Mixed-genotype infections of the rodent malaria *Plasmodium chabaudi* are more infectious to mosquitoes than single-genotype infections

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SUMMARY

Interactions between parasite genotypes sharing a host are poorly understood, but have important consequences for the epidemiology and evolution of the parasite. In mixed-genotype malaria infections, patterns of asexual replication and transmission favoured by natural selection may be different from those in single-genotype infections. The infectivity to mosquitoes of mixed-genotype and single-genotype infections were compared using 2 clones of *Plasmodium chabaudi* inoculated into mice either together or alone. Mice given mixed-clone infections received the sum of the inocula given to the single-clone controls. Mosquitoes were fed on the mice and the numbers of oocysts which developed were counted to assess transmission intensity. For 3 combinations of starting inocula and feed days, mixed-clone infections produced more oocysts per mosquito than the sum of the 2 single-clone infections. This effect was correlated with an increase in gametocyte density, but was less clearly related to asexual infection parameters. The results show that interactions between clones in mixed-clone infections can profoundly affect transmission.

Key words: transmission, competition, malaria, *Plasmodium chabaudi*, gametocytes, oocysts.

INTRODUCTION

The majority of human malaria infections consist of more than 1 genetically distinct genotype of the same *Plasmodium* species (Day *et al*. 1992). In Tanzania, Senegal and Papua New Guinea, for example, PCR analysis has shown that between 70 and 85% of infected people harbour more than 1 parasite genotype (Babiker *et al*. 1994; Ntoumi *et al*. 1995; Paul *et al*. 1995). Unless parasites are regulated by wholly genotype-specific immune responses, clonal parasite populations within hosts will interact, either via the host immune system (apparent competition, *sensu* Holt, 1977), through resource competition, or by other mechanisms (Richie, 1988). These interactions could have a substantial impact on infection dynamics. The kinetics of infection of 1 *Plasmodium* species can be radically altered by the presence of another (Richie, 1988; Snounou *et al*. 1992); where competing populations are distinct genotypes of the same species, niche separation must be less marked and competitive interactions even more severe. Within mixed-genotype infections of *Plasmodium chabaudi* in mice, marked suppression of one clone by another can occur (Snounou *et al*. 1992; A. F. Read and M. Anwar, personal communication). Natural selection should therefore favour parasites with life-histories appropriate for these circumstances.

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As far as we are aware, there have been no studies on how interactions between blood-stage malaria parasite populations in mixed-genotype infections affect transmission to new hosts. Depending on the nature of the interactions, transmission could be unaltered, suppressed or increased. To determine which, we compared transmission from mixed-clone and single-clone infections of the rodent malaria, *P. chabaudi*. Groups of mice were infected with clones CR and ER, either singly or together. Mice given 2 clones received the sum of the starting inocula of the 2 single-clone infections in each experiment, with an initial ratio of either 1:9 or 9:1 of the 2 clones. These starting conditions have previously been shown to result in competitive suppression of the initially rare clone (A. F. Read and M. Anwar, personal communication). We show that transmission rates of mixed-clone infections were over 7 times the sum of the 2 single-clone infections. The increased transmission was correlated with higher gametocyte densities in the mixed-clone infection mice.

MATERIALS AND METHODS

Parasites, mice and mosquitoes

Two cloned lines of *P. chabaudi* denoted CR and ER (Beale, Carter & Walliker, 1978), obtained from the WHO Registry of Standard Malaria Parasites, Edinburgh University, were used. The hosts were male C57BL/6J/Ola mice (Harlan, England) in which *P. chabaudi* is usually non-lethal (Stevenson,

Lyanga & Skamene, 1982). Mice were fed on SDS rat and mouse maintenance diet and drinking water was supplemented with 0.05% para-amino benzoic acid (PABA) to enhance parasite growth. Artificial illumination was provided from 07.00 to 19.00 h. The vector was *Anopheles stephensi*, maintained at 25–28 °C and 70–80% humidity, fed on 10% glucose in water supplemented with 0·05% PABA.

Inoculation of mice with standard numbers of parasites

Parasite densities in infected mice were determined from Giemsa-stained thin blood smears and red blood cell counts made using a Coulter Counter. Blood from these infected mice was diluted in calf serum–Ringer solution (50% heat-inactivated calf serum, 50% Ringer solution (27 mM KCl, 27 mM $CaCl_2$, 0·15 M NaCl), 20 units heparin/ml mouse blood) to give initial dilutions of the 2 parasite clones. These were then further diluted with calf serum–Ringer solution, or mixed together to give the required number of parasites in a 0·1 ml inoculum (see experimental design below).

Parasite counts and mosquito feeds

The number of asexual parasites/ml of blood (asexual density) was calculated from thin blood smears and red blood cell counts taken between 17.45 and 18.15 h on selected days. On the days when mosquitoes were to be fed, asexual parasitaemia (percentage of infected red cells) and gametocytaemia (number of gametocytes/10^3 red blood cells) were calculated. Five-day-old *Anopheles stephensi*, starved for the previous 48 h, were fed on the mice for 20–30 min between 18.00 and 20.00 h. Unfed mosquitoes were discarded, and the remainder were dissected 8–10 days later to obtain counts of the number of oocysts on each mosquito midgut.

Experimental design

Two experiments were conducted, each of which had 3 treatment groups (2 single-clone and 1 mixed-clone). In Experiment 1 (Fig. 1), the mixed-clone treatment group had an initial inoculum of 9×10^5 CR + 1×10^5 ER and the CR and ER treatment groups 9×10^5 CR and 1×10^5 ER parasites respectively. For each experimental block, 2 mice were infected with each of the treatments, and asexual parasite density was measured on days 4, 7 and 10 post-infection (p.i.). On day 14 p.i., thin blood smears were taken to assess asexual parasitaemia and gametocytaemia, and mosquitoes fed on the mice. Each experimental block was repeated 4 times, giving a total of 8 mice per treatment group.

Experiment 2 was broadly similar to experiment 1, except that the relative frequency of the 2 clones was reversed and additional mice were infected for a mosquito feed on day 18 p.i. as well as for that on day 14 p.i.. Thus the basic design for each experimental block was as follows. Three treatment groups each of 4 mice received initial inocula of 1×10^5 CR + 9×10^5 ER, 1×10^5 CR or 9×10^5 ER parasites. Mosquitoes were fed on 2 mice per treatment group on day 14 p.i. and on the remaining 2 mice on day 18 p.i.. The asexual parasite density was measured on days 4, 7 and 10 for all mice and additionally on day 14 p.i. for the mice fed on day 18 p.i.. Thin blood smears were taken to measure asexual parasitaemia and gametocytaemia immediately prior to the feed for all mice. This basic design was again performed 4 times (to give 4 experimental blocks) and a total of 16 mice per treatment group.

