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INFLUENCE OF CHEMOTHERAPY ON THE *PLASMODIUM* GAMETOCYTE SEX RATIO OF MICE AND HUMANS

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Abstract. *Plasmodium* species, the etiologic agents of malaria, are obligatory sexual organisms. Gametocytes, the precursors of gametes, are responsible for parasite transmission from human to mosquito. The sex ratio of gametocytes has been shown to have consequences for the success of this shift from vertebrate host to insect vector. We attempted to document the effect of chemotherapy on the sex ratio of two different *Plasmodium* species: *Plasmodium falciparum* in children from endemic area with uncomplicated malaria treated with chloroquine (CQ) or sulfadoxine-pyrimethamine (SP), and *P. vinckei petteri* in mice treated with CQ or untreated. The studies involved 53 patients without gametocytes at day 0 (13 CQ and 40 SP) followed for 14 days, and 15 mice (10 CQ and 5 controls) followed for five days. During the course of infection, a positive correlation was observed between the time of the length of infection and the proportion of male gametocytes in both *Plasmodium* species. No effects of treatment (CQ versus SP for *P. falciparum* or CQ versus controls for *P. vinckei petteri*) on the gametocyte sex ratio were found for either *Plasmodium* species. This indicates that parasites do not respond to chemotherapy by altering their sex allocation strategy, even though, in the case of *P. falciparum*, they apparently increase their overall investment in sexual stages. This suggests that malaria parasite species respond to different environmental cues for their sex differentiation and sex determination.

INTRODUCTION

Plasmodium species, the etiologic agents of malaria, are obligatory sexual organisms. Transmission of malaria parasites from the vertebrate host to the mosquito host is accomplished solely by gametocytes. Male and female gametocytes develop into gametes in the mosquito midgut, and progress through their sporogonic development following fertilization. *Plasmodium* are hermaphrodites, whereby a single asexual parasite can develop, after several rounds of multiplication, into both male and female gametocytes.[1] Commitment to the sexual pathway is thought to occur prior to the formation of the schizont such that all descendants of a committed schizont will develop into gametocytes.[2] Moreover, it has been recently shown that all gametocytes produced from one sexually committed schizont are of the same sex,[3,4] suggesting that not only may sex be determined at the very beginning of sexual development, but also that exogenous cues may simultaneously affect both sexual differentiation and determination.

In what way *Plasmodium* modulates its production of total gametocytes and the proportion that are male versus female has recently been reappraised within an evolutionary framework, wherein gametocyte allocation is considered as an adaptive phenotype. In particular, the framework proposes two complementary explanations (i.e., ultimate cues) for observed gametocyte sex ratios: 1) the sex ratio is optimized to ensure successful fertilization in the short term, especially important when gametocyte densities are low,[5–7] and 2) the sex ratio is selected over the long term according to the average genetic diversity of infections and thus the level of inbreeding, i.e., the *Plasmodium* population substructure results in deviations from random mating, which favors a more female-biased gametocyte investment.[8–10] That the sex ratios of *Plasmodium* species adhere to evolutionary principles governing eukaryotes in general[11,12] is important because it strengthens the argument for implementing the wealth of evolutionary theory to predict the consequences of an intervention strategy and for instance how will the parasite evolve following implementation of bed nets or a vaccine.[13]

In parallel, the examination of the proximate cues triggering sex allocation[14] suggest that *Plasmodium* species respond to the same host cues but in a species-specific manner,[15,16] which appears to depend on their blood cell preference (e.g., reticulocytes or mature erythrocytes).[17] There is a growing body of evidence showing that parasite sex allocation correlates with the hematologic response of the host to infection and is manifest either through increased gametocyte production or an increased allocation of sexual stages into male versus female gametocytes. For example, in response to anemia, total gametocyte production increases in *Plasmodium chabaudi*,[18] while an increased male investment is observed in *P. vinckei* and *P. gallinaceum*[19]; and in *P. falciparum* both variables were independently shown to increase.[20–23] That sex allocation in *Plasmodium* species responds facultatively to the same exogenous cues facilitates experimentation in controlled laboratory models and consequent extrapolation to *P. falciparum*.

