CHEMOTHERAPY, WITHIN-HOST ECOLOGY AND THE FITNESS OF DRUG-RESISTANT MALARIA PARASITES

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A major determinant of the rate at which drug-resistant malaria parasites spread through a population is the ecology of resistant and sensitive parasites sharing the same host. Drug treatment can significantly alter this ecology by removing the drug-sensitive parasites, leading to competitive release of resistant parasites. Here, we test the hypothesis that the spread of resistance can be slowed by reducing drug treatment and hence restricting competitive release. Using the rodent malaria model Plasmodium chabaudi, we found that low-dose chemotherapy did reduce competitive release. A higher drug dose regimen exerted stronger positive selection on resistant parasites for no detectable clinical gain. We estimated instantaneous selection coefficients throughout the course of replicate infections to analyze the temporal pattern of the strength and direction of within-host selection. The strength of selection on resistance varied through the course of infections, even in untreated infections, but increased immediately following drug treatment, particularly in the high-dose groups. Resistance remained under positive selection for much longer than expected from the half life of the drug. Although there are many differences between mice and people, our data do raise the question whether the aggressive treatment regimens aimed at complete parasite clearance are the best resistance-management strategies for humans.

KEY WORDS: Competitive release, Plasmodium chabaudi, selection coefficient, transmission, within-host fitness.

The evolution of drug-resistant pathogens is a major challenge to modern health care. In malaria, for instance, two widely used drugs, chloroquine and sulphadoxine-pyrimethamine (SP), have become useless against Plasmodium falciparum in many parts of the world and the alternative drugs now in use are threatened by spreading resistance (Hyde 2005; Enserink 2008; Greenwood et al. 2008). The “useful life span of a drug,” the period from deployment to failure, is determined by the time taken for resistance to rise to some threshold frequency. The World Health Organisation (WHO) currently recommends withdrawal of a drug...
when 10% of infections are not responding to treatment, although in practice, governments of poor countries leave it longer (WHO 2006, p. 15). A major determinant of the time it takes for alleles to rise to a given frequency is the strength of selection on them. In the case of drug resistance, the strength of selection depends on a number of factors, not least the in-host ecology of resistant and sensitive parasites in treated and untreated infections (Hastings 1997, 2003, 2006; Mackinnon 1997, 2005; Hastings and D’Alessandro 2000; Koella and Antia 2003; Read and Huijbens 2009).

Most malaria infections contain more than one genotype of malaria parasites (e.g., Arnot 1998; Babiker et al. 1999; Smith et al. 1999; Bruce et al. 2000; Jafari et al. 2004; A-Elbasit et al. 2007; Nwakanma et al. 2008; Vafa et al. 2008; Baruah et al. 2009; Soulama et al. 2009). Mixed infections arise from inoculations of genetically diverse parasites from a single mosquito or contemporaneous bites by multiple mosquitoes infected with different parasites. A consequence of this natural history is that resistant parasites are very likely to share their hosts with susceptible parasites, particularly when resistance is starting to spread in a population. The ecology of genetically diverse infections thus becomes a critical determinant of the relative fitness of resistant and susceptible parasites. If competition occurs, whereby the densities of resistant parasites in a host are reduced when susceptible parasites are present, then drug treatment could, by removing susceptible parasites, make new ecological space available to resistant parasites. Note that we are using the term competition very broadly to mean competition for resources, interference, and immune-mediated apparent competition for enemy-free space. Such “competitive release” following drug treatment would generate total fitness gains for resistant parasites, on top of the relative fitness gains made simply because drugs reduce the fitness of susceptible strains. In theory at least, this competitive release can be a very potent source of selection in favor of resistance, rendering the useful life of a drug much shorter than that expected if competition or co-infections do not occur (Hastings and D’Alessandro 2000; Hastings 2003; Mackinnon 2005; Read and Huijbens 2009). For example, if resistant parasites are able to occupy only 10% of “infection space” when susceptible parasites are present, removal of those competitors by drug treatment could increase the absolute fitness of resistant parasites many times over.

Direct experimental evidence of competition between co-infecting P. falciparum genotypes cannot be ethically obtained from human infections because untreated controls are required. However, a large body of correlational field data is consistent with genotype–genotype competition within malaria-infected humans (Daubersies et al. 1996; Mercereau-Puijalon 1996; Smith et al. 1999; Bruce et al. 2000; Hastings 2003; Talisuna et al. 2006; Bousema et al. 2008; Harrington et al. 2009). Additionally, there is substantial direct experimental evidence for competition in the rodent malaria model P. chabaudi (e.g., Jarra and Brown 1985; Taylor et al. 1997a; de Roode et al. 2004, 2005; Bell et al. 2006; Wargo et al. 2007). There, competitive release of drug-resistant parasites occurs after both prophylactic (de Roode et al. 2004) and therapeutic (Wargo et al. 2007) drug treatment. Competitive facilitation can even occur, where resistant parasites achieve even higher densities after susceptible competitors are removed than when competitors have never been present (Wargo et al. 2007). The transmission consequences of competition have only been examined in the rodent model, but there, competitive release plays out in terms of increased densities of gametocytes, the parasite life stages responsible for transmission to mosquitoes (Wargo et al. 2007). Thus, within-host competition can be a powerful inhibitor of the between-host transmission of resistant parasites, and conversely, the removal of that competition by drug treatment can be a strong driver of the spread of resistant genotypes.