Analysis

Oocyst numbers per mosquito are well described by the negative binomial distribution (Medley *et al.* 1993) which is defined by the mean and the overdispersion parameter (k). In order to deal with this distribution without sacrificing power, generalized linear models are preferable (Wilson & Grenfell, 1997) and were used here (Crawley, 1993). Values of the overdispersion parameter (k) were estimated from the distribution of oocysts between mosquitoes fed on individual mice. Details of the data manipulation and analyses presented in the results section are given in the Appendix.

Throughout the analyses, we use the following definitions. 'Treatment' refers to the clone(s) of parasite with which the mice were inoculated (CR, ER or Mixed). 'Block' refers to the 4 replicates of the basic design (Fig. 1) carried out within each 'Experiment', and experiments 1 and 2 had initial CR:ER ratios in mixed-clone infections of 9:1 and 1:9 respectively. 'Feed day' refers to the day of infection mosquitoes were allowed to feed on the mice within experiment 2 (day 14 p.i. or day 18 p.i.).

RESULTS

Over the 4 blocks of experiment 1, a total of 24 mice were infected and 550 mosquitoes dissected. In experiment 2, 48 mice were infected, and a total of 1257 mosquitoes dissected. An average (±S.D.) of 23·9 (±4·72) and 27·3 (±1·05) mosquitoes were dissected from those fed on each mouse in experiments 1 and 2 respectively.

Patterns of infection

The changes in asexual parasitaemia for all treatment groups through the 2 experiments are shown in Fig. 2.

In experiment 1, the total number of asexuals produced during the main part of the infection (see

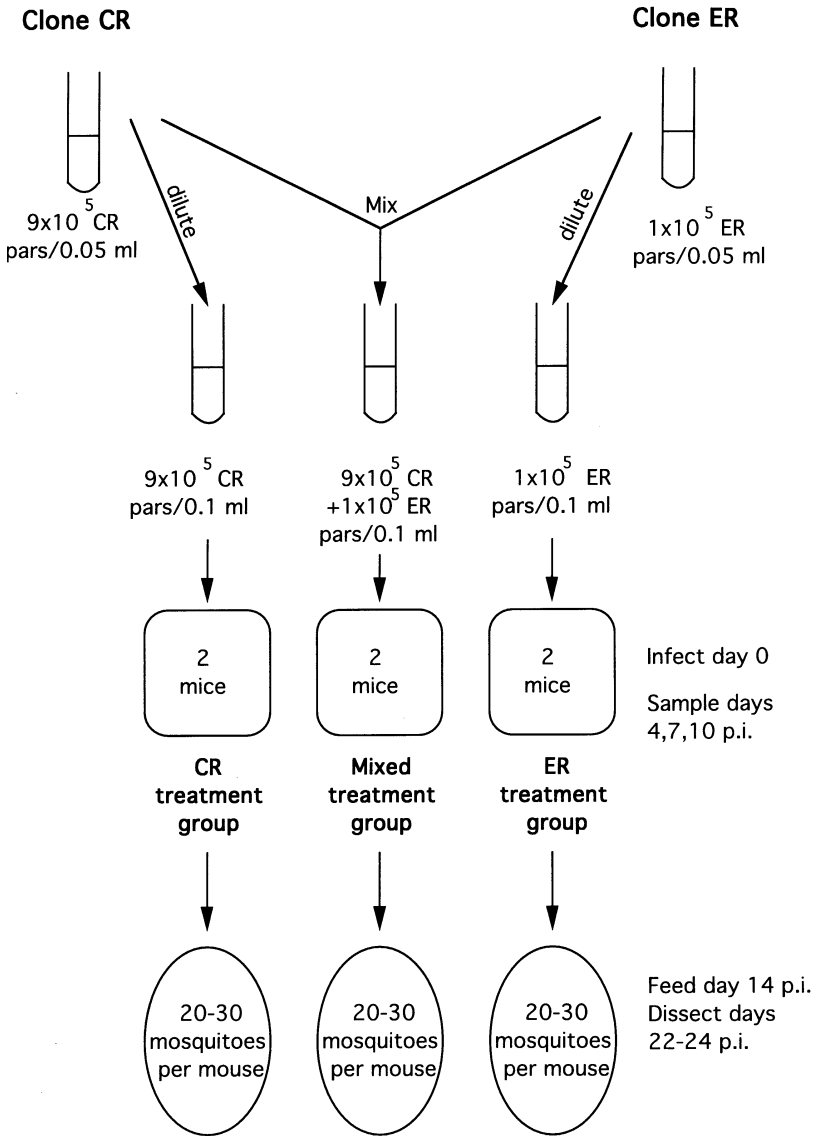

Fig. 1. Schematic representation of an experimental block in experiment 1. Three treatment groups were infected with 9×10^5 CR parasites, 1×10^5 ER parasites or the sum of these. Experiment 1 consisted of 4 experimental blocks, carried out exactly as summarized here. Experiment 2 was similarly designed but the relative frequency of the 2 clones was reversed and extra mice within each block were infected so that feeds could be done on both 14 and 18 days p.i. pars, Parasites; p.i., post-infection of the mice.

Appendix for method of calculation) was significantly lower for ER infections than for CR and mixed-clone infections ($F_{2,22} = 6{\cdot}08$, $P < 0{\cdot}01$). There was no evidence of differences between blocks ($F_{3,20} = 1{\cdot}95$, $P > 0{\cdot}1$) and treatment effects did not vary between blocks ($F_{6,17} = 1{\cdot}22$, $P > 0{\cdot}1$). In experiment 2, the total number of asexuals was higher for the second feed day ($F_{1,40} = 7{\cdot}71$, $P < 0{\cdot}01$) and varied across blocks ($F_{3,42} = 4{\cdot}16$, $P < 0{\cdot}05$), but after controlling for these effects there were no differences between treatment groups ($F_{2,41} = 2{\cdot}80$, $P > 0{\cdot}05$) and no interaction effects were significant (in all cases, $P > 0{\cdot}1$).

All 3 treatment groups had similar asexual densities 4 days prior to the feed in experiment 1 ($F_{2,22} = 2{\cdot}54$, $P > 0{\cdot}05$). For experiment 2, asexual densities altered between days 10 and 14, and so the 2 feeds were analysed separately. Four days prior to the day 14 p.i. feed, the CR infection group showed higher asexual densities than the ER or mixed-clone infection groups ($F_{2,23} = 3{\cdot}59$, $P < 0{\cdot}05$, Fig. 2). Four days prior to the day 18 p.i. feed all the mice had very low asexual densities, and no differences between the treatment groups were significant ($F_{2,21} = 2{\cdot}43$, $P > 0{\cdot}05$). In the analyses of both experiments there was no evidence of differences in asexual density between blocks, and no alteration of the treatment effects between blocks (in all cases, $P > 0{\cdot}1$).