The importance of the sex ratio for transmission of *Plasmodium* species in general, and of *P. falciparum* in particular, has been the subject of discussion.[6,24] In natural infections in both humans and lizards, for example, the sex ratio can vary extensively over a few days with little clear significance for transmission success.[24,25] *In vitro* studies of mosquito infection rates have also failed to demonstrate an effect of the gametocyte population structure, including sex ratio, on infection success.[26] Given our comparative lack of knowledge of the many factors that influence transmission, establishing the significance of variability in sex ratio on transmission success requires careful examination under highly standardized conditions. Under such conditions, clear evidence has arisen from both *in vitro* and *in vivo* studies that the gametocyte sex ratio is important and that transmission success is particularly dependent on the density and proportion of male gametocytes.[1,25,27,28]

The study of the sex ratio phenotype in field populations has been the subject of considerable speculation, but of little empirical investigation. Furthermore most of the *in vivo* studies that established precise counts of *P. falciparum* sex ratios have been performed on populations undergoing chemotherapy.[29–32] Such studies are hampered by the unknown effect of antimalarials on individual gametocyte infectivity and on parasite sex determination. It is therefore of crucial importance to assess the effect of drug treatment on sex ratio to ensure that there was no treatment bias in the sex ratio data previously obtained under chemotherapy. We therefore use field *P. falciparum* data to assess the question of whether anti-malarial treatment has an influence on the sex ratio of gametocytes. Because no untreated controls are possible in the study of *P. falciparum* sex ratios, we extend the study by examining the effect of sub-curative doses of CQ on the sex ratio of a laboratory mouse model, *P. vinckei petteri*. The effect of chloroquine (CQ) treatment on the sex ratio of *P. vinckei petteri* is compared with the effect of CQ and sulfadoxine-pyrimthamine (SP) on natural infections of *P. falciparum* where the infection strain resistance phenotype is additionally characterized.

MATERIALS AND METHODS

Natural human *P. falciparum* infections. A full description of the study is available in the report by Sokhna et al.[33] Here we only describe the facts pertinent to the present study.

Study design. The study was carried out in Diohine, a village approximately 110 km east of Dakar in the Niakhar area of Senegal. This area of mesoendemic malaria is characterized by seasonal transmission that culminates from August to October.

Patients were recruited from September to November 1996 at the dispensary. Children with uncomplicated *P. falciparum* malaria (< 5,000 parasites/μL of blood) who were not using any antimalarial drug at the time were eligible, once the verbal consent of their parents was obtained. Patients were included only if no gametocytes were observed on day 0. The study was reviewed and approved by the Senegalese Ministry of Health.

Treatment and follow-up of children. The children were allocated to two different oral treatment groups: the CQ treatment group (CQ phosphate; Société Industrielle Pharmaceutique de l'Ouest Africain, Dakar, Senegal), 25 mg/kg of body weight given over a three-day period: 10 mg/kg on day 0 and day 1 and 5 mg/kg on day 2, and the SP group (Fansidar®; F. Hoffmann LaRoche, Basel, Switzerland), 25 mg/kg of sulfadoxine and 1.25 mg/kg of pyrimethamine in a single dose.

The children were followed for 14 days post-treatment, and thick blood smears were prepared on days 0, 7, and 14. All children exhibiting signs of clinical failure, severe malaria, or continued parasitemia were given a second-line treatment and excluded from the study.

Determination of sex ratio. Thick blood smears were stained with Giemsa. Gametocyte and asexual parasite densities were calculated from reading 200 microscope fields (magnification ×1,000). It was assumed that 0.5 μL of blood was examined in this way; thus, the sensitivity threshold was two parasites/μL. The thick blood smears prepared for days 7 and 14 were re-read to establish the sex ratios, which were not counted in the original study.[33] Gametocyte sex was determined using the five classic criteria: 1) males are larger than females, 2) the ends of the cells are angular in females and round in males, 3) the nucleus is smaller in females than in males, 4) the granules of malaria pigment are centrally located in females and more widely scattered in males, and 5) the cytoplasm stains deep blue in females and pale purple in males.[28,34] The gametocyte male percentage was calculated as the number of male gametocytes divided by the sum of male plus female gametocytes (the gametocytes that could not be differentiated were not included).

Inclusion criteria. Patients were included in the analysis when their gametocyte densities were 0/μL of blood on day 0 and greater than 20/μL of blood on days 7 or 14.