These findings immediately suggest the hypothesis that the spread of drug resistance in a population can be slowed by treatment regimens that limit the extent of competitive release (Wargo et al. 2007). The logic is as follows. Recommended drug treatment regimens are aimed at eliminating all susceptible parasites from the infection (WHO 2006). Less-aggressive treatment regimens, involving for instance shorter treatment courses or lower drug doses, would leave some susceptible parasites and these could limit the competitive release of resistant parasites until immunity suppresses both resistant and sensitive parasites. This would constrain the transmission of resistant parasites, thus slowing the spread of resistance in a population. In previous work, we tested this idea by treating acute infections of P. chabaudi with drug courses of either one, two, or four days (Wargo et al. 2007). All treatments resulted in competitive release of the resistant parasites, but the release was significantly reduced in the one-day treatment group compared to the longer duration treatments. Importantly, the morbidity of mixed infections was the same for all three regimens.

Here, we test the hypothesis that the extent of competitive release (total fitness gain for resistant parasites) can be restricted still further by reducing drug concentrations in short duration regimens while still alleviating the clinical symptoms of disease. We here also introduce two new analytical methods. First, we more precisely predict the relative transmission success of resistant parasites using functions relating gametocyte density to infectivity (e.g., Stepniewska et al. 2008). Second, to better define the evolutionary consequences of drug treatment, we developed a method to estimate the duration and strength of selection through the course of individual infection. This statistical approach should be applicable to related issues in a wide range of infectious diseases.
Material and Methods

PARASITES AND HOSTS

Two genetically distinct *P. chabaudi* clonal lineages were used to inoculate the hosts: drug-resistant AS12265(pyr-1A) (hereafter referred to as clone R) and drug-sensitive A3714 (hereafter referred to as clone S). These clones were derived by limiting dilutions from isolates from different wild-caught thicket rats Thamnomys rutilans, the natural host of *P. chabaudi*. The *P. chabaudi* model is a well-known model for falciparum malaria, with comparable parasite dynamics and pathogenesis, although lacking cerebral malaria (Carlton et al. 2001; Wykes and Good 2009). Clone R was subsequently made resistant to pyrimethamine after isolation by a single passage with high-dose treatment (Walliker et al. 1975) and has been exposed to pyrimethamine in two later passages as well. Clone S has not been exposed to pyrimethamine since isolation (Beale et al. 1978). In this experiment, we used 6- to 8-week-old female C57Bl/6J laboratory mice (Harlan, UK) maintained on a 41B maintenance diet (Harlan, UK), with their drinking water supplemented with 0.05% para-amino benzoic acid (PABA) to enhance parasite growth (Jacobs 1964).

EXPERIMENTAL DESIGN AND INFECTIONS

Mice were inoculated by intraperitoneal injection of 10⁶ parasites of a given clone. In mixed infections, two separate inoculations were administered so that mixed infections received in total twice as many parasites as single infections. We did this because analyzing competition involves a comparison of the performance of a focal clone in the presence and absence of the other clone, requiring that the focal clone starts from the same initial dose in each case. A twofold difference in dose has a negligible effect on overall parasite dynamics or on morbidity (Timms et al. 2001). Drug treatment was given on day 6 postinfection (PI), when parasite-induced weight loss and anemia became pronounced. Drug treatment consisted of either 8 mg pyrimethamine/kg bodyweight (high dose), 3 mg/kg body weight (low dose), or no drugs (negative control). The first of these treatments was a short duration regimen that we had previously found limited competitive release without compromising clinical protection (Wargo et al. 2007); the second was chosen to be of the same duration but to involve a lower concentration thought likely to effect temporary reductions in susceptible parasites while still achieving clinical improvement. Note that both these drug treatments were expected not to fully clear the susceptible parasites and fall well short of 8 mg/kg pyrimethamine for four successive days, which is the standard drug treatment regimen in this model system to fully clear susceptible parasites (e.g., de Roode et al. 2004; Wargo et al. 2007). Pyrimethamine was dissolved in 100% dimethyl sulfoxide (DMSO) and an inoculum of 50 μL was given intraperitoneal to each mouse. Untreated mice were inoculated with 50 μL of DMSO only.

### Table 1. Experimental set-up of the study. Each treatment group consisted of six mice at the start of the experiment.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No drugs</th>
<th>Low dose</th>
<th>High dose</th>
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<tbody>
<tr>
<td>Single infection R</td>
<td>6</td>
<td>6*</td>
<td>6</td>
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<tr>
<td>Single infection S</td>
<td>6†</td>
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<tr>
<td>Mixed infection R+S</td>
<td>6</td>
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*+ a dead or euthanized mouse, † a mouse excluded because of a substantially low parasite dose.

Each treatment group consisted of six mice, totaling to 54 mice (Table 1).

MONITORING OF INFECTIONS

Mice were monitored daily from day three to 21 PI and thereafter on days 24, 26, and 28 PI. Morbidity was monitored by measuring mouse body mass (to the nearest 0.01 g) and red blood cell density using flow-cytometry (Beckman Coulter) of a 2 μL sample of blood from a tail snip (Taylor et al. 1998; Mackinnon et al. 2002). Quantitative real-time PCR was used to determine asexual parasite and gametocyte densities for each clone using clone-specific assays, for which an additional 5 μL and 10 μL of tail snip blood was taken, respectively.

