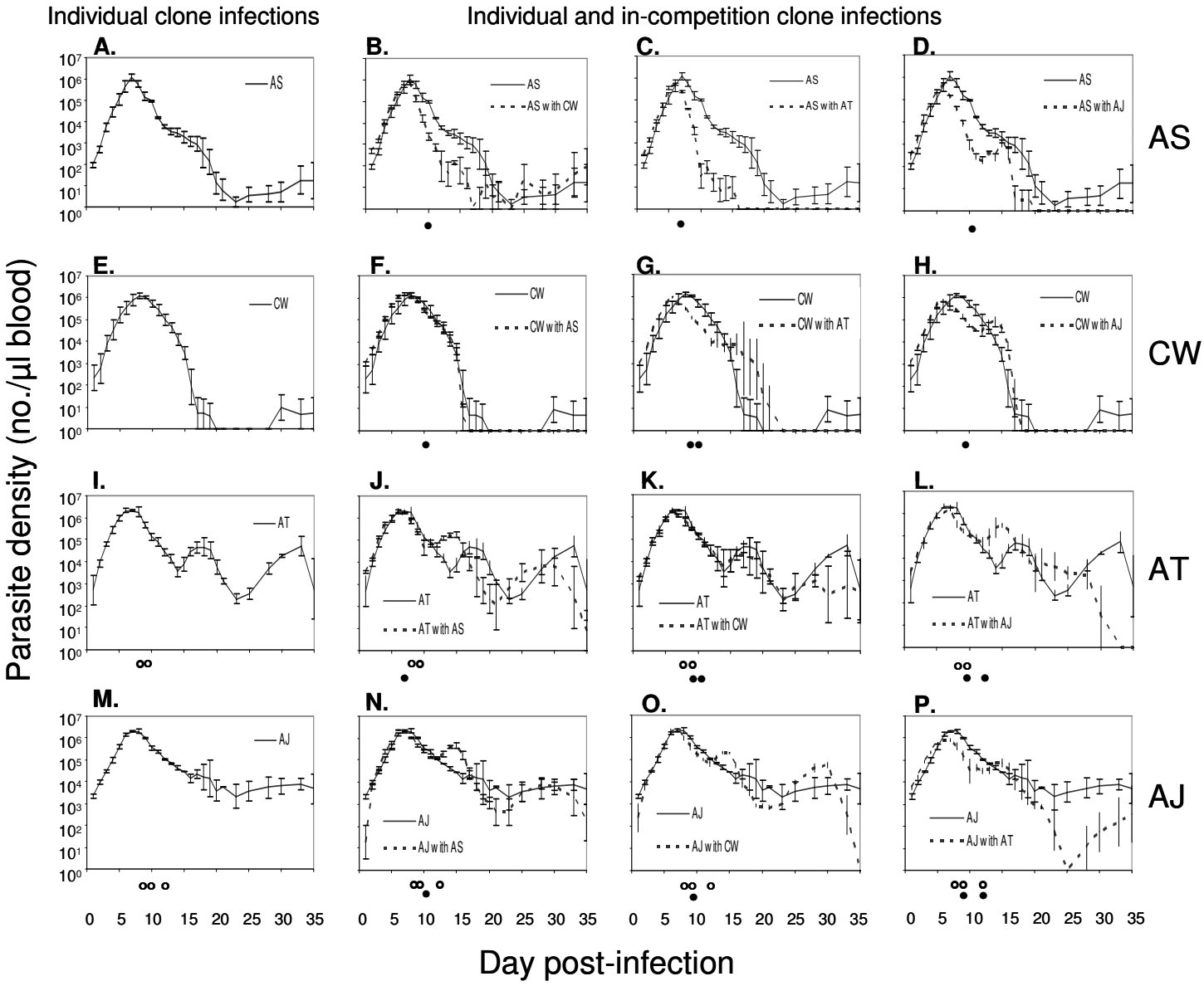

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Fig. 3a