Statistical analysis. Due to a non-normal distribution of values, quantitative variables (patient age of treatment groups, sex ratio) were compared between treatment groups with a non parametric Mann-Whitney U test. For comparison of sex ratio at days 7 and 14 within treatment groups, data were analyzed with a non-parametric Wilcoxon Z test. The change in sex ratio over time across both treatment groups was analyzed with Pearson's correlation coefficient.

Mouse parasite. *Plasmodium vinckei petteri* (strain 106HW) (courtesy of I. Landau, Museum National d'Histoire Naturelle, Paris, France), was used. This strain is an isolate from the rodent *Thamnomys rutilans* in the Central African Republic in 1988. This strain has a drug-sensitive phenotype and CQ treatment has previously been shown to have no effect on gametocyte conversion (GC) rates. Parasite development is synchronous and schizogony occurs 24 hours following the injection of frozen infected blood.[35] The time between merozoite invasion and appearance of gametocytes was estimated to be 24–36 hours depending on the maturation type, and the first identifiable gametocytes appear at 27 hours.[36] The life span of mature gametocytes (more than 36 hours of age) is unknown but probably does not exceed 24 hours.

Infection of mice. An intraperitoneal injection of 10^5 parasites/mouse (8–10-week-old female Swiss albino mice; Charles River/IFFA-CREDO, Lyon, France) was carried out in 20 mice at day 3. Fifteen were selected after blood smears on day 0 to be included in the study; selection aimed at standardizing parasitemias and thus having similar infection parasitemias in each of the three groups. In practice, this meant elimination of two high parasitemia infections and three very low parasitemia infections. All experimental animals were maintained according to European Union guidelines.

Drug administration. Preparations of CQ diphosphate (Sigma, St. Louis, MO) were diluted in 1× phosphate-buffered saline (PBS). The 15 mice were randomly assigned in three different treatment groups on day 0. Two groups of five mice were injected intraperitoneally with CQ at sub-curative doses of 5 mg/kg of body weight (CQ5) and 10 mg/kg of body weight (CQ10), and the five remaining mice were injected with 1× PBS on day 0.

Follow-up of parasitamia and determination of sex ratio. Thin blood smears (tail blood) were prepared daily at the same time each morning, fixed with 100% methanol, and stained with 5% Giemsa in phosphate buffer (0.1 M $NaPO_4$, pH 7.2) for five minutes. Parasitemias were calculated as percentages observed in a minimum of 100 red blood cells by reading a fixed subset of 10 different microscope fields; gametocytemias were calculated from observations on 10,000

red blood cells Mature male and female gametocytes are distinguishable after staining with Giemsa: males stain pink and females stain blue with a distinct red nucleus.[37] Sex ratios based on counts of 50–75 gametocytes were found to be representative.[19] We calculated sex ratios from either 50,000 red blood cells or 100 gametocytes, whichever was smaller. Sex ratios are given as the proportion of males.

Statistical analysis. Statistical analyses were conducted using the statistical package Genstat 6.1 (VSN International Ltd., Oxford, United Kingdom). Because each individual mouse was included in the data set many times, we corrected for repeated measures by fitting a generalized linear mixed model that nested day within individual mouse in the random model. Sex ratio, gametocytemia, percentage of reticulocytes, and the GC rates were analyzed specifying a binomial error structure. The effect of CQ treatment groups were considered separately (control versus CQ5 versus CQ10) and combined (control versus CQ). For the overall change in sex ratio with time, the three treatment groups were combined because there was no difference in their sex ratios at any time point. Statistical significance is presented as Wald statistics, which approximate to a chi-square distribution. For the sex ratio analyses, the data were over-dispersed; they were corrected for by estimating a dispersion parameter for each analysis.

In the analyses, only the data from the day of treatment (day 0 of study) and the two days following were considered. This was because the immediate effect of CQ was considered to have an impact on the parasites up to two days post-treatment. In addition, the majority of control mice died on day 3 of the study (day 6 post-infection). The GC rates were calculated in two ways according to the estimated lifespan of mature gametocytes (> or < 12 hours). Based on the assumption that mature gametocytes take up to 36 hours to mature and subsequently live for more than 12 hours, gametocytes from the previous time period will overlap with young developing gametocytes; in this case the GC rate GC_{Low} was calculated as the ratio of [(gametocytemia$_{(\text{time t})}$ − gametocytemia$_{(\text{time t} - 1)}$)/ parasitemia$_{(\text{time t})}$]. Based on the assumption that gametocytes live less than 12 hours, the GC rate GC_{High} was calculated as the ratio of [gametocytemia$_{(\text{time t})}$/ parasitemia$_{(\text{time t})}$]. These rates provide upper and lower estimates of the GC rate.