DNA was extracted from 5 μL blood using the BloodPrep kit (Applied Biosystems, Foster City, CA) on the ABI Prism 6100 Nucleic Acid PrepStation according to manufacturer’s instructions. DNA was eluted in a total volume of 200 μL and stored at −80°C until quantification (Bell et al. 2006). RNA was extracted from 10 μL of blood, using the “RNA Blood-DNA” method (Applied Biosystems) on the ABI Prism®6100 Nucleic Acid PrepStation, following the manufacturer’s protocol, and eluted in 100 μL elution solution. Following RNA extraction, single-stranded cDNA was synthesized using the High-Capacity cDNA Archive Kit (Applied Biosystems) and stored at −80°C until quantification (Wargo et al. 2006, 2007).

PCR was performed on DNA to quantify the respective total parasite densities of each clone, and subsequently also on cDNA-converted RNA to quantify the number of gametocytes (transmission stages) of each clone (Drew and Reece 2007). Densities of asexual parasites, the nontransmissible replicative parasite stages that cause disease and make up the vast majority of parasites in an infection, were calculated by subtracting gametocyte counts from total parasite counts. The PCR reaction was identical for both clone-specific assays. Each reaction, with a final volume of 25 μL, consisted of 7 μL of DNA or cDNA, 900 nM forward and reverse primers, 250 nM probe and 1× concentration TaqMan® Universal PCR Master Mix (Applied Biosystems). All reactions were run on the ABI Prism® 7000 Sequence Detection System.
using the assay: 50°C for 2 min, 95°C for 10 min, then 45 cycles of: 95°C for 15 s and 60°C for 1 min. For primer and probe sequences, see Drew and Reece (2007). Quantification was based on serial dilutions of DNA and cDNA standards of known parasite and gametocyte density, respectively, determined beforehand by microscopy.

DATA ANALYSIS
Selection coefficients
To estimate the temporal pattern of the strength and direction of within-host selection, the coefficient of selection on the resistant clone was estimated. In a two-clone infection, the selection coefficient is the difference in the per-capita growth rate of each clone. This strength of selection, denoted by \( s_i(t) \), can be calculated from the frequency of the competitors

\[
s_i(t) = \frac{dp_i(t)}{dt} \left( \frac{1}{p_i(t) (1 - p_i(t))} \right),
\]

where \( p_i(t) \) is the relative abundance of competitor \( i \) at time \( t \) (Nelson et al. 2005). Essentially, selection reflects how quickly competitor proportions are changing, modified by how close they are to loss or fixation. The approach is to fit a time-series model to the parasite dynamics, which allows a statistical representation of the dynamics in relative abundance and, by using eq. (1), a statistical description of the selection dynamics. A time-series model was fitted to the asexual parasite and gametocyte dynamics for each clone in each mouse. This resulted in four time-series models from each mouse, and two estimates of selection dynamics—one for selection between the strains in the asexual stage and one for selection between strains in the gametocyte stage. The time-series model was a Generalized Additive Model (GAM) with a quasi-likelihood distribution (McCullagh 1983).

\[
y_i(t) = s_i(t)
\]

\[
Y_i(t) \sim Q(y_i(t), V(y)),
\]

where \( s(t) \) is a smoothing cubic spline that will represent the fit parasite dynamics. The GAM is set-up with the standard modified objective function that includes a likelihood term that measures how well the model fits the data, as well as the wiggliness term that adds a penalty for excessive curvature (Wood 2001, 2006). The trade-off parameter for the modified objective function was estimated by generalized cross-validation. The expected parasite dynamics are denoted by \( y(t) \), and the observed parasite dynamics by \( Y(t) \). The observed parasite dynamics are distributed as \( Q(y(t), V(y)) \), which has a quasi-likelihood distribution with an expectation \( y(t) \) and a mean–variance relationship denoted by \( V(y) \).

Because parasite dynamics span 12 orders of magnitude in natural log units (6 in log\(_10\) units), we need to pay particular attention to the mean–variance function \( V(y) \). To estimate this relationship, we conducted a series of additional mouse trials in which replicate samples were taken on days 3–15 PI from mice infected with clone R (see Supporting Information). These data suggest a power relationship between the mean \( y \) and variance \( V(y) \) of both asexual parasite and gametocyte densities. The function has the form

\[
V(y) = ay^b,
\]

which is the general form for Tweedie distributions, where \( a \) is the dispersion parameter and \( b \) determines the specific class of distribution (Jorgensen 1987). For asexual parasites, \( b = 1.59 \), which falls within the range of compound Poisson distributions. For gametocytes, \( b = 2.206 \), which falls into the Gamma distribution class. Noninteger values of \( b > 2 \) yield compound distributions that are difficult to implement in a GAM framework. To accommodate the implementation, we assumed \( b = 2 \) for both asexual and gametocyte distributions, which still provides a good fit to the raw data (see Supporting Information). The dispersion parameter \( (a) \) was estimated from the time-series data during the fitting process.