The estimates of the overdispersion parameter, k, of the negative binomial model used in the generalized linear models were 0·47 and 0·91 for experiments 1 and 2 respectively (S.E.S were 0·052 and 0·088 respectively). The relationship between the

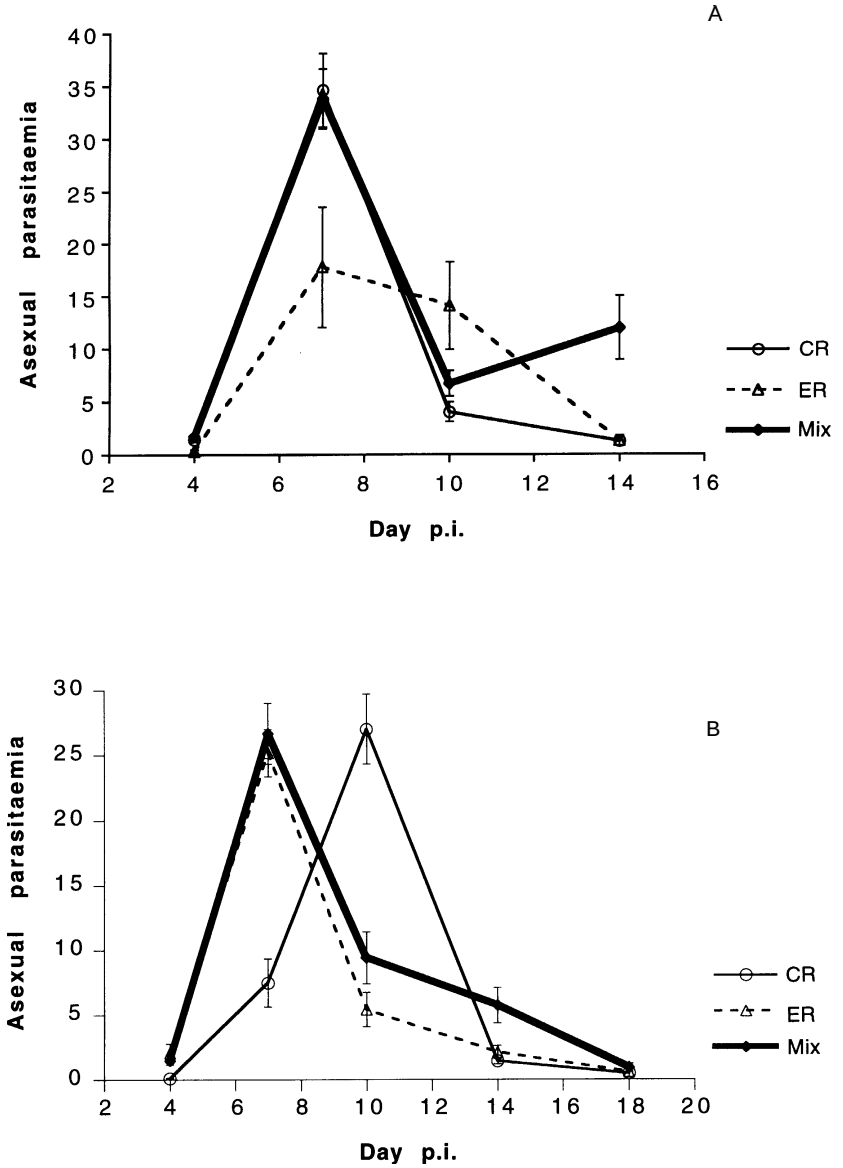

Fig. 2. The course of asexual infections in experiment 1 (A) and experiment 2 (B). Error bars are ±1 S.E.

proportion of mosquitoes infected and oocyst burden generated by these estimates provides a good fit to the data (Fig. 3).

Infectivity to mosquitoes

(*i*) *Oocyst burdens*. In experiment 1, oocyst burdens in mosquitoes differed significantly between treatment groups ($F_{2,17} = 8{\cdot}44$, $P < 0{\cdot}01$). Mixed-clone infections generated higher oocyst burdens than the CR infection group, which in turn generated higher oocyst burdens than the ER infection group (Fig. 4A). Mean oocyst burdens fell across successive blocks ($F_{3,17} = 6{\cdot}47$, $P < 0{\cdot}01$), but treatment effects did not differ between blocks (treatment.block interaction, $F_{6,11} = 0{\cdot}88$, $P > 0{\cdot}1$). The mixed-clone infection group and the most infectious single-clone group (CR) differed in their mean oocyst burdens ($F_{1,10} = 5{\cdot}11$, $P < 0{\cdot}05$). Therefore, mixed-clone infections were more infectious to mosquitoes than either of the single-clone groups.

In experiment 2, the day of feed had a strong effect on oocyst burdens ($F_{1,40} = 29{\cdot}29$, $P < 0{\cdot}001$). Far fewer oocysts resulted from the day 18 p.i. feed than from the day 14 p.i. feed (compare the scales of Fig. 4B and C). After controlling for this effect, the treatment groups differed as in experiment 1 ($F_{2,40} = 8{\cdot}33$, $P < 0{\cdot}001$). Mice with mixed-clone infections produced higher oocyst burdens than mice with CR which in turn were more infectious than mice with ER (Fig. 4B and C). Blocks did not differ significantly ($F_{3,40} = 1{\cdot}83$, $P > 0{\cdot}05$), and all second order and third order interactions between day of feed, treatment and block were insignificant (in all cases $P > 0{\cdot}05$). As in experiment 1, the mixed-clone group produced higher oocyst burdens than the most infectious single-clone group ($F_{1,15} = 9{\cdot}00$, $P < 0{\cdot}01$).

