CHANGES IN *PLASMODIUM FALCIPARUM* GAMETOCYTAEMIA IN CHILDREN WITH CHLOROQUINE-SENSITIVE ASEXUAL INFECTIONS

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Summary:

A non-compartmental pharmacokinetic model was used to describe the changes in gametocytaemia in nine children with chloroquine-sensitive *Plasmodium falciparum* malaria in whom asexual parasitaemia cleared within 72 h of chloroquine treatment. Peak gametocytaemia was 74 ± 19.9 (se), range 24-198, geometric mean 58 sf (sexual forms)/µl. Time to peak gametocytaemia was 43.2 ± 14.4, range 0-120 h. Following peak gametocytaemia, gametocytes persisted in blood for a period of 168-504 h. The decline from peak gametocytaemia was exponential with a half-life of gametocytaemia of 43.2 ± 20.4, range 13.1–206 h. The mean pre-treatment sex ratio was male-biased and remained so till complete elimination of gametocytaemia. Peak microgametocytaemia, area under the curve of microgametocytaemia versus time, and the half-life of microgametocytaemia were significantly higher than those of macrogametocytaemia. The volume of blood completely cleared of macrogametocytaemia per unit time was significantly higher than that of microgametocytaemia. Macrogametocytes are cleared from the circulation faster than microgametocytes but chloroquine treatment of chloroquine-sensitive infections has little or no significant effect on gametocyte sex ratios in this group of children.

KEY WORDS : *P. falciparum*, gametocyte, sex ratio, chloroquine, children, Nigeria.

Résumé : MODIFICATION DE LA GAMÉTOCYTÉMIE CHEZ DES ENFANTS INFECTÉS PAR UNE SOUCHE CHLOROQUINO-SENSIBLE DE *PLASMODIUM FALCIPARUM*

Un modèle pharmacocinétique non compartementalisé a été employé pour décrire les modifications de la gamétocytémie chez neuf enfants infectés par une souche de Plasmodium falciparum *et chez qui les formes asexuées ont disparu de la circulation avant 72 h au cours d'un traitement à la chloroquine. Un pic de gamétocytes apparaît après une durée moyenne de 43.2 ± 14.4 h (valeurs extrêmes 0-120 h). La valeur moyenne du nombre de gamétocytes est 74 ± 19.9 (valeurs extrêmes 24 et 198), et le mode de 58 formes sexuées par µl. Après ce pic, les gamétocytes persistent dans la circulation pendant 168 à 504 h. La décroissance de la gamétocytémie est exponentielle avec une demi-vie de 43.2 ± 20.4 h (valeurs extrêmes 13.1 et 206 h). La moyenne des rapports microgamètes (mâles)/macrogamètes (femelles) avant traitement est biaisée en faveur des microgamètes. Le pic de microgamètes (mâles), l'aire sous la courbe (f (t)) de la microgamétocytémie, et la demi-vie de cette dernière sont significativement plus hauts que ceux des macrogamètes (femelles). Le volume de sang débarassé des macrogamètes par unité de temps est significativement plus grand que celui des microgamètes. Les macrogamètes sont plus vite éliminés de la circulation que les microgamètes, et le traitement par la chloroquine de ces souches CQ-sensibles a peu d'effet sur le rapport macrogamète/microgamète*

MOTS CLÉS : P. falciparum, *gamétocyte, sex ratio, chloroquine, enfant, Nigeria.*

INTRODUCTION

Infections in *Plasmodium* spp. can only be transmitted from the vertebrate host to the mosquito through the male and female gametocytes that developed from committed asexual parasites in the vertebrate host obtained from the blood meal by the mosquito. In the mosquito, the female *Plasmodium falciparum* gametocyte (macrogametocyte) release one gamete, whereas the male gametocyte (microgametocyte) may release up to eight gametes (Sinden *et al.*, 1978). Exflagellation of the microgamete and fertilization of the macrogamete by the microgamete form the zygote that later develops to the sporozoite. The sporozoite is eventually injected through the mosquito bite to the vertebrate host when the female mosquito obtains a blood meal.

Immature but not mature *P. falciparum* gametocytes which develop from proliferating asexual parasites in man can be eradicated by a number of antimalarial drugs including chloroquine (CQ) and pyrimethamine sulphadoxine (PS) (Sinden, 1982, 1983). If CQ promptly and completely clears asexual parasites following treatment of CQ-sensitive (CQ-S) *P. falciparum* infections, then further recruitment of gametocyte is completely inhibited and the changes in the mature gametocytes in circulation can be conveniently followed (Smalley & Sinden, 1977).