Parametric bootstrapping was used to estimate the confidence bands around the estimated selection dynamics for the asexual parasites and gametocytes in each mouse. The upper and lower 95% confidence bands for selection dynamics were calculated from 5000 bootstraps replicates. For examples of model fits to individual mice, see Supporting Information.

Predicted infectiousness
Ideally, the outcome of within-host selection would be directly measured in terms of parasite frequencies in mosquitoes. Such logistically complex experiments require repeated mosquito feeds through time on the same mouse, or greatly increased number of mice in the study, and were beyond the resources we had available. Instead, we inferred between-host transmission from the kinetics of gametocyte densities. In earlier work, we typically used cumulative gametocyte densities over the course of infection as a measure of potential transmission (e.g., Wargo et al. 2007). However, infectiousness (proportion of mosquitoes infected) is unlikely to be a simple linear function of gametocyte density, not least because there must be a saturation effect at high gametocyte densities as well as less-efficient transmission at low densities as mates become limiting (gametocytes are dioecious sexual stages) (Barnes and White 2005; Paul et al. 2007; Sinden et al. 2007). Therefore, we used a density–infectivity function (or q-function) to estimate the number of mosquitoes potentially infected with each clone of the following general form (Sinden et al. 2007):
Figure 1. Density–infectivity q-functions used in the analysis: probability of a single mosquito becoming infectious based on gametocyte density. Function \( q_1 \) (black dashed line) is based on data from Barnes and White (2005), \( q_2 \) (red solid line) is based on data from Carter and Graves (1988). Probability of infection was calculated using eq. (3) and parameters \( \alpha = 0.03, \beta = 0.6, \alpha/\gamma = 0.85 \) for \( q_1 \) and \( \alpha = 1 \times 10^{-6}, \beta = 2, \alpha/\gamma = 1 \) for \( q_2 \). Infectivity saturates at \( \alpha/\gamma \). Gametocyte density is plotted on a log scale.

\[
q = \frac{\alpha N^\beta}{1 + \gamma N^\beta},
\]

with gametocyte density denoted as \( N \), probability of infection by \( q \) and \( \alpha, \beta, \) and \( \gamma \) as parameters defining the shape of the relationship. The exact shape of the q-function is not well resolved and it may vary with epidemiological settings (Stepniewska et al. 2008). We used two different curves (Fig. 1) both derived from experimental studies with \( P. falciparum \). One comes from data compiled by Barnes and White (2005), and is based on feeding experiments on artificial \( P. falciparum \) infections of 88 neurosyphilitic patients using two different parasite strains, one from South Carolina, the other from Panama (Jeffery and Eyles 1955). The other dataset comes from six different studies on natural \( P. falciparum \) infections from West Africa, summarized by Carter and Graves (1988). Ideally, we would have included a curve estimated from experimental work on \( P. chabaudi \) (e.g., Buckling et al. 1997; Taylor et al. 1997b; Mackinnon and Read 1999). However, relevant data on \( P. chabaudi \) come from experiments designed to maximize transmission, so that there are too few data to estimate transmission success at the lower gametocyte densities relevant here (posttreatment). We assess the sensitivity of our conclusions to our choice of q-function in the discussion.

Posttreatment gametocyte densities (day 7–28) were used to calculate probability of infection through time using the q-function. To translate these dynamical probabilities into a single transmission potential parameter, the number of infected mosquitoes posttreatment from a sample of 100 equally distributed across time was calculated for each mouse. These calculations assume that any subsequent interaction between resistant and sensitive parasites in mosquitoes is uncorrelated with relative fitness in the subsequent vertebrate host, and that infectivity per gametocyte remains equal over the course of infection. Again, we revisit these assumptions in the discussion.

**Statistical analysis**

Analyses were performed in R 2.9.0 (R Development Core Team 2009). Morbidity was summarized as arithmetic mean mouse body mass and arithmetic mean red blood cell density, with minimum red blood cell density and minimum body mass taken as a measurement of morbidity during the acute infection. Because asexual parasite densities range over many orders of magnitude, we summarize average asexual densities using the geometric mean over time. This reduces the impact on the mean density of very high counts on a few days. Morbidity and parasite densities were calculated for each mouse over the posttreatment infection period (day 7–28 PI). To ensure treatment groups with the same clonal infections did not differ prior to drug treatment, day 6 PI values on asexual parasite density, body mass, and red blood cell density were evaluated for significant differences. No significant differences were found.

General linear modeling was used to fit the above response variables with the following factors: competition (single/mixed) and drugs (no drugs/low dose/high dose). Maximal models with the interaction terms were fitted first and subsequently removed if not significant. When comparing the effect of drugs on single or mixed infections only, one-way ANOVA was performed. To test for differences among drug treatment groups, Tukey’s HSD (Honestly Significant Difference) posthoc multiple comparison tests were carried out and the adjusted \( P \)-values are reported. The analysis on the predicted number of infected mosquitoes was done separately for single and mixed infections using factors drugs (no drugs/low dose/high dose) and clone (R/S). Gametocyte densities did differ significantly between treatment groups with the same clonal infections prior to treatment on day 6, therefore, day 6 gametocyte densities were included in the model as a covariate.

Six mice were excluded from the analysis (Table 1). Four mice died or were euthanized during the acute phase of the infection and two evidently received a substantially lower parasite dose (parasite density at time of treatment two orders of magnitude lower than expected; Table 1). Note that we set out to minimize risk of death by our choice of mouse strain, sex, and size; to the point of death, the infection kinetics in the mice that died were very similar qualitatively to those in mice that survived.