RESULTS

Sex ratio of *P. falciparum* gametocytes after CQ or SP treatment. The data set was composed of 53 children (27 boys and 26 girls) with mean age of 5.3 years (range = 0.5–12.5 years). There was no significant difference in the ages of the two treatment groups ($P = 0.99$, by Mann-Whitney U test). The sex ratios and the gametocyte densities did not correlate with age on either days 7 or 14 (sex ratio: $P = 0.57$ for day 7 and $P = 0.79$ for day 14; gametocyte density: $P = 0.42$ for day 7 and $P = 0.12$ for day 14). Parasitologic responses after CQ treatment were characterized: 4 patients among 13 treated with CQ (31%) and 10 patients among 40 treated with SP (25%) had resistant phenotypes. The total number of gametocytes counted was 14,682, which consisted of 2,238 males (15.2%), 12,418 females (85.6%), and 26 of undetermined sex (0.2%).

No statistical difference was found between treatments and the gametocyte sex ratio was shown to be the same between the two treatments, both at days 7 and day 14 (Table 1). When the sensitive and resistance phenotype infection groups were compared, no statistical difference was found at either days 7 and 14 for either treatment (day 7 for SP and CQ combined $P = 0.50$; n = 50, for CQ $P = 0.44$; n = 13, and for SP $P = 0.92$; n = 37; day 14 for SP and CQ combined $P = 0.77$; n = 40, for CQ $P = 0.99$; n = 9, and for SP $P = 0.71$, n = 31, by Mann-Whitney U test).

The gametocyte sex ratio clearly increased during the course of infection (Figure 1). The median percentages of male gametocytes at days 7 and 14 were 21.3% and 20.4%, respectively, in the CQ group and 15.0% and 20.0%, respectively, in the SP group (Table 1). A positive correlation was observed between time (days 7 and 14) and the gametocyte sex ratio for the SP group and the SP and CQ groups combined (Pearson's coefficient r = 0.179; n = 22; $P = 0.43$ for CQ; r = 0.254; n = 68; $P = 0.036$ for SP; and r = 0.236; n = 90; $P = 0.025$ for CQ plus SP).

Sex ratio of *P. vinckei petteri* gametocytes after CQ treatment. The sub-curative doses of CQ (5 and 10 mg/kg) injected on day 3 post-inoculation (day 0 of the study) resulted in a rapid dose-dependent decrease in parasite load by the

TABLE 1

Percentage of *Plasmodium falciparum* gametocytes that are male in children with uncomplicated malaria, treated with chloroquine (CQ) or sulfadoxine-pyrimethamine (SP) in Diohine, Senegal and comparison of this percentage as 1) a function of time post-treatment (days 7–14) within the treatment group and 2) a function of treatment (CQ versus SP) at defined times (days 7 and 14)*

		Day 0	Day 7	Day 14	P† (Days 7–14)
CQ	No. of children	13	13	9	
	Mean		16.97%	20.93%	
	Median		21.26%	20.41%	0.14 (n = 9 pairs)
	Minimum		0.64%	8.33%	
	Maximum		42.86%	37.04%	
SP	No. of children	40	37	31	
	Mean		15.23%	21.26%	
	Median		15.00%	20.00%	0.10 (n = 28 pairs)
	Minimum		0.00%	2.46%	
	Maximum		40.82%	55.56%	
Total	No. of children	53	50	40	
	Mean		15.68%	21.18%	0.033 (n = 37 pairs)
P‡ (CQ vs. SP)	Mean %		0.53	0.88	

* With the exception of three individuals, all patients who were positive on day 14 were also positive on day 7.
† By the Wilcoxon Z paired test.
‡ By Mann-Whitney U test.

