

# Adaptive changes in *Plasmodium* transmission strategies following chloroquine chemotherapy

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## SUMMARY

Both theory and data suggest that malaria parasites divert resources from within-host replication to the production of transmission stages (gametocytes) when conditions deteriorate. Increased investment into transmission stages should therefore follow subcurative treatment with antimalarial drugs, but relevant clinical studies necessarily lack adequate control groups. We therefore carried out controlled experiments to test this hypothesis, using a rodent malaria (*Plasmodium chabaudi*) model. Infections treated with a subcurative dose of the antimalarial chloroquine showed an earlier peak and a greater rate of gametocyte production relative to untreated controls. These alterations led to correlated changes in infectivity to mosquitoes, with the consequence that chloroquine treatment had no effect on the proportion of mosquitoes infected. Treatment of human malaria commonly does not result in complete parasite clearance. If surviving parasites produce compensatory increases in their rate of gametocyte production similar to those reported here, such treatment may have minimal effect on decreasing, and may actually increase, transmission. Importantly, if increased investment in transmission is a generalized stress response, the effect might be observed following a variety of antimalarial treatments, including other drugs and potential vaccines. Similar parasite life history counter-adaptations to intervention strategies are likely to occur in many disease-causing organisms.

## 1. INTRODUCTION

Strategies of resource allocation that maximize fitness can differ between benign and stressful environments. Consequently, natural selection often favours phenotypic alteration of reproductive effort in response to stress (Minchella & LoVerde 1981; Crowl & Covich 1990; Roff 1992; Stearns 1992). In disease-causing organisms, such adaptive alterations could render intervention strategies against parasitic diseases less effective than anticipated. During the course of an infection, malaria parasites (*Plasmodium* spp.) are capable of modulating the proportion of replicating parasites (asexuals) that develop into non-replicating transmission stages (gametocytes). If this modulation is stress-induced, medical interventions, such as chemotherapy, could lead to greater investment in gametocyte production, thus offsetting much of the transmission-reducing benefits of killing parasites. Here we test this idea experimentally using a rodent malaria–mouse model.

Extrapolating from metazoan life history studies, there are two reasons to expect that malaria parasites will switch investment from asexuals to gametocytes when conditions deteriorate. First, increased reproductive effort should occur following cues associated with decreased probability of future survival or reproduction. For example, enhanced egg production has been demonstrated in snails following exposure to

castrating trematode infections (the ‘fecundity compensation’ hypothesis; Minchella & LoVerde 1981). Increased investment by malaria parasites into gametocytes should therefore occur in response to environmental cues associated with the decline in future transmission potential, such as clearance of the infection or the onset of transmission-blocking immunity (Koella & Antia 1995; Taylor & Read 1997). Second, if conditions change such that one life history stage becomes relatively more vulnerable than another, increased investment into the least vulnerable stage is predicted (the ‘safe harbour’ hypothesis; Shine 1978). In the Jerusalem artichoke (*Helianthus tuberosus* L.), for example, inhibition of sexual reproduction by removing flowers results in increased investment in asexual reproduction via tuber development (Westley 1993). If conditions become less favourable for asexuals relative to gametocytes (for example as stage-specific immunity develops), increased investment into transmission is again predicted.

The environmental cues that stimulate gametocyte production (gametocytogenesis) are poorly understood (Sinden 1983; Carter & Graves 1988; Alano & Carter 1990), but there is some evidence that the rate of gametocyte production increases in response to conditions unfavourable for asexual replication. *P. falciparum* (the most common and virulent human malaria parasite) produces more gametocytes *in vitro* when there is a high density of parasitized red blood cells

(Carter & Miller 1979; Brockelman 1982; Bruce *et al.* 1990). By definition, antimalarial drugs acting against blood-stage parasites impose considerable stress, and some also have a greater inhibitory effect on asexual parasites than on gametocytes. Increased gametocytogenesis following treatment that greatly reduces parasite numbers is therefore to be expected. However, here the evidence is more ambiguous.