**Results**

**ASEXUAL PARASITE DYNAMICS**

In untreated infections, densities of clones R and S increased similarly to peak parasitaemia, after which clone R densities declined...
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Figure 2. Parasite densities of the drug-resistant clone R (left panels) and the drug-sensitive clone S (right panels) in single (top panels) and mixed (bottom panels) infections that were either left untreated (dashed red line), received a low drug dose (solid blue line) or high dose (thick black line). Black diamonds indicate the timing of drug-treatment at day 6 postinfection. Data are geometric means (±standard error) for up to six mice (Table 1).

more rapidly than the densities of clone S (Figs. 2 and 3). Subsequently, clone S typically increased in densities again around day 13 and day 25. These dynamics are likely a result of resource abundance, immune control, and antigenic escape, although the exact mechanisms have not been fully determined. Competitive suppression was observed, whereby fewer R parasites were produced when susceptible parasites were present (Fig. 2C, competition: $F_{1,27} = 15.6, P < 0.001$). The performance of clone S was unaffected by the presence of clone R, with as many susceptible parasites persisting for as long in single and mixed infections in the absence of drug treatment (Fig. 2D, competition: $F_{1,28} < 0.1, P = 0.94$).

As expected from previous studies (de Roode et al. 2004; Wargo et al. 2007), parasites from clone R and from clone S were indeed, respectively, resistant and susceptible to treatment with pyrimethamine. In single-clone infections, the densities of asexual parasites of clone R were unaffected by drug treatment (Fig. 2A, drugs: $F_{2,13} = 0.2, P = 0.78$), whereas clone S densities were reduced (Fig. 2B, drugs: $F_{2,12} = 26, P < 0.001$). The high drug dose killed more S parasites than the low drug dose (Single-clone infections—Tukey’s HSD, high dose vs. low dose: $P_{adj} < 0.001$). The low drug dose resulted in an initial kill of S parasites, but over the period following drug treatment, an equal amount of S parasites were produced compared to untreated infections (Tukey’s HSD, low dose vs. no treatment: $P_{adj} = 0.85$).

As in single-clone infections, densities of clone S parasites in mixed infections were greatly reduced by drug treatment. The two drug dosages had similar rates initially, but after two days the decline of S parasites was greater following high-dose treatment (Figs. 2B,D and 3).

Three days after treatment, there were as many or more resistant parasites in drug-treated mixed infections than in single-clone infections (competitive facilitation; Figs. 2C, 3, and 4A, competition × drugs: $F_{2,27} = 5.8, P = 0.008$). The higher drug dose resulted in a more than 200-fold increase in resistant parasites after treatment than in untreated infections and a 160-fold increase compared to low-dose treatment (mixed infections—Tukey’s HSD, no drugs vs. high dose: $P_{adj} = 0.002$, low dose vs. high dose: $P_{adj} = 0.098$).

Together, these data show that densities of resistant parasites were suppressed when drug-sensitive parasites were present. Removing those sensitive parasites with drug treatment led to competitive release, and subsequently greatly increased densities of resistant parasites compared to untreated mixed infections.
Figure 3. Asexual parasite dynamics of individual mice that were infected with a mixed infection of clone R (dashed blue line) and clone S (solid black line), which received no drug treatment (left column), a low dose of pyrimethamine (middle column) or a high dose of pyrimethamine (right column). Each group consisted of six mice at the outset of the experiment; however, one mouse in the high-dose treatment group received a much lower parasite dose than intended and was excluded (Table 1). Drug treatment was given on day 6 postinfection, as indicated by the black diamonds.

TRANSMISSION POTENTIAL

In untreated single-clone infections, gametocytes of both clones peaked on or around day 6 and subsequently again around day 13 (Figs. 5 and 6). Clone S peaked at higher gametocyte densities than clone R and typically had an additional gametocyte peak after day 20 postinfection (Fig. 5A,B). As with the asexual dynamics, gametocyte densities for clone R were, in the absence of drug treatment, suppressed when sensitive parasites were present (Fig. 5C), while clone S achieved similar gametocyte densities in untreated single-clone and mixed infections (Fig. 5D). The day after high-dose drug treatment, clone S had higher gametocyte densities, both in single and mixed infections (Fig. 5B,D). On subsequent days however, gametocyte densities from the sensitive clone were dramatically reduced. Following low-dose treatment, gametocyte densities a day later were not elevated. Clone S in mixed infections failed to produce the second gametocyte peak around day 13 following high-dose treatment but it did following low-dose treatment. All mixed infections demonstrated a third S gametocyte peak (Figs. 5D and 6). In line with the competitive release of asexual densities following chemotherapy, resistant gametocyte densities in mixed infections were also elevated, particularly following high-dose treatment (Figs. 5C and 6).

With high drug dose, the number of mosquitoes predicted to be infected from mixed infections was increased for R parasites
and decreased for S parasites (Fig. 7A; Table 2, drug × dose interaction). Using q1 (Fig. 1), the greater than threefold advantage of clone S over clone R in untreated infections was reduced by high-dose drug treatment to parity (Fig. 7A). In single-clone infections, drug treatment did not affect predicted infectivity of clone R, but it did for clone S (Fig. 7B; Table 2, drug × dose interaction), but not as a simple reduction, a consequence of the elevated gametocyte densities the day after high-dose treatment and elevated second gametocyte peaks in low-dose treatment (Fig. 5B).