In West Africa, children are the most susceptible to falciparum malaria. In these children gametocyte rate

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prior to drug treatment may be up to 17 % (von Seidlein *et al.*, 2001) or even higher (Robert *et al.*, 2000). In much of West Africa, CQ still remains the drug of first choice but despite the sex ratio being crucial to transmission success by the mosquito (Robert *et al.*, 1996; Paul *et al.*, 2002), there is little or no recent information on gametocyte sex ratios or their changes following CQ treatment in this at risk group.

The aims of the present study were: to evaluate the changes in *P. falciparum* gametocytaemia during treatment of CQ-S infections in children, and to determine if there are differences in the changes involving macro- and micro-gametocytes during treatment with CQ.

PATIENTS AND METHODS

Patients

The study took place at the University College Hospital in Ibadan, a hyperendemic area for malaria in southwestern Nigeria (Salako *et al.*, 1990), between August 2000 and December 2002 and was approved by the local ethics committee.

The details of the clinical component of the study are as previously described (Sowunmi, 2002, 2003; Sowunmi & Fateye, 2003a). Briefly, children with symptoms compatible with acute falciparum malaria who fulfilled the following criteria were enlisted in the study: age below 120 months, pure *P. falciparum* parasitaemia greater than 2,000 asexual forms/µl blood, gametocytaemia ≥ 12 sexual forms/µl blood negative urine tests for antimalarial drugs (Dill-Glazko and lignin tests), absence of concomitant illness, no evidence of severe malaria and written informed consent given by parents or guardians. An important additional criterion was: following CQ treatment clearance of parasitaemia within 72 h and with no recurrence within 672 h of follow-up.

After detailed clinical and parasitological assessment, CQ was given orally at a dose of 30 mg/kg of the base over 3 d, that is, 10 mg/kg at presentation (day 0, 0 h) and daily for additional two days. The patients were observed for three hours to ensure that the drug was not vomited. Follow-up with clinical and parasitological evaluation was at 24, 48, 72, 96, 120, 144, 168, 336, 504 and 672 h. Clinical evaluation consisted of a general clinical examination including measurement of weight, core temperature and physical examination. Thick and thin blood films prepared from a finger prick were Giemsa-stained and quantification of parasitaemia was done as previously described (Sowunmi, 2002, 2003; Sowunmi & Fateye, 2003a). Giemsa-stained blood films were examined by light microscopy under an oil-immersion objective, at × 1,000 magnification, by two independent assessors. Parasitaemia in thick films was estimated by counting asexual parasites relative to 1,000 leukocytes, or 500 asexual forms, whichever occurred first. From this figure, the parasite density was calculated assuming a leukocyte count of 6,000/µl of blood.

Parasite deoxyribonucleic acid (DNA) was extracted by methanol fixation and heat extraction (Plowe *et al.*, 1995) from blood samples collected on to filter paper at 0, 24, 48, 72, 96, 120, 144, 168, 336, 504 and 672 h and the merozoite-surface-protein (MSP1 and MSP2) the K1, MAD20, and RO33 allelic families of MSP1, the ICI and FC27 allelic families of MSP2, and region II of glutamine-rich-protein (GLURP) were analysed, using the primer sequence and PCR conditions described by Snounou & Beck (1999). The regions of the CQ resistance transporter (*pfcrt*) and the parasite's multidrug resistant 1 (*pfmdr1*) genes surrounding the polymorphisms of interest in pre- and post-treatment samples were amplified by nested polymerase chain reaction (PCR) (Djimde *et al.*, 2001) and analysed by RFLP, so that the *pfcrt* (GenBank accession number AF030694) K76T and *pfmdr1* (GenBank accession number X56851) N86Y and D1246Y mutations could be detected, if present, as previously described (Happi *et al.*, 2003).