The increased transmission level from the mixed-clone infections may be due simply to the larger number of parasites in their initial inocula. Alter-

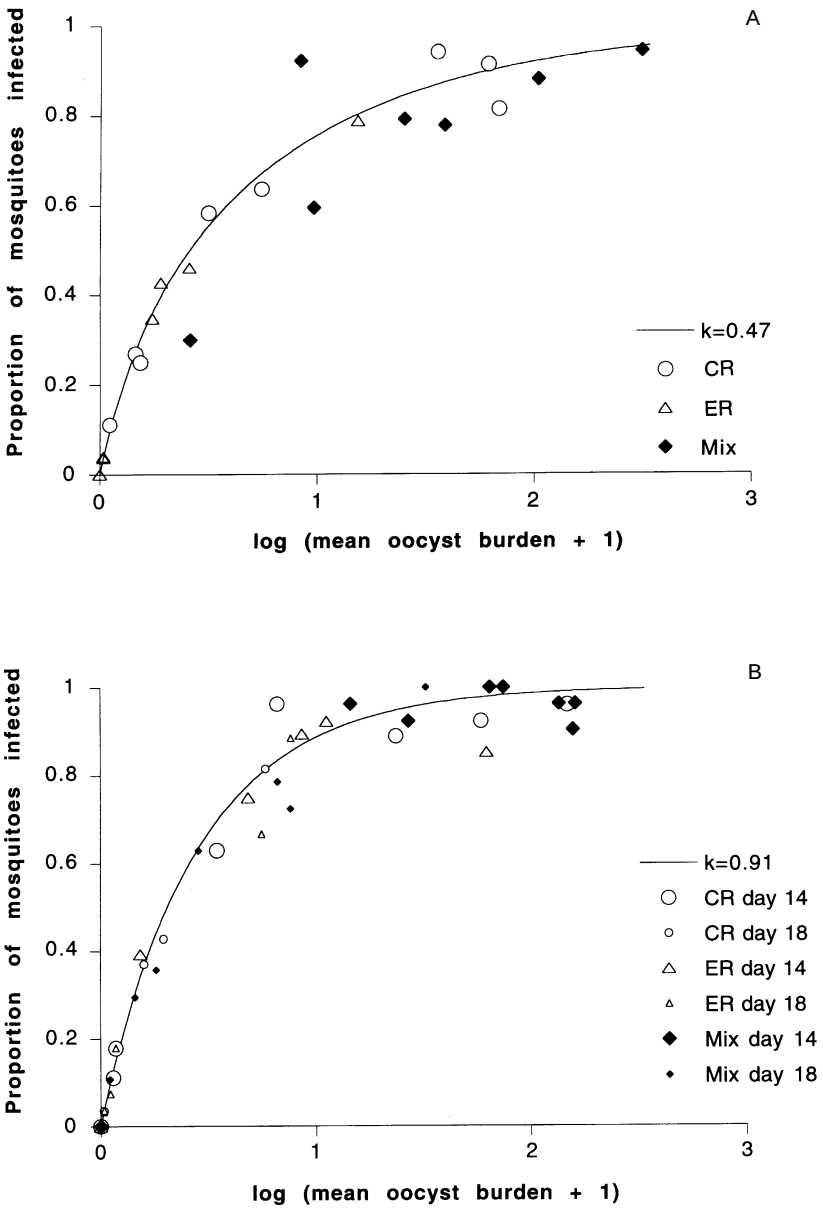

Fig. 3. The relationship between the proportion of mosquitoes infected and the mean oocyst burden for experiment 1 (A) and experiment 2 (B). Each datapoint represents 1 mouse and the fitted line is the relationship expected from a negative binomial distribution with $k = 0{\cdot}47$ for experiment 1 and $k = 0{\cdot}91$ for experiment 2.

natively, mixed-clone infections may be more infectious for other reasons. To differentiate between these explanations we constructed the following hypothesis. If the 2 clones were behaving independently within mixed-clone infections, the oocyst burdens generated by these infections would equal the sum of the oocyst burdens generated by the corresponding single-clone infections. To test this (see Appendix for details), a total of 12 independent comparisons (from the 4 blocks of experiment 1 and the 4 blocks for each feed in experiment 2) were used. Across the whole dataset there was a significant difference between the oocyst burden generated from the mixed-clone infections and that expected from the sum of the single-clone infection groups (Wilcoxon signed rank test, $Z = 2{\cdot}20$, $P < 0{\cdot}05$). The mixed-clone infection group produced higher oocyst burdens in 10 out of the 12 comparisons. An assumption is made in this analysis that oocyst burdens in mosquitoes are not limited within the range of infection levels found here, so that mixed-clone infection mosquitoes could carry the sum of the burdens of the 2 single-clone infection mosquito groups. In fact the mixed-clone infection mosquitoes are able to carry more than this sum, so it is clear that the assumption is justified.

The ratio of the oocyst burdens from the mixed-clone infections to that expected (the sum of the 2 control group oocyst burdens) was calculated for each comparison. The mean ($\pm$S.E.) of these ratios showed that, on average, oocyst burdens generated by mixed-clone infections were 7·6 ($\pm$2·7) times higher than those generated by the 2 single-clone infection groups together. When mixed-clone in-

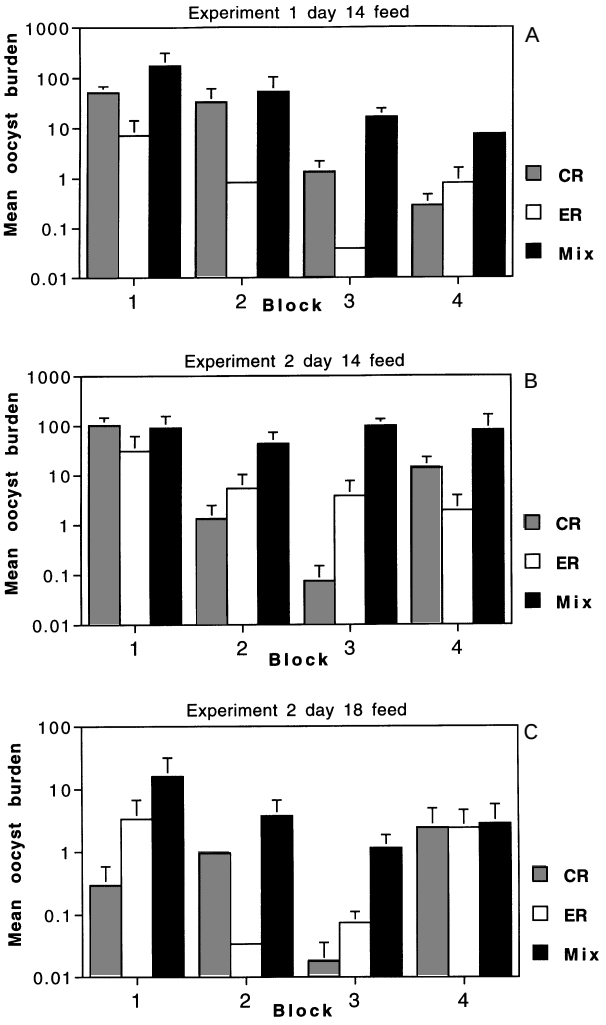

Fig. 4. Mean (+1 S.E.) oocyst burdens for each treatment group in each experimental block in experiment 1 (A) and experiment 2 (B and C), showing the two feeds of the latter separately.