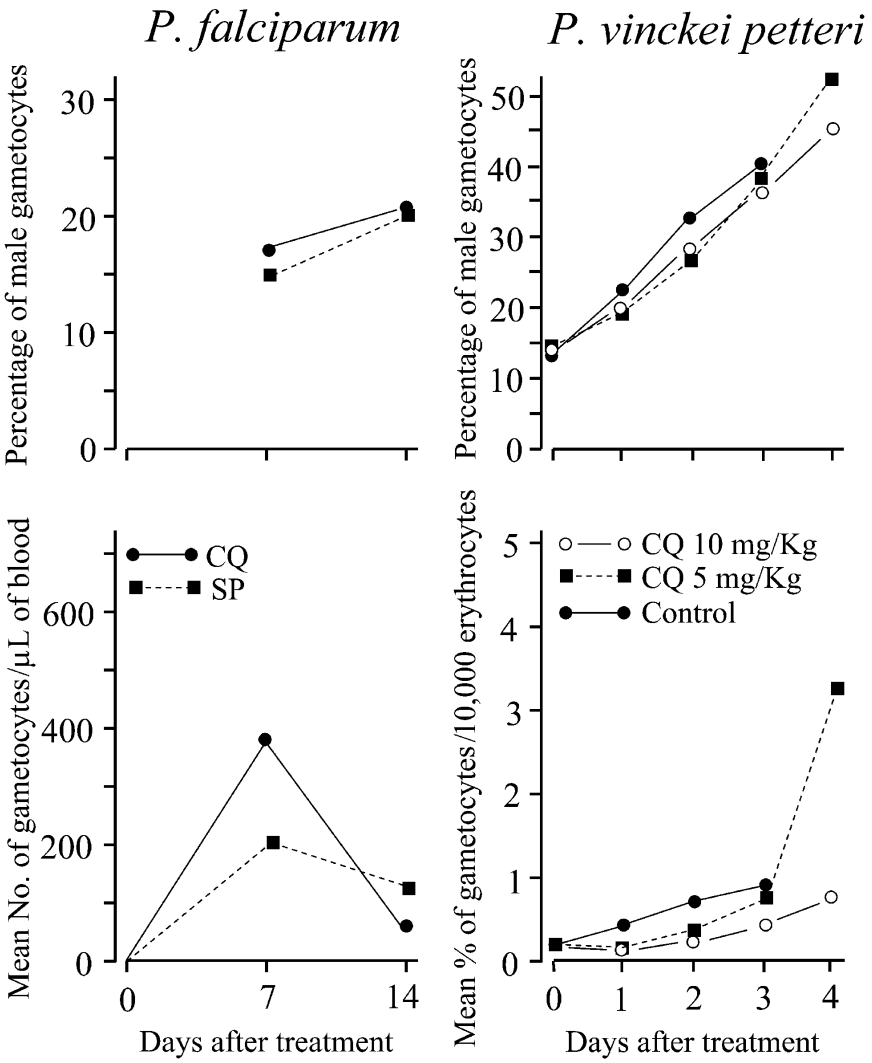

FIGURE 1. Variation in the density of gametocytes and the gametocyte sex ratio over the course of the malarial infection, as a function of two chloroquine (CQ) treatment doses versus untreated controls in *Plasmodium vinckei petteri* experimental infections in mice and CQ or sulfadoxine-pyremethamine (SP) treatment of natural *Plasmodium falciparum* infections in humans.

following day. Drug treatment consequently delayed mouse death by 1–3 days. A similar reduction in gametocytemia was observed (combined CQ treatments versus controls: $\chi^2_1 = 11.9$, $P = 0.0007$).

Treatment with CQ had no effect on the gametocyte sex ratio (control versus CQ5 versus CQ10, $\chi^2_2 = 2.52$, $P = 0.28$; combined CQ versus control, $\chi^2_1 = 2.07$, $P = 0.15$). Neither of the CQ treatments had any significant effect on GC rates (CQ treatment on GC_{Low}: control versus CQ5 versus CQ10, $\chi^2_2 = 3.44$, $P = 0.18$; combined CQ versus control, $\chi^2_1 = 0.84$, $P = 0.36$; CQ treatment on GC_{High}: control versus CQ5 versus CQ10, $\chi^2_2 = 4.52$, $P = 0.11$; combined CQ versus control, $\chi^2_1 = 1.51$, $P = 0.28$). However, the sex ratio was found to be influenced by the density of reticulocytes independently of treatment (% reticulocytes on sex ratio: $\chi^2_1 = 6.44$, $P = 0.011$), as previously found.[18] A positive correlation between time and sex ratio was observed: 14.5% of the gametocytes were male at day 0 (99% confidence interval [CI] = 11.8–17.2%), 37.5% at day 3 (99% CI = 35.6–39.4%), and 47.8% at day 4 (99% CI = 43.3–52.3%) ($\chi^2_1 = 10.8$, $P = 0.001$).