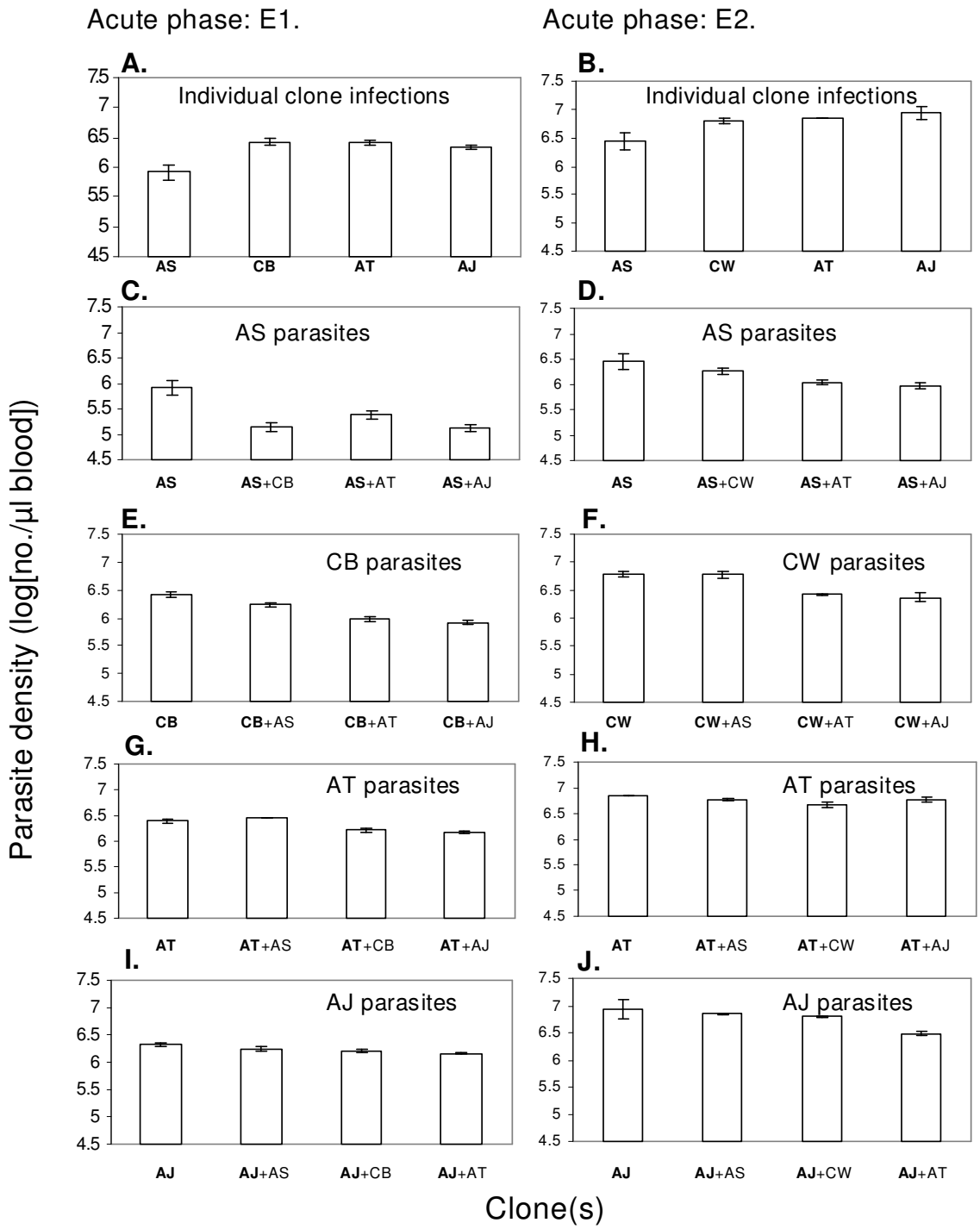

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Fig. 3b