Quantification of gametocytaemia and determination of gametocyte sex

Gametocytaemia was quantified as previously described (Sowunmi, 2002, 2003; Sowunmi & Fateye, 2003a) at 0, 24, 48, 72, 96, 120, 144, 168, 336, 504 and 672 h. Gametocytes were counted in thick blood films against 1,000 leukocytes assuming an average leukocyte count of 6,000/µl of blood. Gametocytes were sexed if gametocytaemia was ≥ 12 sexual forms/µl. Gametocyte sex determination was based on following criteria (Carter & Graves, 1988; Robert *et al.*, 1996): males are smaller than females; the nucleus is bigger in males than females; the ends of the cells are round in males and angular in females; the cytoplasm stains pale purple in males and deep blue in females; and the granules of malaria pigment are centrally located in females and more widely scattered in males.

Gametocyte sex ratio was defined as the proportion of gametocytes in peripheral blood that were microgametocytes (Pickering *et al.*, 2000).

Kinetics of micro-and macrogametocytaemia

Gametocyte kinetic parameters were estimated from gametocyte and gametocyte sex densities (gametocyte and gametocyte sex concentrations or gametocytaemias) by a non-compartmental method using the computer programme *Turbo Ken* (Clinical Pharmacology Group, University of Southampton, UK, through the courtesy of Professor A.G. Renwick). The following

gametocyte kinetic parameters were calculated from the curve of gametocytaemia by using the real times of sampling from each patient. Areas under the curves of gametocytaemia versus time until the last detectable gametocyte concentration (C_{tgm}), (AUC_{gmlast}), were calculated using the trapezoidal method. Area under the gametocytaemia-time from zero to infinity ($AUC_{gm0-\infty}$) was calculated by adding to AUC_{gmlast} the extrapolated AUC_{gm} calculated as C_{tgm}/k_{el}, the elimination rate constant derived from the semilogarithmic plot of gametocytaemia versus time (visual inspection of the final part of the gametoctaemia – time curve was used to identify the elimination phase). C_{maxgm} (maximum gametocytaemia) and t_{maxgm} (time to C_{maxgm}) were noted directly from the data. Terminal elimination half-life, $t_{1/2\beta}$, was calculated as $0.693/k_{el}$. The volume of blood completely cleared of gametocytes (CLB_{gm}) was calculated as the gametocytaemia at enrolment/AUC_{gm}.

STATISTICAL ANALYSIS

Data were analysed using version 6 of the Epi-Info software (Anon., 1994). Normally distributed, continuous data were compared by Student's t-tests and analysis of variance (ANOVA). Data not conforming to a normal distribution were compared by the Mann-Whitney U-tests and the Kruskal-Wallis tests (or by Wilcoxon ranked sum test). All tests of significance were two-tailed. P-values of < 0.05 were taken to indicate significant differences. The values presented below are generally means and standard deviations (sd) or standard error (se).

RESULTS

A total of 96 children with acute symptomatic uncomplicated falciparum malaria was initially evaluated. Gametocytes were seen in peripheral blood in 15 children at presentation but in only 10 children did parasitaemia clear within 72 h of commencing CQ treatment. One of the 10 children was excluded because of uncontrolled additional antimalarial intake during follow-up. The demographic and other characteristics at enrolment and the responses to CQ treatment of the nine patients who completed the study are shown in Table I. Parasite DNA was demonstrable at 0-48 h only confirming clearance of asexual parasitaemia within 72 h of commencing CQ treatment. The parasites from all nine children did not carry mutant alleles of *pfcrt or pfmdr1*.

A total of 78 gametocytes was counted on day 0 (0 h) and 34, 64, 106, 74, 74, 55, 53, 20, and 10, gametocytes were counted at 24, 48, 72, 96, 120, 144, 168, 336 and 504 h, respectively. Of these, gametocytes could be sexed in 70, 27, 56, 99, 79, 79, 58, 58, 22, 10 and 4 at 0, 24, 48, 72, 96, 120, 144, 168, 336, 504 and 672 h, respectively. Gametocytes were found in one patient at 672 h.

Age (years)	
mean ± sd	5.9 ± 2.9
range	2.4-9.5
Weight (kg)	
mean ± sd	17.1 ± 7.1
range	7-28.0
Duration of symptom (d)	
mean ± sd	3.8 ± 2.3
range	1-8
Axillary temperature (° C)	
mean ± sd	37.6 ± 1.2
range	36.3-39.8
Parasite density (/µl)	
geometric mean	15,669
range	4,860-87,600
Fever clearance time (d)	
mean ± sd	1 ± 0 (n = 7)
range	1-1
Parasite clearance time (h)	
mean ± sd	55.2 ± 12.0
range	48-72

Table I. - Summary of clinical and parasitological characteristics at enrolment and responses to treatment.