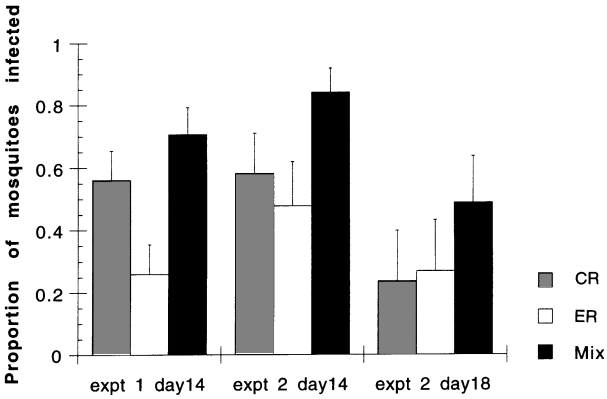

Fig. 5. Mean (+1 S.E.) proportion of mosquitoes infected for the treatment groups in experiments 1 and 2.

fection mice are compared to the single-clone group with the largest starting innoculum, very large increases in transmission are seen. Mixed-clone infections produced oocyst burdens 10·7 (±5·5) times higher than the CR infections in experiment 1, and oocyst burdens 27·4 (±12·9) times higher than the ER infections in experiment 2.

Table 1. Correlations of infectivity with blood-stage infection parameters

(Summary of the analyses of covariance of infectivity (oocyst burden) and parameters of the blood-stage infections (for statistical details, see Appendix). For experiment 2, the day of the feed was controlled for first, then the correlation tested. In no case did blocks differ, and no interactions between models terms were significant.)

	Correlation with infectivity	
Parameter	Experiment 1	Experiment 2
Total number of, asexuals	$F_{1,22} = 2{\cdot}64$, N.S.	$F_{1,44} = 0{\cdot}10$, N.S.
Asexual parasitaemia, 4 days before the feed	$F_{1,22} = 0{\cdot}07$, N.S.	$F_{1,44} = 0{\cdot}48$, N.S.
Asexual density, 4 days before the feed	$F_{1,22} = 1{\cdot}37$, N.S.	$F_{1,44} = 3{\cdot}02$, N.S.
Asexual parasitaemia, 2 days before the feed	$F_{1,16} = 1{\cdot}96$, N.S.	$F_{1,44} = 0{\cdot}02$, N.S.
Asexual parasitaemia, on the day of the feed	$F_{1,16} = 4{\cdot}41$, †	$F_{1,43} = 10{\cdot}51$, **
Gametocytaemia, on the day of the feed	$F_{1,16} = 5{\cdot}29$, *	$F_{1,44} = 23{\cdot}24$, **

N.S., Non-significant; † $0{\cdot}1 > P > 0{\cdot}05$; * $P < 0{\cdot}05$; ** $P < 0{\cdot}01$.

(*ii*) *Proportions of mosquitoes infected.* In experiment 1, a higher proportion of mosquitoes was infected by mixed-clone infection than by single-clone infections ($\chi^2_2 = 10{\cdot}65$, $P < 0{\cdot}01$; Fig. 5). Mice from later blocks produced lower oocyst burdens, but this effect was only marginally significant ($\chi^2_3 = 7{\cdot}75$, $P \approx 0{\cdot}07$), and there was no evidence of treatment effects differing between blocks ($\chi^2_6 = 5{\cdot}50$, $P > 0{\cdot}1$).

In experiment 2, a greater proportion of mosquitoes became infected when fed on mice on day 14 p.i. than on day 18 p.i. ($\chi^2_1 = 7{\cdot}10$, $P < 0{\cdot}01$, Fig. 5). After controlling for this effect, treatments differed only marginally ($\chi^2_2 = 5{\cdot}42$, $P \approx 0{\cdot}07$). However, mixed-clone infections gave rise to a significantly higher proportion of infected mosquitoes than did the single-clone infections when treatment was reduced to a 2-level factor (mixed-clone versus single-clone infections; $\chi^2_1 = 5{\cdot}42$, $P < 0{\cdot}05$). There was no significant difference between experimental blocks ($\chi^2_3 = 2{\cdot}74$, $P < 0{\cdot}1$) and all the 2- and 3-way interactions between the day of feed, treatment and block were insignificant ($P > 0{\cdot}1$).

The higher infectivity of mixed-clone infections to mosquitoes was less marked when measured as the

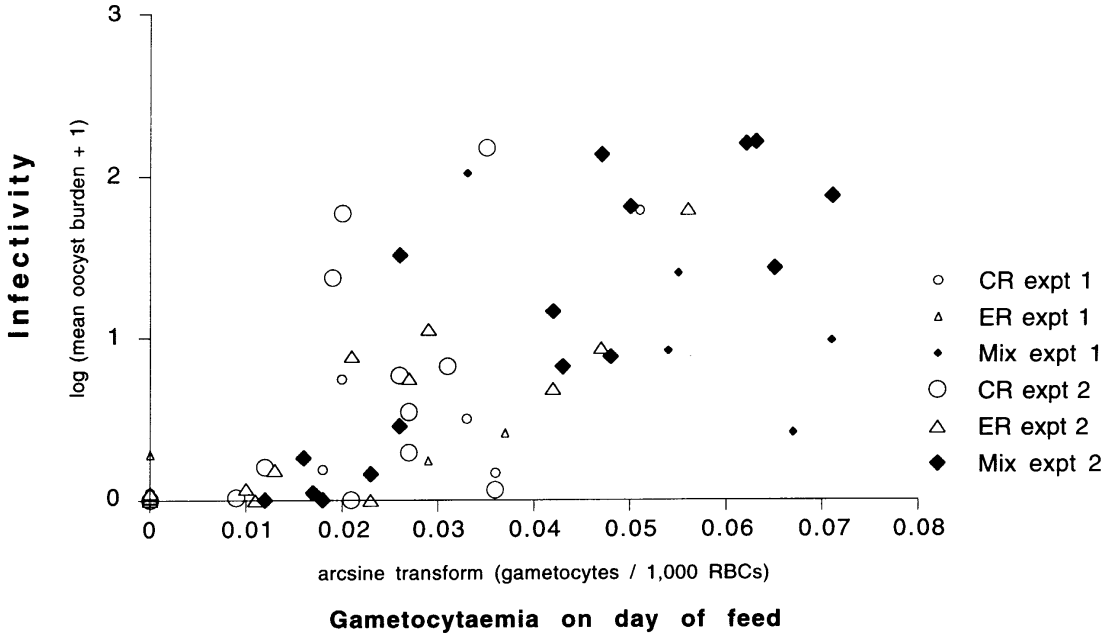

Fig. 6. Data from both experiments showing the relationship between infectivity and the gametocytaemia on the day of the feed.