Early *in vivo* clinical trials with human *Plasmodium* frequently reported more gametocytes following treatment with drugs inhibiting folate metabolism and hence DNA synthesis (e.g. paludrine, pyrimethamine and the sulphonamides; Findlay *et al.* 1946; Mackerras & Ercole 1948; Shute & Maryon 1951; Foy & Kondi 1952; Ramakrishnan *et al.* 1952; McCarthy & Clyde 1973). More recently, increased gametocyte production has also been reported in *P. falciparum* infections following treatment with Fansidar, a synergistic combination of pyrimethamine and sulphadoxine (Tin & Nyunt-Hlaing 1984; Marwoto *et al.* 1986). However, these increases occurred more rapidly than the 8–10 day maturation period of *P. falciparum* gametocytes (Smalley 1976; Jensen 1979), and other studies using Fansidar with longer follow-up periods do not support the hypothesis (Strickland *et al.* 1986; Hogh *et al.* 1995). With other blood-stage antimalarials, such as chloroquine or quinine, most clinical studies have found no increases in gametocytogenesis following subcurative treatment (e.g. Mackerras & Ercole 1949*a*; Jeffery *et al.* 1956; Jeffery 1958; Hogh *et al.* 1995). These data have given rise to the conventional wisdom that only drugs which inhibit DNA synthesis are capable of inducing increased gametocytogenesis (Carter & Graves 1988; Alano & Carter 1990), even though there may be exceptions (Mackerras & Ercole 1949*b*).

But all these data have a common problem: chemotherapy may have been used when increases in gametocyte production would have occurred anyway. This is particularly likely if the conditions stimulating both gametocytogenesis and symptoms (and hence treatment) approximately coincide, as would be the case if, for example, high parasite densities or host stress were a common trigger. Understandably, no studies included appropriate untreated control infections, so that the effect of subcurative antimalarial chemotherapy on gametocyte production has yet to be resolved.

If enhanced gametocytogenesis can occur following subcurative treatment, reductions in infectivity might not be nearly as great as reductions in morbidity. This may have implications for malaria control and epidemiology, particularly if increased gametocytogenesis is a generalized stress response stimulated by a variety of treatments. We therefore carried out controlled experiments using a *P. chabaudi*–mouse model to test the life history prediction of increased gametocytogenesis following subcurative chloroquine chemotherapy, and to determine any impact on subsequent infectivity to a mosquito vector. Chloroquine (CQ) was used because of its wide availability in malaria-endemic areas and the general view that its use does not result in increased gametocytogenesis (Carter &

Graves 1988; Alano & Carter 1990). In addition, it only affects asexual parasites and immature gametocytes (Smalley & Sinden 1977), so that increased gametocytogenesis might be expected on account of both the fecundity compensation and safe-harbour hypotheses.

## 2. MATERIALS AND METHODS

### (a) *Parasites and hosts*

Male C57/BL/6J mice (Harlan-Olac, England) aged 8–14 weeks were infected with  $1 \times 10^6$  red blood cells infected with a CQ-naive *P. chabaudi* clone (either CR or ER, from the WHO Registry of Standard Malaria Parasites maintained at the University of Edinburgh, UK) in a 0.1 ml intraperitoneal inoculum of 50% Ringer's solution (27 mM KCl, 27 mM CaCl<sub>2</sub>, 0.15 M NaCl), 45% heat-inactivated calf serum and 5% 200 units ml<sup>-1</sup> heparin solution. Mice, age-matched within experiments, were housed in cages of 2–4 animals at a temperature of  $25 \pm 1$  °C with a 0700 to 1900 hours light cycle, and provided with 41B rat and mouse maintenance diet (Harlan-Teklad, England) and water containing 0.05% pABA, *ad libitum*.

### (b) *CQ preparation and administration*

CQ solution was prepared from a stock of 40 mg ml<sup>-1</sup> CQ sulphate (Nivaquine<sup>TM</sup>) diluted in distilled water and administered orally using a lubricated catheter in approximately 0.1 ml doses of 12 mg kg<sup>-1</sup> of mouse weight in all cases. Preliminary experiments revealed this dose to be subcurative. Control mice were given 0.1 ml distilled water. All treatment took place between 1600 and 1700 hours.