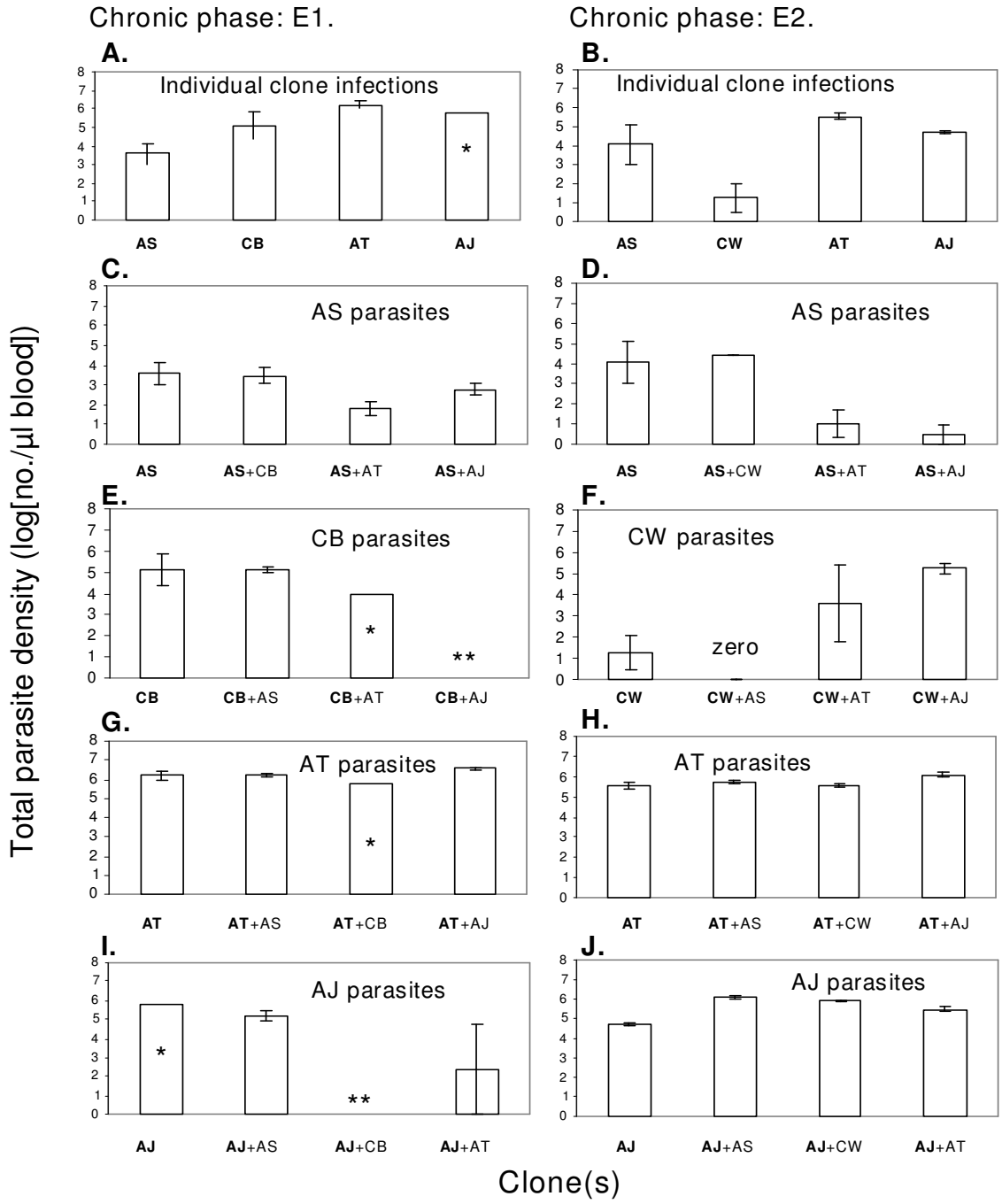

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Fig 4.