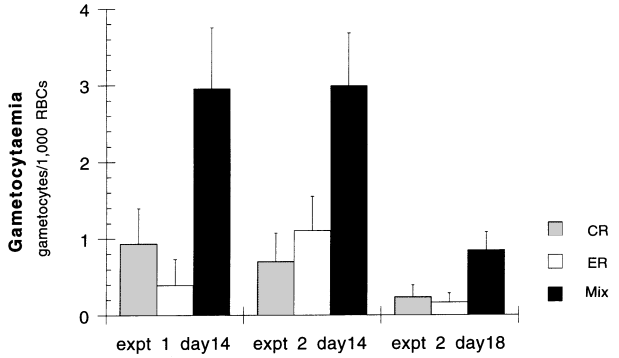

Fig. 7. Mean (+ 1 S.E.) gametocytaemias for the treatment groups in experiments 1 and 2.

proportion of mosquitoes infected rather than by the oocyst burden. This could be for 2 reasons. First, although these 2 variables are tightly correlated (see Fig. 2) the relationship is non-linear. At mean oocyst burdens above 10, there is relatively little variation in the proportion of mosquitoes infected, but oocyst burdens can vary by over an order of magnitude. Second, estimates of the proportion of infected mosquitoes from sample sizes of around 25 mosquitoes involve larger errors than do estimates of oocyst burden (Medley *et al.* 1993). This error would increase within-group variance and obscure between-group differences. For these reasons we only use oocyst burden in the following analyses.

Predicting transmission from blood-stage infection parameters

Our results show that mixed-clone infections are more infectious to mosquitoes than are single-clone infections. We used analyses of covariance (ANCOVA) to identify parameters of the blood-stage infections that could be used to predict these patterns of infectivity. Log(mean oocyst burden + 1) calculated for each mouse was used as the response variable throughout, and we refer to this as 'infectivity'.

This type of analysis is less powerful than the generalized linear models relying on individual mosquito data used above (Wilson & Grenfell, 1997). Despite this, treatment effects were still evident. In experiment 1, block was just marginally significant ($F_{3,20} = 3{\cdot}05$, $P \approx 0{\cdot}07$), but treatments differed significantly ($F_{2,22} = 4{\cdot}08$, $P < 0{\cdot}05$), with mixed-clone infections showing the highest oocyst burdens. In experiment 2, the day 14 p.i. oocyst burdens were again higher than on day 18 p.i. ($F_{1,43} = 11{\cdot}13$, $P < 0{\cdot}01$), but after controlling for this, treatments differed significantly ($F_{2,44} = 4{\cdot}20$, $P < 0{\cdot}05$) with mixed-clone infections producing higher mean oocyst burdens. In both experiments, no interaction terms between treatment, block and feed day were significant (in all cases $P > 0{\cdot}1$).

Six parameters of the blood infection were calculated for each mouse. (i) The total number of parasites produced during the main part of the asexual infection, referred to as 'total asexuals' (see Appendix for method of calculation). (ii) An estimate of the asexual parasitaemia 2 days prior to the feed. This was made by averaging the parasitaemias on the day of the feed and 4 days previously. (iii) Asexual density 4 days prior to the feed. (iv) Asexual parasitaemia 4 days prior to the feed. (v) Asexual parasitaemia on the day of the feed. (vi) Gametocytaemia on the day of the feed. Among the 6 parameters of the blood-stage infection, only gametocytaemia and the asexual parasite density on the day

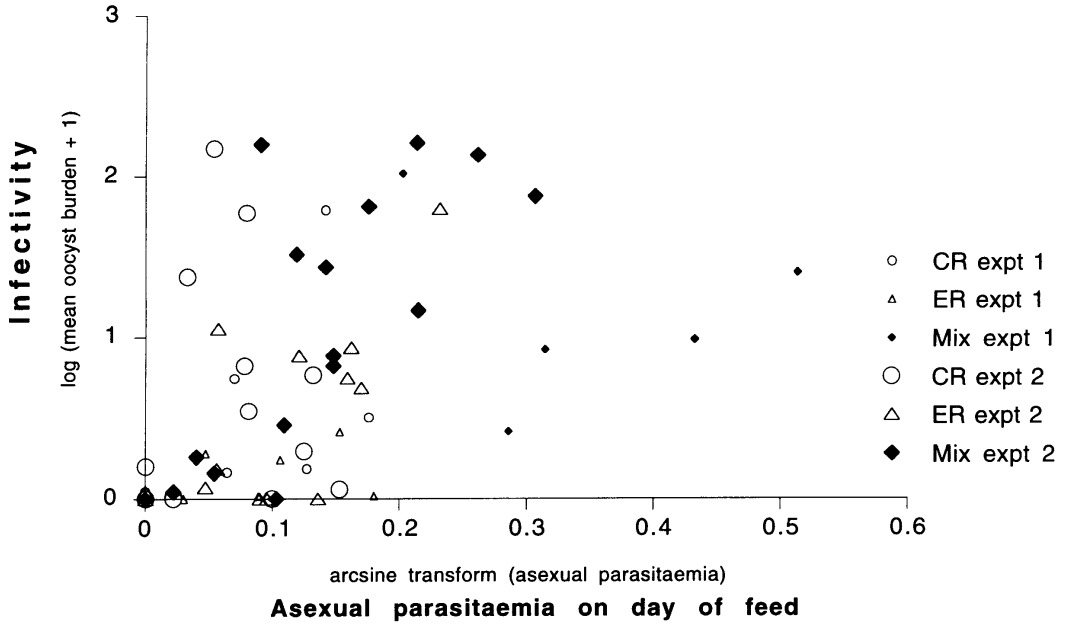

Fig. 8. Data from both experiments showing the relationship between infectivity and the asexual parasitaemia on the day of the feed.

of the feed showed significant correlations with infectivity (Table 1). These two were analysed in more detail to see if they could explain the observed treatment effects.