### (c) *Parasite counts*

From day 4 post-infection (p.i.), daily thin blood smears from the tail vein were Giemsa-stained and asexual parasites counted per  $1.5 \times 10^3$  red blood cells (RBCs). At low asexual densities, parasites were counted per  $10^4$  RBCs. Mature gametocytes were counted per  $1.25 \times 10^4$  RBCs. Parasite densities were calculated from RBC densities, measured by flow cytometry (Coulter Electronics<sup>TM</sup>) every second day, multiplied by parasites per RBC.

### (d) *Mosquito feeds and dissections*

Mice were anaesthetized by an intramuscular injection of 0.5 ml per 20 g mice 3:2:1 distilled water: Vetalar<sup>TM</sup>: Rompun<sup>TM</sup>, and placed onto pots covered with nylon mesh containing about 40 4–5 day-old female *Anopheles stephensi*, which had been starved for the previous 24 h. Mosquitoes

Table 1. *Details of experiments*

experiment	clone used	day p.i. of CQ treatment	no. of mice infected	
			CQ	control
1	CR	6	6	5
2	CR	5	6	6
3	CR	4	5	6
		5	6	
		6	5	
4	ER	5	6	6

p.i., post-infection; CQ, chloroquine.

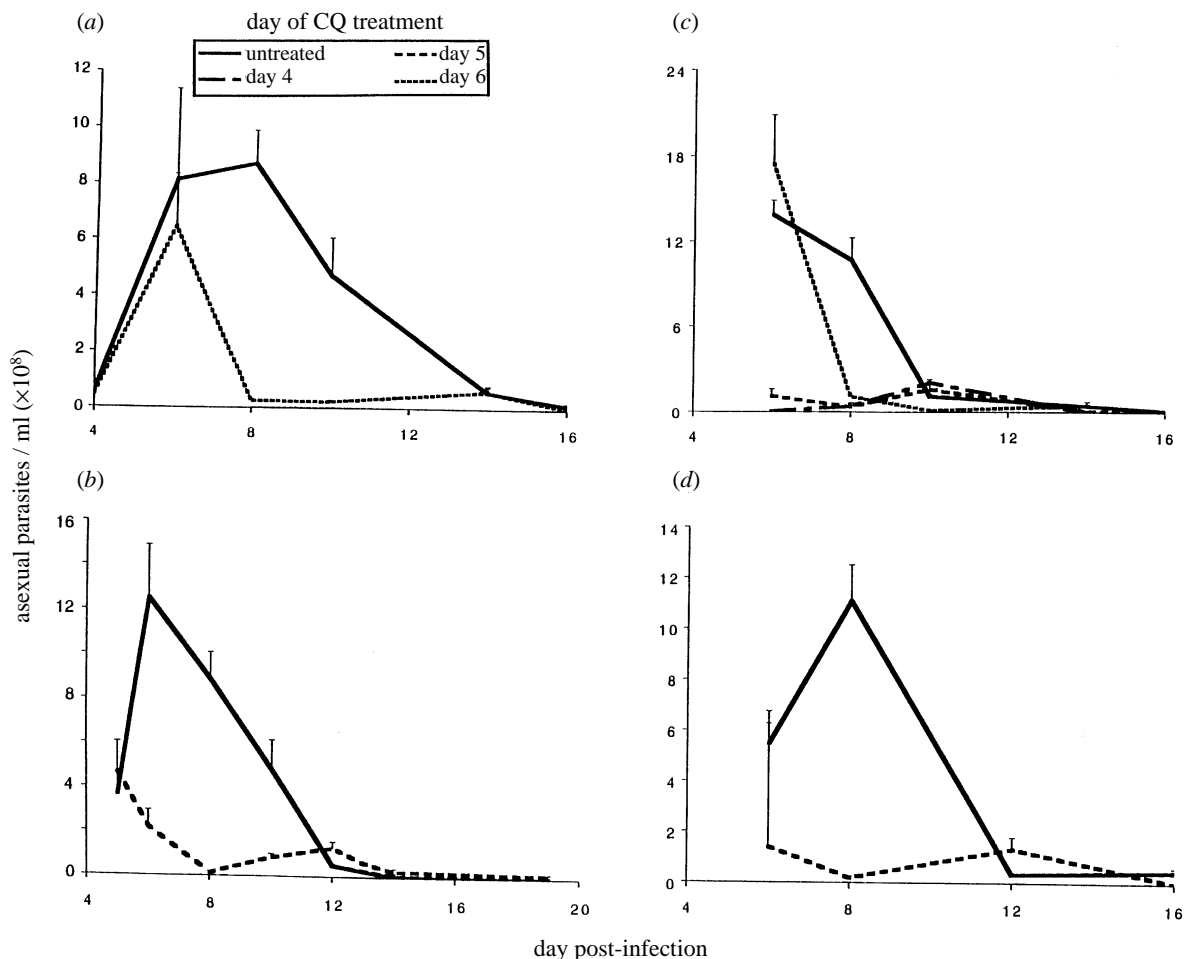


Figure 1. Mean asexual parasite densities ( $\pm 1$  s.e.) during infections for experiments 1-4 (*a-d* respectively). CQ, chloroquine.

were left to feed in the dark for 30 min, between 1845 and 1945 hours. They were subsequently maintained at 25–30 °C, 70–80% humidity, with a 12-h light cycle, and provided with 5% glucose, 0.05% pABA solution *ad libitum*. After 8–9 d, approximately 25 mosquitoes per mouse were dissected to determine the presence of oocysts on midguts.

#### (e) Experiments

Details of individual experiments are shown in table 1. In experiments 1, 2 and 4, infectivity was assayed by exposing half the mice in each experimental group to mosquitoes on day 12 p.i., and the other half on day 14 p.i. Preliminary experiments and other data (Taylor *et al.* 1997) showed that infectivity peaks during this period.

#### (f) Statistical analysis

Comparisons of control and CQ-treated infections were made using univariate analyses of the following summary measures for each infection:

(1) *Total asexuals*. This was estimated from parasite densities between days 8 and 16 p.i. Parasite densities prior to day 8 were not used because it was unclear whether parasites in the CQ-treated groups were dead or alive at the time of the smear, and by day 18 p.i. parasite densities were at very low levels. Estimates of total parasites between these days were obtained by integrating under the parasite density through time curves for each infection. This is a reasonably accurate

measure of total numbers of asexuals (between days 8 and 16 p.i.) because the asexual cycle of *P. chabaudi* is known to be 24 h (reviewed in Cox 1988).

(2) *Total gametocytes*. Gametocytes of *P. chabaudi* take approximately 2 d to mature (A. G. J. Buckling *et al.*, unpublished data; Gautret *et al.* 1997). An index of the total number of gametocytes produced by asexuals between eight and 16 days p.i. was therefore obtained by integrating under the gametocyte density through time curves between days 10 and 18 p.i. This measure is not a direct estimate of total gametocyte numbers because gametocyte longevity is uncertain, though the data presented below suggest few survive longer than 24 h. However, the sum of the gametocyte densities on each day is likely to be well correlated with total transmission probability: gametocyte densities at any point in time correlate with both the proportion of mosquitoes infected and oocyst densities within mosquitoes (Taylor & Read 1997).

(3) *Index of gametocytogenesis (IG)*. This was calculated as the ratio of total gametocytes (2) to total parasites [(1) + (2)] for each mouse.

(4) *Day of peak gametocyte density*.

(5) *The proportion of mosquitoes infected*.

Prior to analysis, estimates of total asexuals and total gametocytes were  $\log_{10}$ -transformed and indices of gametocytogenesis (IG) square-root arcsin-transformed. All analyses were carried out using generalized linear models (GLIM; Crawley 1993). Starting with the highest order interactions, all factors (experiment, treatment (CQ+, CQ-) and, where relevant, day of CQ treatment or day of mosquito feed) and their interactions were individually removed in turn from the

maximal model. Non-significant factors were removed and test statistics obtained from the resulting minimal model. A binomial error structure, applying Williams's correction for overdispersion, was used for the logistic regression of infection probability (Crawley 1993).