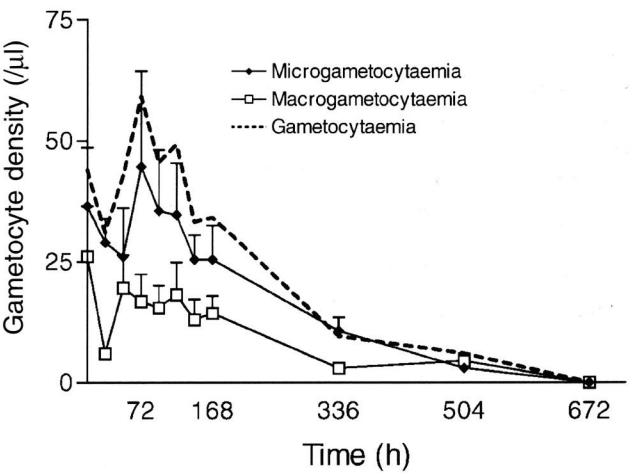

Fig. 1. – Changes in total gametocytaemia, microgametocytaemia and macrogametocytaemia in children with chloroquine-sensitive *Plasmodium falciparum* infections. Bars represent standard error of mean.

The temporal changes in gametocytaemia are shown in Figure 1. The individual model output data are shown in Table II. Following peak gametocytaemia, gametocytes persisted in blood for 72-504 h with a terminal elimination half-life of 43.2 ± 20.4, range 13.1-206 h. Figure 2 shows the prevalence and the temporal changes in sex ratios during follow-up. Pre-treatment sex ratio was female-biased in three and male-biased in six children. Overall, the pre-treatment mean sex ratio was

No	Age (y)/Sex	C_{maxap} (/µl)	C_{maxgm} (/µl)	T_{maxgm} (h)	$t1/2_{gm}$ (h)	AUC_{gm} (sf/µl.h)	CLB_{gm} (µl/kg/h)
151/00	4.1 M	4,860	72	120	22.2	9,400.0	0.00012
152/00	9.0 M	10,890	36	0	13.1	4,438.5	0.00048
175/00	9.5 M	15,630	198	72	26.8	24,931.5	0.00013
200/00	9.5 M	9,390	48	72	206.0	13,352.4	0.00011
35/01	2.4 M	16,034	48	72	21.6	8,794.4	0.00019
130/01	7.0 M	24,857	24	0	23.8	4,450.9	0.00024
39/02	2.7 F	97,684	30	48	26.8	5,274.7	0.00029
45/02	4.7 M	1,116	60	0	27.0	12,816.1	0.00026
76/02	4.2 M	36,267	150	0	21.9	17,608.1	0.00065
Mean ± se	5.9 ± 2.9	2,4081 ± 9,853	74 ± 19.9	43.2 ± 14.4	43.2 ± 20.4	11,267 ± 2,262	0.0003 ± 0.00006
95 % CI	3.7-8.1	1,361-48,601	28-120	9.6-76.8	– 3.7-90.3	6,051-16,843	0.00013-0.00041

C_{maxap}: peak asexual parasitaemia (occurred in all patients at enrolment); C_{maxgm}: peak gametocyte density; T_{maxgm}: time to peak gametocyte density; $t1/2_{gm}$: apparent half-life of gametocytaemia; AUC_{gm}: area under the curve of gametocytaemia versus time; CLB_{gm}: volume of blood completely cleared of gametocytes per unit time; sf: sexual forms: y: year.

Table II. – Output data of a non-compartment model of the changes in gametocytaemia in children enrolled in the study.

male-biased and it remained so throughout the duration of the study (Fig. 2). In the three children with pre-treatment female-biased sex ratio, there was a change to male-biased sex ratio by day 7 and these remained so till day 14. In these three children, a sex ratio of 0.5 was attained on days 2, 4 and 6. In the remaining six children, the pre-treatment male-biased sex ratio remained unchanged till days 7 and 14. The model output data for macro- and micro-gametocytaemias are shown in Table III. Both peak macro- and micro-gametocytaemia occurred between 0-120 h, but compared with macrogametocytaemia, C_{maxgm}, $t1/2_{gm}$ and AUC_{gm} were significantly higher for microgametocytaemia and clearance (CLB_{gm}) was significantly slower.