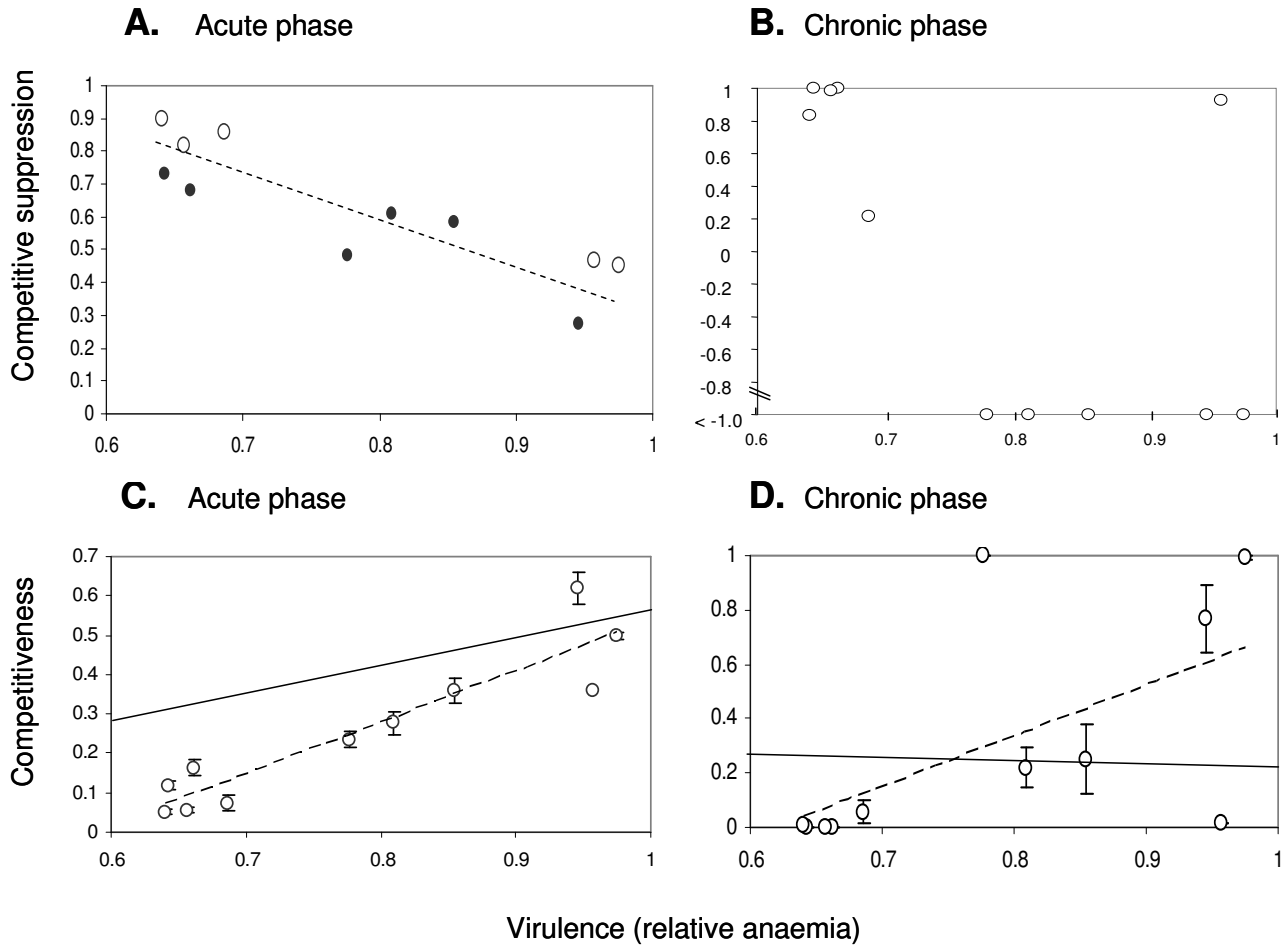
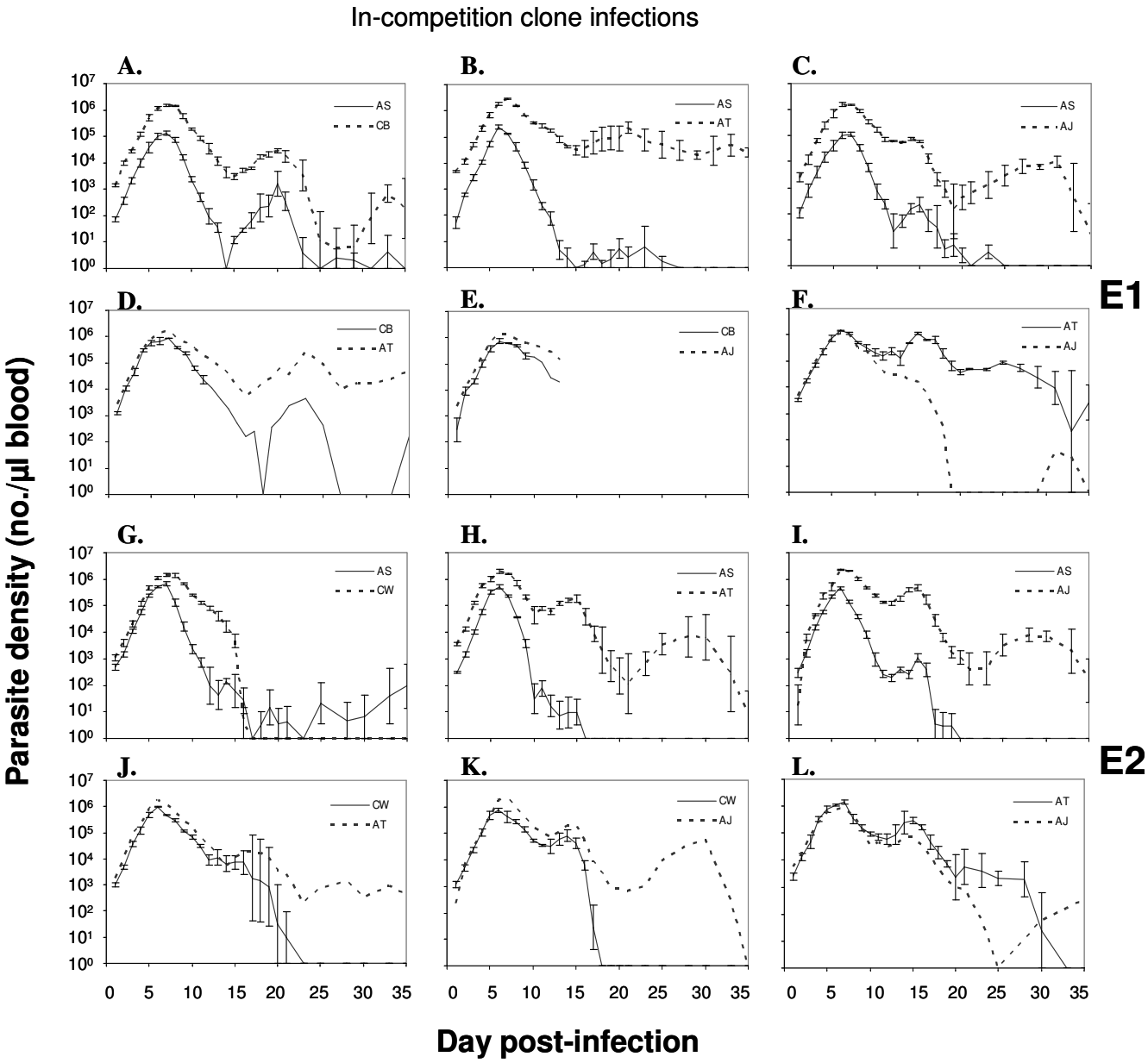


Fig. 5


In-competition clone infections
A.
B.
C.
D.
E.
F.
G.
H.
I.
J.
K.
L.
AS
CB
AT
AJ
CW
E1
E2
Parasite density (no./µl blood)
Day post-infection