### 3. RESULTS

Total asexuals and total gametocytes did not differ between experiments ( $F_{3,52} = 0.67$ ,  $p = 0.58$ ;  $F_{3,52} = 0.44$ ,  $p = 0.73$ , respectively). CQ treatment, however, had a significant effect, reducing asexuals to 20% of controls and gametocytes to 50% (figure 1,  $F_{1,52} = 114.40$ ,  $p < 0.0001$ ; figure 2,  $F_{1,52} = 7.10$ ,  $p = 0.01$ , respectively). The effect of CQ did not differ between experiments (treatment by experiment interactions:  $F_{3,49} = 1.26$ ,  $p = 0.3$ ;  $F_{3,49} = 1.87$ ,  $p = 0.15$ , asexuals and gametocytes, respectively).

The index of gametocytogenesis (IG) was about 2.5 times greater in CQ-treated infections than in control infections ( $F_{1,52} = 16.00$ ,  $p < 0.0001$ ). There were no differences between experiments, nor did treatment effects differ between experiments ( $F_{3,52} = 0.76$ ,  $p = 0.52$ ;  $F_{3,49} = 1.54$ ,  $p = 0.22$ , respectively). Figure 3 shows the relationship between total gametocytes and total asexuals for all infections.

Peak gametocyte densities occurred approximately 2 d earlier in CQ-treated infections than in control

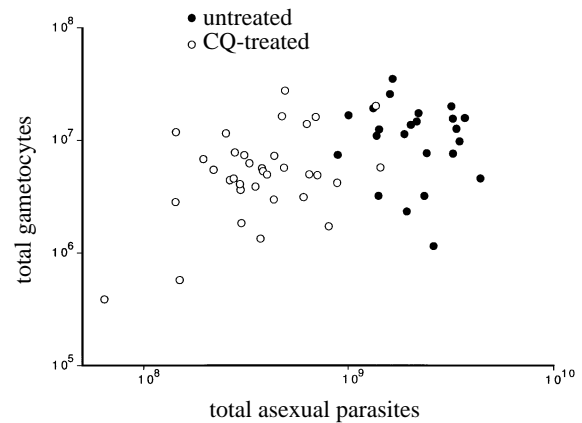


Figure 3. Total asexuals and total gametocytes (between days 8 and 16, and 10 and 18 p.i., respectively) for chloroquine-treated and untreated infections in experiments 1–4. Mean ( $\pm$ s.e) index of gametocytogenesis (IG): CQ-treated =  $0.0180 \pm 0.0026$ ; control =  $0.0066 \pm 0.0012$ .

infections for the experiments using CR (experiments 1–3:  $F_{1,39} = 54.86$ ,  $p < 0.0001$ ; figures 2*a–c*) and 3 d earlier in the experiment using ER (experiment 4:  $F_{1,10} = 19.89$ ,  $p = 0.001$ ; figure 2*d*). For experiments 1–3, the effect of treatment on timing of peak gametocyte densities did not differ between experiments (treatment by experiment interaction:  $F_{2,39} =$