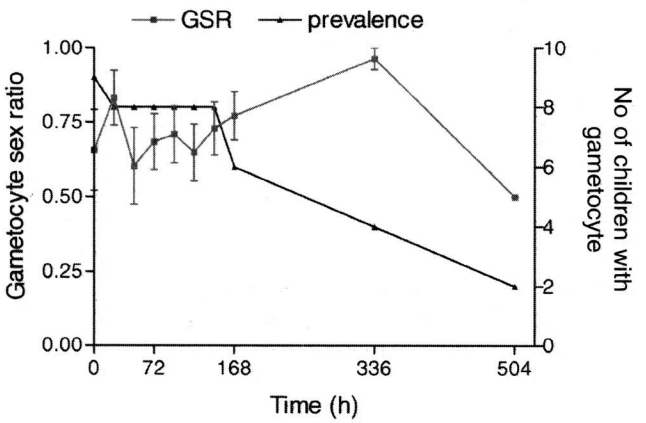

Fig. 2. – Prevalence of gametocytaemia and changes in gametocyte sex ratios (gsr) in children with chloroquine-sensitive *Plasmodium falciparum* infections. Bars represent standard error of mean.

DISCUSSION

Given the results of the molecular analyses and the treatment outcome, it seems certain that the asexual parasites and the gametocytes that arose from them were from CQ-S infections and there was no recurrence of sub-patent parasitaemia during the entire duration of the study. The molecular analyses we did became necessary for two reasons: children with clinical cure of their infections by chloroquine can harbour and transmit gametocytes arising from parasites carrying resistance genes (Sutherland *et al.*, 2002a); sub-patent infections can continue to generate more gametocytes resulting in multiple gametocyte genotypes post treatment (Sutherland *et al.*, 2002b).

Although a cohort of asexual parasites destined to become gametocytes will be initiated synchronously at schizogony, it is unlikely that all cohorts of parasites present in a particular patient will be initiated at the same time. This is more so since different stages of asexual parasite development can be readily encountered and recognized in the blood films obtained from patients in this endemic area (Sowunmi & Oduola, 1998). Thus, recruitment of a particular cohort of gametocytes would be synchronous but in most natural infections, maturation of all cohorts should be continuous and therefore asynchronous. By enrolling patients in whom asexual parasitaemia cleared within 72 h of initiating CQ treatment, we tried to ensure that no further recruitment of gametocytes occurred during follow-up of the patients. Thus, only the maturing and mature gametocytes were estimated, and these would, with time, die without replacement (in the absence of asexual parasitaemia during the 28 d period of follow-up). This method allows estimation of gametocyte half life (Smalley & Sinden, 1977; Reece *et al.*, 2003) since

	Microgametocytaemia	Macrogametocytaemia	P. value⁺
C_{maxgm} (/ul)			
GMGD	48	22	0.008
range	14-158	6-70	
interquartile range	30-84	12-41	
T_{maxgm} (h)			
mean ± sd	2.3 ± 0.7	1.6 ± 0.5	0.52
range	0-6	0-4	
95 % CI	0.6-4.0	0.6-2.8	
AUC (sf/µl.h)			
mean ± se	8,347 ± 1,828	2,987 ± 527	0.005
range	2,435-19,102	448.5-5,564	
95 % CI	4,132-12,563	1,771-4,202	
$t_{1/2}$ (h)			
mean ± se	41.8 ± 18.7	17.4 ± 6.1	< 0.0001*φ
range	13.1-191.2	3.4-65.0	
95 % CI	– 1.4-84.9	3.4-31.4	
CLB_{gm} (µl/kg/h)			
mean ± se	0.00025 ± 0.00006	0.00045 ± 0.00008	0.019
range	0.00002-0.0006	0.00002-0.00076	
95 % CI	0.0001-0.0004	0.00025-0.00064	

GMGD: geometric mean gametocyte density, ⁺paired t-test, *by paired t-test excluding one pair of outliers- 191.2 and 65.0 h for micro- and macrogametocytaemia, respectively, and by φ Wilcoxon ranked sum test without excluding outliers (P = 0.004).
C_{maxgm}: peak gametocyte density; T_{maxgm}: time to peak gametocyte density; AUC_{gm}: area under the curve of gametocytaemia versus time; $T1/2_{gm}$: apparent half-life of gametocytaemia; CLB_{gm}: volume of blood completely cleared of gametocytes per unit time; sf: sexual forms.