DISCUSSION

Our experiments used two very different *Plasmodium* species, *P. falciparum* (subgenus *Laverania*) and *P. vinckei petteri* (subgenus *Vinckeia*) that infect humans and rodents, respectively. To our knowledge, this is the first designated study of the effect of chemotherapy on the gametocyte sex ratio. We found no effect of CQ on the sex ratio of *P. vinckei petteri*. For *P. falciparum*, no significant differences were found between the drug treatments, but given the absence of an untreated control, we are unable to strictly evaluate the effect of drug treatment on the parasite sex ratio. Nevertheless, the fact that CQ and SP have drastically different modes of action, coupled with the demonstrated increase in sex ratio over the course of natural *P. falciparum* infections observed previously in the absence of treatment,[23] allow us to reasonably conclude that treatment does not influence parasite sex allocation. These conclusions indicate that previously published results on male versus female gametocytes counts from patients undergoing chemotherapeutic treatment were not biased by drug treatment.

This contrasts with the recognized effect of drug treatment on the induction of gametocytogenesis in *P. falciparum*, which was indeed also noted in this study (day 7 post-treatment, Figure 1). This induction has been previously observed following treatment with either CQ or SP in both *P. falciparum* and animal model malaria parasite species infections.[33,38–42] Indeed, treatment using other antimalarial drugs also induces gametocytogenesis,[43] but the significance for gametocyte infectivity remains uncertain.[44,45] Whereas SP generally reduces gametocyte infectivity,[39,46] the infectiousness of CQ-treated gametocytes is variable and is probably dependent on the parasite strain, the dose of CQ administered, and additional vertebrate host immune factors.[38,39,44,45,47] Drug resistance and increased gametocytogenesis have been previously found to be correlated in strains of *P. chabaudi* and *P. vinckei petteri*[48] and thought to be so in *P. falciparum*.[32,33] However, studies using the mouse model with *P. yoelii nigeriensis* demonstrated that drug resistance and increased gametocytogenesis are not necessarily linked.[49] Indeed, our data did not show any significant differences in sex ratios between sensitive and resistant phenotypes in *P. falciparum*; this suggests that resistance to drugs *per se* is not a factor influencing sex determination. A similar conclusion cannot be drawn for *P. vinckei petteri* because the strain used was sensitive to CQ. Given the differential mode of action of CQ and SP, this would suggest that the drugs themselves have no direct biochemical action on the pathways involved in sexual development. It should be noted, however, that there is considerable cross-communication among signaling pathways involved in cell fate, thus enabling the same response from multiple cues.[50] However, the fact that the stress induced by chemotherapy has an influence on the rate of commitment to sex in *P. falciparum* but not on sex ratios, strongly suggests that induction of gametocytogenesis and sex determination operate using distinct pathways.

For both *Plasmodium* species examined here, the sex ratio increased over the course of the infection (Figure 1). One plausible explanation is that male gametocytes have a longer half-life than females. A recent study by Reece and others[51] addressed sex-specific gametocyte longevity using the rodent malaria parasite *P. chabaudi* and curative CQ drug treatment to remove the asexual population (and thus the supply of new gametocytes). This study concluded that male gametocytes have a higher natural longevity. This contrasts with findings from a similar study on *P. falciparum* infections, in which no change in sex ratio was observed following drug treatment, albeit based on a small number of individuals.[30] An alternative explanation concerns the response of the parasite to anemia: previous studies have demonstrated a positive correlation between sex ratio and reticulocytosis[19]; this was again borne out here for *P. vinckei petteri*. The absence of an effect of sub-curative doses of CQ on sex ratio of would suggest that any differential sex mortality is insignificant compared with the natural shift to increased male sex allocation in response to hematologic recovery. These differential species effects of exogenous cues on sex ratio increasingly highlight the importance of an appreciation of the characteristics associated with the ecology of the species. Therefore, particular attention must be paid to parasite life history characteristics to interpret the gametocyte phenotypic response to exogenous cues or during an infection.

The parasite seems to have evolved two distinct ways in which to respond to the environment with respect to their transmission to the mosquito host: gametocyte production, and sex allocation. However, the cues that activate one do not necessarily act upon the other. If anemia influences both, it now seems probable that drug treatment only affects gametocyte production.

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