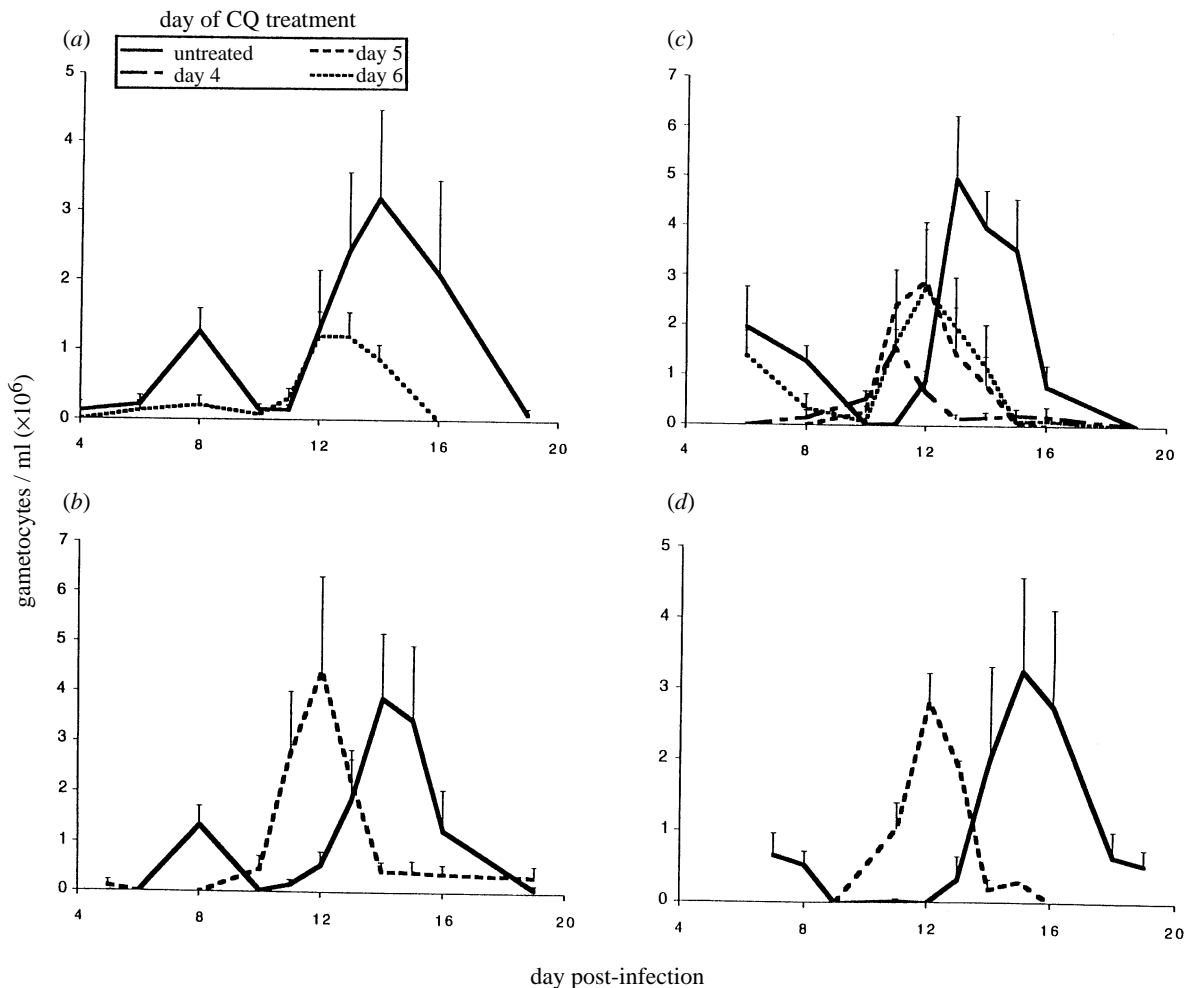


Figure 2. Mean gametocyte densities ( $\pm$ 1 s.e.) during infections for experiments 1–4 (*a–d* respectively). CQ, chloroquine.

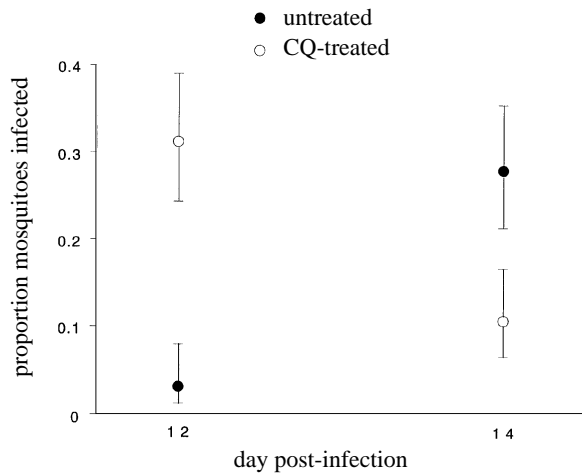


Figure 4. Mean proportions of mosquitoes infected ( $\pm 1$  s.e.) for chloroquine-treated and untreated mice on days 12 and 14 post-infection.