Gametocytaemia was positively correlated with infectivity in both experiments 1 and 2, explaining 33 and 62% of the total variance in infectivity respectively (Table 1 and Fig. 6). Treatment did not account for any additional variation for either experiment ($F_{2,15} = 1{\cdot}19$, $P > 0{\cdot}05$ and $F_{2,43} = 0{\cdot}25$, $P > 0{\cdot}05$, for experiments 1 and 2 respectively). Likewise, block, day of feed and all the interactions between these, treatment and gametocytaemia did not explain any additional variance in infectivity ($P > 0{\cdot}05$ in all cases). Gametocytaemia was higher in the mixed-clone infection group of mice than in either of the single-clone infection groups for both experiments ($\chi^2_2 = 15{\cdot}51$, $P < 0{\cdot}001$, $\chi^2_2 = 27{\cdot}68$, $P < 0{\cdot}001$ for experiments 1 and 2 respectively, Fig. 7). Furthermore, gametocytaemia in the mixed-clone infections was significantly higher than the sum of the gametocytaemias of the 2 single-clone groups (Wilcoxon signed rank test , $Z = 2{\cdot}93$, $P < 0{\cdot}01$). On average, mixed-clone infection mice had a mean ($\pm$S.E.) gametocytaemia 3·3 ($\pm$1·3) times higher than that of the sum of the 2 single-clone infections. Taken together, these analyses suggest strongly that the mixed-clone infections are more infectious to mosquitoes due to increases in their gametocytaemias relative to those of the single-clone infections.

Asexual parasitaemia on the day of the feed was strongly and positively correlated with infectivity in experiment 2, but only marginally in experiment 1. The weaker effect in experiment 1 may have been due to the smaller sample sizes involved. Asexual parasitaemia explained 27 and 38% of the variance in infectivity for the 2 experiments respectively (Table 1 and Fig. 8). Treatment explained no additional variation ($F_{2,15} = 1{\cdot}55$, $P > 0{\cdot}05$ and $F_{2,42} = 2{\cdot}16$, $P > 0{\cdot}05$ for Exps 1 and 2 respectively) nor did block, day of feed or any of the interactions between these factors ($P > 0{\cdot}05$ in all cases). Mixed-clone infections had higher asexual parasitaemias on the day of the feed than the single-clone infections ($F_{2,16} = 5{\cdot}56$, $P < 0{\cdot}05$, $F_{2,43} = 4{\cdot}33$, $P < 0{\cdot}05$ for Exps 1 and 2 respectively). On average the mixed-clone infection mice had an asexual parasitaemia 4·5 times higher than that of the sum of the 2 single-clone infections.

DISCUSSION

These results show that mixed-clone infections of *P. chabaudi* were more infectious to mosquitoes than single-clone infections and gave rise to more oocysts than the sum of the single-clone infections. Interactions occurring between clones in mixed-clone infections strongly and positively affected transmission for all 3 combinations of starting inocula and feed day. Mixed-clone infections produced on average over 7 times as many oocysts as the sum of the corresponding single-clone infections. In both experiments, the addition of a second clone as a minority of the inoculum (10%) increased the overall transmission of an infection to more than 10 times that achieved by the initally dominant clone on its own.

Based on ecological and evolutionary theories of competition, within-host interactions between clones could have several outcomes, depending on the mechanisms involved. (1) Where oocyst burden is

regulated entirely by genotype-specific immune responses, no interactions will occur within mixed-clone infections. Total oocyst burden from mixed-clone infections would be equivalent to the sum of that which each genotype would have produced on its own. (2) If some factor acts to limit oocyst burdens, irrespective of their genotype, mixed-clone infections will have transmission rates similar to those of the single-clone infections. (3) Competitive interactions which alter asexual parasite dynamics within mixed-clone infections might also alter gametocyte and hence oocyst production. Increased asexual density, or a prolonged infection, may lead to increased transmission. (4) If a genotype is suppressed by competition within a host, natural selection should favour variants which reallocate resources from asexual replication to gametocyte production. Oocyst burdens in mixed-clone infections would therefore be higher than that from single-clone infections, and may reach levels higher than the sum of the 2 control infections.

In our experiments, transmission from mixed-clone infections was higher than the sum of the control infections, ruling out the first and second of these mechanisms. However, the third and fourth are both consistent with our results.

The increased infectiousness of the mixed-clone infections was almost certainly due to greater gametocyte densities on the day of the feed. There was no evidence that treatment, or the day of infection that mosquitoes fed on the mice, had any additional effect once gametocyte density had been controlled for, showing that the infectivity of gametocytes was not influenced by these factors.

Experiments with splenectomized rats suggested that *P. chabaudi* gametocytes take 4 days to become functionally mature (Cornelissen & Walliker, 1985), but more recent work in mouse hosts has suggested it may be only 2 days (Gautret *et al*. 1997). This makes it difficult to determine the population of asexual parasites which gave rise to the gametocytes transmitted to mosquitoes during the feeds in these experiments.

If conversion to functionally mature gametocytes takes around 4 days, then increased conversion rates from asexual parasites to gametocytes must have occurred in the mixed-clone infections. The asexual density of the mixed-clone infections 4 days before the feed was always the same as, or lower than, the highest single-clone infection. There must therefore have been suppression of the asexual population of at least 1 of the clones. Re-allocation of resources from asexual replication to gametocyte production should be favoured by natural selection when asexual growth is limited by competition, as is thought to occur in response to drug pressure (Findlay, Maegraith & Holden, 1946; Foy & Kondi, 1952; Buckling *et al*. 1997). If suppressed clones can increase their investment into gametocytes, this would explain the higher gametocyte density in the mixed-clone infections on the day of the feed.

If gametocyte maturation takes around 2 days, another explanation is possible. Higher gametocyte densities may arise due to similar conversion rates as in single-clone infections, but from higher asexual parasitaemias in the mixed-clone infection mice. Our observation that asexual parasitaemia on the day of the feed correlates with infectivity, unlike any other measure of asexual density, is consistent with this mechanism, but it also requires marked dynamics in asexual parasitaemia just prior to the feed. Previous work suggests that strain-specific immune clearance could play an important role in generating such dynamics in mixed-clone infections (Snounou *et al*. 1989; A. F. Read & M. Anwar, personal communication). As the initially dominant clone becomes cleared by the immune system, the subdominant clone could be sufficiently distinct antigenically to be able to increase in numbers so that the population size of the whole infection rises. Such changes in the dynamics of the asexual infection are predicted by one of the few models dealing with superinfection (Nowak & May, 1994) and underlie presumptions about how antigenic variation prolongs infections. An analysis of the clonal composition of these infections should increase our understanding of these interactions. Samples suitable for genetic and immunological analysis were taken from the mice in these experiments and the results are presented elsewhere (Taylor, Walliker & Read, 1997).

Implications for theory

Recently developed models have shown that interactions between different parasite genotypes of the same species within a host can have important consequences not only for the duration and outcome of the infection (Frank, 1992; Hellriegel, 1992; Antia, Nowak & Anderson, 1996) but also for the epidemiology and evolution of the species (Bremermann & Pickering, 1983; Nowak & May, 1994; Gupta & Day, 1996). There is a clear need to understand these interactions more fully (Bull, 1994; Read, 1994; Schmid-Hempel & Koella, 1994) and they are likely to vary considerably according to the ecology of the host–parasite system being studied (May & Anderson, 1990; Read, 1994; Ewald, 1995). However, it is also clear that in no system do we understand how genetic variation between parasites within hosts might affect the transmission from them - the underlying basis of many of these models (May & Anderson, 1990; Bull, 1994; Gupta & Day, 1996).