Together, these data show that the relative fitness of resistant parasites in mixed infections was increased by drug treatment in two ways. First, susceptible parasite fitness was decreased,
Figure 6. Gametocyte dynamics of individual mice that were infected with a mixed infection of clone R (dashed blue line) and clone S (solid black line), which were either left untreated (left column), received a low drug dose (middle column) or high dose (right column). Each group consisted of six mice at the outset of the experiment; however, one mouse in the high-dose treatment group received a much lower parasite dose than intended and was excluded (Table 1). Drug treatment was given on day 6 postinfection, as indicated by the black diamonds.

particularly at the high-dose treatment. Second, the fitness of resistant parasites increased as a consequence of competitive release (Fig. 7A). In single-clone infections, the fitness of resistant parasites relative to the fitness of sensitive parasites was increased but only because treatment suppressed the fitness of sensitive parasites, and then, only in the high-dose treatment (Fig. 7B).

TOTAL PARASITE DYNAMICS
The total parasite burden (clone R + clone S) of the mixed infections was unaffected by drug treatment (Fig. 8A, drugs: $F_{2,14} = 0.1, P = 0.90$). This is because the susceptible parasites, which dominate in the absence of treatment, were replaced by resistant parasites if drug treatment was administered. Similar dynamics were seen for total gametocyte densities in mixed infections (Fig. 8B), with resistant gametocytes dominating in drug-treated infections and susceptible gametocyte dominating in untreated infections. Consequently, drug treatment did not affect overall predicted infectiousness (drugs, q1: $F_{2,13} = 1.3, P = 0.30$; q2: $F_{2,13} = 1.6, P = 0.23$).

MORBIDITY
Unsurprisingly, drug treatment of infections consisting of only resistant parasites did not improve morbidity, because clone R was resistant to treatment (Fig. 9A,B, drugs- maximum RBC loss: $F_{2,13} = 0.2, P = 0.84$; maximum weight loss: $F_{2,13} = 0.1, P = 0.90$). In contrast, drug treatment alleviated morbidity in all
infections containing S parasites (Fig. 9C–F, drugs: maximum RBC loss: \( F_{2,29} = 15, P < 0.001 \); maximum weight loss: \( F_{2,29} = 2.9, P = 0.073 \)). Following drug treatment, mixed infections were less anemic than untreated infections (drugs: mean RBC density: \( F_{2,14} = 6.9, P = 0.008 \)). Importantly, this was independent of drug dose (Tukey’s HSD, low dose vs. high dose—mean RBC density: \( P_{adj} = 0.46 \), so that mice given the low-dose treatment were no more anemic than those given the high dose. Drug treatment had no impact on mean body mass of mice (drugs: mean body mass: \( F_{2,14} = 0.7, P = 0.53 \)).

### Selection Dynamics

Drug treatment had a pronounced effect on the dynamics of selection on drug-resistant parasites throughout the infection. The rate of selection on clone R through time for each mouse is shown in Figure 10A–C (selection on asexual parasite densities) and Figure 10D–F (selection on gametocyte densities). Selection was acting on the resistant clone even in the absence of drugs, but that selection varies through time and between mice.

In the absence of treatment, resistant parasites in five of six mice were under negative selection on asexual parasite densities from approximately day 7 to day 12, after which selection became positive in half the mice. When infections were treated with a single high dose of pyrimethamine, the opposite occurred: resistant parasites were under positive selection for the period immediately following drug treatment, and negative selection later on. Under low-dose treatment, there was no indication of significant positive selection, with the exception of a few short periods in two mice. Later on in the infection, resistant parasites in this treatment group were also selected against. Positive selection of resistant gametocytes was very strong in all infections that received a high drug dose. In untreated and low-dose treatment infections, little positive or negative selection on R gametocytes was observed.

### Discussion

We found that in the absence of drug treatment, drug-sensitive parasites competitively suppressed resistant parasites, as has been found in previous studies (de Roode et al. 2004; Wargo et al. 2007). Drug treatment led to competitive release of the drug-resistant

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**Table 2.** Analysis of variance table of mixed and single infections to test for an effect of drug treatment (drugs: no drugs/low dose/high dose), parasite clone (clone: R/S), and the interaction between them, on predicted number of infected mosquitoes based on two density-infectivity functions (Fig. 1; q1 and q2) using the posttreatment gametocyte densities (day 7–28). Gametocyte densities on day 6 were included as a covariate.