0.15,  $p = 0.86$ ), but the timing of peak gametocyte densities differed between experiments ( $F_{2,39} = 4.46$ ,  $p = 0.02$ ). This was probably due to differences in timing of CQ treatment between experiments; there was no significant difference between experiments when CQ-treated groups were separated on the basis of specific day of treatment ( $p > 0.25$  for controls and days 5 and 6 p.i. CQ treatment). Interestingly, there was a significant positive relationship between the day of CQ treatment and the day of peak gametocyte density for days 4, 5 and 6 p.i. CQ-treated groups in experiments 1–3 (ordered heterogeneity test (Rice & Gaines 1994):  $r_s P_c = 0.96$ ,  $p < 0.01$ ).

There was no difference in total gametocytes between days 4, 5 and 6 p.i. CQ-treated groups in experiment 3 ( $F_{2,13} = 0.20$ ,  $p = 0.83$ ). IG, however, significantly differed between the groups ( $F_{2,13} = 4.38$ ,  $p = 0.035$ ); pairwise comparisons revealed the IG to be greater in day 6 p.i. CQ-treated infections than in those treated on day 4 p.i., but there were no other significant differences (Scheffe analysis  $p = 0.04$ ,  $p > 0.2$  for both other comparisons).

Proportions of mosquitoes infected were greater in CQ-treated mice than control mice on day 12 p.i. and vice versa on day 14 p.i. (figure 4; day by treatment interaction:  $\chi^2_1 = 15.74$ ,  $p < 0.001$ ). There were no significant differences in overall proportion of mosquitoes infected between experiments, day and treatment, and neither treatment nor day effects, and their interaction, differed between experiments ( $p > 0.2$  in all cases).

#### 4. DISCUSSION

Infections treated with CQ produced significantly fewer asexuals and gametocytes than untreated infections, but for a given number of asexuals, the number of gametocytes was about 2.5 times greater in CQ-treated infections. This is most likely the result of increased gametocytogenesis following subcurative CQ treatment, consistent with our hypothesis.

Changes in the relative mortality rates of gameto-

cytes and asexuals cannot adequately explain the results. First, stage-specific mortality caused by CQ cannot be involved. The half-life of whole blood CQ-concentration in mice heavily infected with *P. chabaudi* (21–25% parasitized RBCs) is in the order of 7 h (Cambie *et al.* 1994). Parasite numbers were first assayed at least 2 d after treatment, when the CQ level must have been less than  $0.5 \text{ mg kg}^{-1}$ ; preliminary experiments revealed twice this concentration to have no noticeable effect on parasite numbers or infection dynamics. Moreover, virtually all the gametocytes counted must have been produced from post-treatment asexuals: any long-lived gametocytes present during the period of CQ activity would have made up only a tiny fraction of the gametocytes used to estimate subsequent gametocytogenesis because they were at such low densities when infections were drug-treated (figure 2). Second, differential mortality resulting from differences in immune response between the groups not only requires a gametocyte-specific clearance mechanism, for which there is currently no evidence (Taylor & Read 1997), but also one that is suppressed by CQ.

The best explanation for the relative increase in numbers of gametocytes following CQ treatment is therefore increased gametocytogenesis, as predicted by both the safe-harbour and the fecundity compensation hypotheses. Nevertheless, the delay between CQ treatment (hence peak asexual density) and gametocyte production (figures 1 and 2) is not entirely consistent with these adaptive hypotheses. Increased gametocytogenesis in response to stress might be expected to be immediate, resulting in mature gametocytes 2–3 days later. Instead, we observed a delay of about a week. However, it is striking that in control infections there is also a delay of approximately a week between peak asexual density and peak gametocyte density. An intriguing possibility, consistent with the hypotheses, is that a sudden drop in parasite numbers might be acting as the cue for delayed gametocytogenesis in both CQ-treated and control mice. In untreated infections, such a strategy would have the consequence of minimizing production of gametocytes during crisis, an immune-mediated response that down-regulates parasite numbers (Jarra & Brown 1989) and during which gametocyte infectivity is greatly suppressed (Wery 1968). It is feasible that CQ treatment results in an artificially early occurrence of the same cue that stimulates this crisis-avoiding delay.