Table III. – Comparison of output data of a non-compartment model of the changes in macro- and microgametocytaemia.

CQ eradicates both the asexual parasites and immature gametocytes in CQ-S infections (Sinden, 1982, 1983). However, the method and the non-compartment model for estimating the output, cannot measure gametocyte sequestration time, which a recent modelling data suggest could be as short as four days *in vivo* in patients not previously exposed to malaria (Eichner *et al.*, 2001). Following treatment, peak gametocytaemia occurred on day 0 in approximately half of the patients, and the remainder between 2-5 d and gametocytes persisted for a period of 3-21 d with an average half life of 1.8 d. These observations are in keeping with those of earlier and more recent studies in West African children (Smalley & Sinden, 1977; Sowunmi & Fateye, 2003a), but the average terminal elimination half-life of gametocytaemia in the present study appears to be slightly lower than 2.4 d reported by Smalley & Sinden (1977). The mean sex ratio at enrolment was male-biased and in contrast to that seen in natural populations (West *et al.*, 2001, 2002). There are possible reasons for this:
1 - The initial sex ratio could have been female-biased and the male-biased ratio could have been due to the changes accompanying the progression of infection including the anaemia of infection (Paul *et al.*, 2000).
2 - Gametocytaemia is often lower in CQ-S than CQ-R infections (Robert *et al.*, 2000; Sowunmi & Fateye, 2003a) and multiple infections are not uncommon. In a recent study, Happi *et al.* (2003) found the average multiple infections with *P. falciparum* per child from this endemic area was over four. Since low gametocytaemias and multiple infections are associated with less female-biased sex ratio (West *et al.*, 2002), these may, in general, explain the relatively more male-biased sex ratio seen in natural infections in the area. In addition, malaria anaemia is also common and may further contribute to the maleness.
3 - Although considered relatively non-immune (five of the nine children were aged less than five years), immunity could still have influenced the observed sex ratio.
4 - There could have been an early release and subsequent early clearance of macrogametocyes from the circulation before presentation by the children for treatment. Finally, macrogametocytes may have been selectively sequestered in the internal organs because of their relatively large size, before presentation by the children.
Does CQ significantly alter sex ratio during the course of treatment? Or contribute to the observed sex ratio in the cohort of children evaluated? In contrast to our previous finding in children treated with pyrimethamine-sulhadoxine (PS) (Sowunmi & Fateye, 2003b), the sex ratios in the majority of the children remained relatively unchanged during and after CQ treatment. A similar finding has been documented in a group of Gambian children with female-biased sex ratio treated with CQ (Smalley & Sinden, 1977). However, it is possible that CQ may have influenced the change from female to male-biased sex ratio seen in the minority of the children since CQ stimulates erythropoietin

secretion (el Hassan *et al.*, 1997); but it is also possible that the change was due to the course of infection (Paul *et al.*, 2000).

Gametocytes sex ratios and their disposition have been evaluated before in a limited number of African children (Smalley & Sinden, 1977). However, details of the kinetics of the disposition of sex gametocytes in children have not been provided before now. The finding that macrogametocytes are lost from the circulation faster than microgametocytes was not unexpected. In synchronized *P falciparum* gametocyte culture *in vitro*, microgametocytes are longer lived (Ponnudurai *et al.*, 1986). Similarly in *P. chabaudi* infection in black mice, microgametocytes are also longer-lived (Reece *et al.*, 2003). We have also found (Sowunmi & Fateye, 2003b) that following PS treatment of *P. falciparum* infections in children, microgametocytaemia was significantly higher than macrogametocytaemia on days 7 and 14, and microgametocytes are longer lived.

There are possible reasons for the differential clearance of macrogametocytes in our cohort of children: although most of the children are relatively non-immune, immune killing rate may differ between gametocyte sex, as is clearance by the spleen; sex differences in capillary sequestration may occur; finally, a combination of these or other factors may operate.

It is not clear whether in chloroquine-resistant (CQ-R) infections there would be alteration in sex ratio or their disposition following CQ treatment, but studies are under way to address this issue. However, gametocyte half-life appears to be longer in CQ-R than in CQ-S infections in children from this endemic area (Sowunmi & Fateye, 2003a).

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