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<th>q1</th>
<th>q2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mixed infections</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drugs</td>
<td>( F_{2,27} = 1.7, P = 0.20 )</td>
<td>( F_{2,27} = 1.8, P = 0.19 )</td>
</tr>
<tr>
<td>Clone</td>
<td>( F_{1,27} = 90, P &lt; 0.001 )</td>
<td>( F_{1,27} = 100, P &lt; 0.001 )</td>
</tr>
<tr>
<td>Drugs×clone</td>
<td>( F_{2,27} = 22, P &lt; 0.001 )</td>
<td>( F_{2,27} = 11, P &lt; 0.001 )</td>
</tr>
<tr>
<td>Day 6 gametocyte density</td>
<td>( F_{1,27} = 5.6, P = 0.025 )</td>
<td>( F_{1,27} = 8.1, P = 0.008 )</td>
</tr>
<tr>
<td><strong>Single infections</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drugs</td>
<td>( F_{2,24} = 14, P &lt; 0.001 )</td>
<td>( F_{2,24} = 28, P &lt; 0.001 )</td>
</tr>
<tr>
<td>Clone</td>
<td>( F_{1,24} = 47, P &lt; 0.001 )</td>
<td>( F_{1,24} = 53, P &lt; 0.001 )</td>
</tr>
<tr>
<td>Drugs×clone</td>
<td>( F_{2,24} = 7.2, P = 0.004 )</td>
<td>( F_{2,24} = 9.3, P &lt; 0.001 )</td>
</tr>
<tr>
<td>Day 6 gametocyte density</td>
<td>( F_{1,24} = 7.1, P = 0.014 )</td>
<td>( F_{1,24} = 11, P = 0.002 )</td>
</tr>
</tbody>
</table>
Figure 8. Total asexual parasite (left graph) and gametocyte (right graph) dynamics of mixed infections that were either left untreated (dashed red line), received a low drug dose (solid blue line) or high dose (thick black line). Black diamonds indicate the timing of drug-treatment at day 6 postinfection. Data are geometric means (±standard error) for up to six mice (Table 1).

clone, seen as increased asexual parasite densities. Importantly, we found here that competitive release of drug-resistant parasites was more restricted following low-dose therapy than high-dose treatment, even though the lower dose treatment reduced morbidity to the same level as high-dose treatment. The high-dose treatment thus exerted stronger positive selection on resistant parasites for little clinical gain. Most importantly, resistant parasites in mixed infections that received high-dose drug treatment had the highest transmission potential. Note that the drug treatments in this experiment were given on a single day only, while treatment aimed at full parasitological cure consists of high-dose treatment given for four or more consecutive days. Selection under such a treatment regime will be much more extensive than the treatments used in this experiment. Our results confirm the findings in Wargo et al. (2007), extend the results to transmission potential, and provide proof of principle that less-aggressive treatment regimes than are currently recommended could help slow the spread of drug-resistant malaria parasites. Before discussing this provocative proposition, we first discuss various methodological issues.

So far as we are aware, the selection coefficient analysis we presented here, which is derived from ecological statistics (Nelson et al. 2005), has not been previously used to measure the strength and direction of selection on pathogen traits. We have shown that using longitudinal data on single infections, it is possible to quantitatively compare the strength of selection for resistance imposed by different drug regimens (Fig. 10). We note that the selection coefficients presented here are conservative estimates of the strength of selection. With our approach, a selection coefficient cannot be calculated when one of the parasite clones is below the threshold of detection, because selection is a function of the abundance of both clones. When a clone was not detected at some time point, but then subsequently observed, that clone must have been present at the earlier time point. Positive selection on the more abundant clone could be very strong on these earlier time points, but would not be captured in the model. Therefore, the selection coefficients shown in Figure 10, both positive and negative, may in reality be more extreme. Future technological developments may lower detection thresholds; not only would this allow estimates of possibly more extreme selection, it would also enable estimates of the strength of selection to be made later in infections when densities of one or both clones are below detection.

It seems likely that the selection coefficient approach will be useful for measuring in-host selection on a wide range of pathogen traits in diverse host–pathogen systems. This should help reveal or better define poorly studied phenomena. In our data, for example, the analysis demonstrated that positive selection for resistance following a high-dose treatment lasted for approximately 5 days. Interestingly, the half-life of pyrimethamine in a mouse is estimated to be approximately 4.5 h (Coleman et al. 1986). If that half-life estimate is correct, our data suggest that positive selection for resistant parasites continued even after drug concentrations became essentially homeopathic (from the half life, 0.001 mg/kg after just 2.5 days after drug treatment). The factors that continue to drive positive selection for resistance even after drug clearance are unknown. One possibility is that the more abundant resistant clone is capable of temporarily suppressing the less-abundant susceptible parasites. Another possibility is that genotype-specific immunity transiently suppresses the parasite strain that dominated numerically when the immune response was first provoked (discussed in Wargo et al. 2007). Whatever the explanation, susceptible parasites are not increasing in frequency as soon as drug pressure wears off (Fig. 2D), which provides an extended fitness advantage to the resistant parasites.

Over the course of infection, both positive and negative selection was exerted on drug-resistant parasites following drug
treatment. Naturally, resistant parasites experienced positive selection following drug treatment, but resistant parasites were eventually selected against. Similar selection dynamics are seen over the course of a year in *P. falciparum* dynamics in areas with seasonal malaria. In eastern Sudan for instance, the frequency of resistance genes rises during the rainy season when malaria intensity and hence drug usage is high. When malaria transmission and hence drug use declines, so too does the frequency of resistance genes in the parasite population (Abdel-Muhsin et al. 2004; Ord et al. 2007; Babiker 2009). Such an outcome is usually attributed to “the cost of resistance.” Our experiments demonstrate that a fitness disadvantage in the absence of drugs is unlikely to be some fixed value (Read and Huijben 2009): it is greatly exacerbated by competitors and even varies during the course of an infection.