One assumption commonly used, especially in models seeking to explain levels of parasite virulence, is that transmission is correlated with within-host replication rate. For parasites in general (Bremermann & Pickering, 1983, see reviews by

Bull, 1994; Frank, 1996) and malaria parasites in particular (Hellriegel, 1992; Gupta & Day, 1996), it is assumed that increased within-host proliferation allows a genotype to increase its population size relative to within-host competitors, so that it dominates the transmission population from that host. Our experimental data do not support this assumption: there was no correlation between the total number of parasites produced during the main part of the infection and transmission from their hosts. However, there was a positive (albeit weak) association between asexual density on the day of the feed and transmission. In all infections, the bulk of gametocytes were produced around day 14 post-infection. A high asexual density during this period could therefore be important for epidemiological persistence. If clone-specific clearance of parasites within the mixed-clone infections is occurring, then the initially suppressed clone may be able to increase its asexual density towards the end of the infection and might even dominate the transmission from these infections.

Whatever the mechanism involved, at least 2 pressing questions arise. First, why are so many asexual parasites produced early in infections if they have little consequence for transmission? Second, why do gametocytes in all infections constitute such a low proportion of all the parasites in an infection (Taylor & Read, 1997)? This second problem is especially acute in single-clone infections where gametocyte densities are demonstrably lower than they could be.

Relevance to the field

In natural situations of malaria transmission, oocyst burdens very rarely reach the intensities seen in this experimental situation (Muirhead-Thomson, 1954; Lyimo & Koella, 1992) and instead fall nearer to the origin of Fig. 2, where variation in mean oocyst burdens corresponds to variation in proportion of mosquitoes infected. This has been shown for mosquitoes naturally infected with *P. falciparum* in Tanzania (Billingsley *et al*. 1994). If processes involved in determining transmission in the human malarias are similar to those occurring in *P. chabaudi*, then both the proportion of mosquitoes infected and their oocyst burdens would be increased from mixed-genotype compared to single-genotype infections. This may have important consequences for attempts to control malaria. For example if initial reductions in transmission lead to fewer mixed-genotype infections in a population, then the average transmission level from future infections may become reduced even further. This self-reinforcing process could also work in reverse if changing environmental conditions (e.g. global warming) increase transmission rates and hence the frequency of mixed-genotype infections.

APPENDIX

Estimating k *of the negative binomial distribution*. We used GLIM macros to estimate k for oocysts derived from individual mice using maximum-likelihood (Crawley, 1993). Mice where fewer than 2 mosquitoes became infected were excluded, and the rest ($n = 18$ and 24 for experiments 1 and 2 respectively) averaged to give a mean k value for each experiment. GLIM macros were also used to specify the negative binomial error structure in log-linear models (Crawley, 1993).

Total asexual data. These were calculated for each mouse individually by calculating the area under the curve of asexual density through time between days 4 and 10 p.i. (for mice fed on day 14 p.i.) or days 4 and 14 p.i. (for mice fed on day 18 p.i.). As *P. chabaudi* replicates once every 24 h, this represents a reasonable approximation to the total number of asexuals produced during this period of infection.

Oocyst burdens. Oocyst burdens from each mosquito were used in generalized linear models with negative binomial error structures. All factors and their interactions were fitted to the model, with 'mouse' added last as a nested factor. Where interaction terms were sufficiently insignificant (Sokal & Rolf, 1981, p.285), their mean squares were combined with the nested mouse term and used as the error term. This results in the total degrees of freedom for the analysis equalling the number of mice in the experiment minus 1, thus avoiding pseudoreplication. Significance of the remaining effects in these models was assessed by calculating an F-ratio from the change in deviance per degree of freedom divided by the residual mean square deviance as terms were removed from the minimal model (Crawley, 1993).

Proportion data. For the proportion of mosquitoes infected, general linear models with binomial error structures were used, together with William's correction for overdispersion (Crawley, 1993). After correction, changes in residual variance were tested using the Chi-squared distribution with corresponding degrees of freedom. Where proportion data were used as a predictor variable (e.g. asexual parasitaemia, gametocytaemia), they were fitted as arcsine(square-root(p)), where p is the untransformed proportion.

Mean oocyst burdens produced by individual mice. Negative binomial models (with the appropriate k value for the experiment) were fitted to the oocyst burden per mosquito data with each mouse as a separate factor level. This gave the mean oocyst burden produced in a mosquito fed on each mouse. After adding 1 to these measures of mean oocyst burden, they were $\log_{10}$ transformed and used in the analyses of covariance.

Mixed-clone infections versus the sum of the controls. Two mice were inoculated with each treatment 4 times during experiment 1 and 4 times for each of the 2 feeds in experiment 2. This allows 12 independent comparisons to be made between the mixed-clone infections and the sum of the 4 single-clone infections initiated at the same time. First, the relevant parameters (mean oocyst burdens or gametocytaemias) for the 2 mice in each of the 3 treatment groups were averaged for each comparison separately. The

averages for the CR and ER infection groups for each comparison were then summed to give an estimate of the expected transmission assuming no interaction between clones. This estimate of expected transmission was compared to the actual transmission from the mixed-clone infections for the same comparison using a Wilcoxon-Sign Rank Test.

Correlations with blood-stage parameters. Initial ANCOVAs were performed with $\log_{10}$(mean oocyst burden + 1), calculated as above, as the response variable. In the detailed analyses of the relationship between gametocyte and asexual densities, all possible interactions between feed day, block, treatment, and the particular asexual parameter were included in a general linear model. Then, beginning with the maximal model, stepwise omission of non-significant factors was used, leaving treatment and the blood-stage parameter until last (Crawley, 1993). Effectively this tests for additional significant effects of treatment above the blood stage parameter. The results presented are the minimal model explaining infectivity in each case. The residuals after each minimal model had been fitted were examined to ensure the data conformed to assumptions of normality and homogeneity of variance.

Missing data points. During the course of the experiments 3 mice died. One mixed-clone infection mouse in experiment 1 and 1 ER mouse in experiment 2 died during feeds on day 14 p.i. and 1 CR mouse in experiment 2 (due to be fed on day 18 p.i.) died on day 13. As there was no evidence of abnormal infections in these mice, they were included in the analyses of data up to the point that they died. For the first block of experiment 1 blood smears were not taken prior to the feed, so measurements of the asexual parasitaemias and gametocytaemias on the day of the feed for these mice were not available.

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