Gametocytogenesis frequently follows a substantial reduction in overall parasite numbers (Sinden 1983; Carter & Graves 1988; Alano & Carter 1990; Sinden *et al.* 1996; figures 1 and 2), suggesting a positive relationship between gametocytogenesis and factors that correlate with parasite destruction. Such factors might explain how gametocytogenesis was triggered in control and CQ-treated infections despite radically different parasite dynamics (figures 1 and 2) and host condition in the two groups. Increased relative gametocytogenesis following CQ treatment might be explained by the resulting short-term very high rate of parasite destruction. This relationship would also explain why gametocytogenesis was greater in mice treated with CQ on day 6 p.i. compared with day 4 p.i.

The later treatment inevitably resulted in greater parasite death, both because by that stage there were more for CQ to kill (figure 1c), and because of enhanced immune activity as a result of greater exposure, in terms of both time and numbers, to parasite antigens.

Whatever the mechanism involved, these results clearly demonstrate increased gametocytogenesis following subcurative CQ treatment. As far as we are aware, this is the first fully controlled demonstration that subcurative treatment with an antimalarial drug can increase gametocytogenesis in *Plasmodium*, and thus alter patterns of infectiousness. The dose of CQ used in this study is equivalent to half the commonly recommended dose for treatment of people with *P. falciparum* (25 mg kg<sup>-1</sup> over 3 d; Desjardins *et al.* 1988). Because treatment often terminates following clinical improvement, and drug-resistant parasites are common, subcurative treatment frequently occurs (Wernsdorfer 1994). The results are therefore potentially relevant to the treatment of *Plasmodium* with CQ in the field. First, even large reductions in the numbers of asexual parasites may have a much less dramatic effect on infectivity because of compensatory investment into gametocytes. It is notable that in our transmission experiments, the proportion of mosquitoes infected was similar from treated and untreated infections (figure 4). Second, transmission between hosts might actually be faster where subcurative treatment is common, because of the earlier timing of peak infectivity (figure 4). Transmission may be further enhanced by the general reduction in host immunity because of decreased exposure to the parasite (Graves *et al.* 1988) and by the increased infectiousness of gametocytes apparently induced by very low level CQ treatment (Ramkaran & Peters 1969; Wilkinson *et al.* 1976; Ichimori *et al.* 1990). Finally, if the effect is a generalized stress response, it might be observed following treatment with most blood-stage anti-malarials and even potential vaccines.

Here we have only considered phenotypic modifications of gametocytogenesis. Consistent differences in gametocyte production between isolates of *P. falciparum* *in vitro* (Burkot *et al.* 1984; Graves *et al.* 1984) imply natural genetic variation in gametocytogenesis on which drug-imposed selection could act to generate long-term changes in resource allocation between asexuals and gametocytes. Parasites will probably increase their fitness under drug pressure by producing gametocytes in greater quantities earlier in the infections. Lines *et al.* (1991) suggested that just such adaptation may underlie increases in malaria transmission in Tanzania following long-term CQ use. This is supported by a recent study in Sri Lanka that showed that infections of CQ-resistant parasites are more likely to be gametocyte-positive than infections of sensitive parasites (Handunnetti *et al.* 1996); under drug pressure, adaptive life history changes are likely to evolve in parallel with the development of drug resistance. Both short- and long-term parasite life history changes in response to intervention strategies are to be similarly expected in many disease-causing organisms.

We thank Alan Gemmill, Margaret Mackinnon, Ben Sheldon, David Walliker and three anonymous referees for useful comments. The work was funded by the BBSRC and the mosquito facilities by the MRC. A.B. and L.T. were supported by MRC studentships, J.C. by an MRC grant to D. Walliker, and A.R. by a BBSRC Advanced Research Fellowship.

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Received 4 November 1996; accepted 11 December 1996