We estimated the transmission potential of both clones from gametocyte densities using a density–infectivity function (Fig. 1). The precise form of the q-function is not well established. We used two functions based on different datasets (Carter and Graves 1988; Barnes and White 2005) and our conclusions were qualitatively similar for both q-functions. We also analyzed a variety of other hypothetical functions (data not shown) and our conclusions were again unaffected, except when the threshold for infectivity exceeded the posttreatment gametocyte densities we observed, in which case the resistant clone would have never transmitted after treatment. Further refining the precise nature of the q-function.

**Figure 9.** Body mass (±standard error, left panels) and red blood cell density (±standard error, right panels) of mice infected with drug-resistant clone R (top panels), drug-sensitive clone S (middle panels), and mixed infections of both clones (bottom panels) for untreated infections (dashed red line), low-dose treatment (solid blue line) and high-dose treatment (thick black line). Black diamonds indicate timing of drug treatment at day 6 postinfection. The inset bar charts show the mean body mass and mean red blood cell density posttreatment (±standard error) for untreated infections (red bars—“N”), low-dose treatments (blue bars—“L”) and high-dose treatments (black bars—“H”). All data are arithmetic means for up to six mice (Table 1).
would be useful, although other issues are also relevant. For instance, we are unaware of any information on the competitive interactions between genotypes within a mosquito. Competition between different parasite species within mosquitoes can occur (Paul et al. 2002) and so it is likely that there will be competition between presumably ecologically more similar members of the same species. How this might be related to resistance, and the consequences for onward transmission to the next vertebrate host are unclear. Similarly, gametocyte infectivity is known to vary through time (Drakeley et al. 2006), not least in response to transmission-blocking immunity, and there is also evidence of strain-specific transmission-blocking immunity independent of anti-gametocyte immunity (Buckling and Read 2001). So for malaria parasites, as with many organisms, considerable work needs to be done to fully define fitness. Nonetheless, we believe the analysis reported here is an improvement of the somewhat arbitrary cumulative gametocyte densities through time used previously. Until sufficient resources are available to directly measure transmission to and from mosquitoes in experiments of this scale, our approach provides a tool to qualitatively compare of the fitness of resistant parasites following different drug treatments. But we emphasize: fitness is difficult to measure because transmission success is dependent on many more factors than merely producing transmission stages, therefore, the normal cautions associated with fitness measures necessarily apply here too.

The susceptible parasite clone in this study showed increased gametocyte densities on the day following high-dose drug treatment. On subsequent days, however, gametocyte densities dropped rapidly. Transient increases in gametocyte densities were not elicited by low-dose treatment and nor were they seen in the resistant clone. Drug-induced gametocytogenesis has been observed before with pyrimethamine use in \emph{P. chabaudi} in vivo (Buckling et al. 1999a) and in \emph{P. falciparum} following chloroquine treatment in vitro (Buckling et al. 1999b). Many authors have reported gametocytogenesis following SP drug treatment of human falciparum infections (Puta and Manyando 1997; Robert et al. 2000; Osorio et al. 2002; Sowunmi and Fateye 2003; Talman et al. 2004; Ali et al. 2006; Sowunmi et al. 2006), although field
data are difficult to interpret in the absence of untreated controls. It has been proposed that drug-induced gametocytogenesis is a facultative response to conditions unfavorable to in-host replication (Buckling et al. 1997), and our data are consistent with that. The quantitative impact of this transient phenomenon on the relative fitness of drug-resistant parasites and hence the spread of drug resistance has yet to be determined.

We found that, for no detectable clinical gain, the higher dose treatment led to stronger and more prolonged positive selection for resistance than did the low-dose treatment. Therefore, compared to the treatment regimes currently employed in the field, which are aimed at removing all sensitive parasites as fast as possible (WHO 2006), milder drug dosages may achieve better resistance management, and thus prolong the useful life span of drugs, without clinical compromise. We suggest that this possibility, which flies contrary to orthodox thinking in a wide range of infectious diseases, is worthy of further investigation. A few recent studies from other disease models reach the same conclusion. In the treatment of both cancer (Gatenby et al. 2009) and Staphylococcus aureus infections (Drsano et al. 2009), it appears that reduced treatment eases the proliferation of resistant agents and in the former even reduces mortality. In both cases, the underlying mechanism appears to be competitive release.

Clearly many more studies regarding the effects of drug regimes on competition between susceptible and resistant parasites and their effect on disease epidemiology are needed before any recommendations are made for public health practices. Mice are not people, and the results we present have to be interpreted with extreme caution (for further discussion of perils and merits of are not people, and the results we present have to be interpreted any recommendations are made for public health practices. Mice asites and their effect on disease epidemiology are needed before mechanism appears to be competitive release.

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Supporting Information
The following supporting information is available for this article:

Figure S1. Variance (σ) to mean (μ) relationship for asexual and gametocyte malaria parasites.

Figure S2. Model fits for asexual parasite densities (left panels) and gametocyte densities (right panels) on natural log scale for three arbitrarily chosen example mice that were either untreated (upper panels), received a low dose (middle panels) or a high dose (bottom panels) of pyrimethamine on day 6 postinfection.

Supporting Information may be found in the online version of this article.

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