Effect of gametocyte sex ratio on infectivity of *Plasmodium falciparum* to *Anopheles gambiae*

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Abstract

Insectary-reared *Anopheles gambiae* were experimentally fed with the blood of 90 naturally infected human volunteers carrying gametocytes of *Plasmodium falciparum*. At least one mosquito was successfully infected in 74% of experiments. The probability that a gametocyte carrier was infective, the probability that a mosquito became infected, and the number of oocysts harboured were related to gametocyte density. The mean proportion of male gametocytes was 0·217 (i.e., 3·6 females for every male). Sex ratios differed significantly between gametocyte carriers. Variation in sex ratio was not related to the probability that a gametocyte carrier was infective. Among infective people whose sex ratio estimates were based on a reasonable number of gametocytes, sex ratio significantly predicted the proportion of infected mosquitoes and mean oocyst load, with infectivity rising as the proportion of male gametocytes increased towards 50%. There was no indication that infectivity reached a peak at some intermediate sex ratio, as would be expected if random mating of gametes was the primary determinant of fertilization success. These results raise 2 interesting questions: why should higher sex ratios be more infective, and why is the observed population sex ratio lower than that which produces the greatest infectivity?

Keywords: malaria, *Plasmodium falciparum*, gametocyte sex ratio, infectivity to anophelines

Introduction

The change from a human to an anopheline environment is a critical phase in the life cycle of malaria parasites. Mature gametocytes are the only stage able to initiate this transition. Gametocytes are morphologically and physiologically distinguishable as males or females. As single haploid asexual parasites can give rise to both sexes, sex determination cannot be due to segregation of chromosomes (CARTER & GRAVES, 1988). Several factors have been identified as influencing the success of the infection of mosquitoes. Considering only *Plasmodium falciparum*, both gametocyte density (BOYD, 1949) and the sickle cell trait status of gametocyte carriers (ROBERT *et al.*, 1996) increase infectivity; those acting negatively include specific and non-specific transmission blocking factors (SINDEN & SMALLEY, 1976; MULDER *et al.*, 1994). Despite the sexual reproduction of *P. falciparum* in the midgut of mosquitoes, the sex ratio of gametocytes has generally not been considered a factor influencing infectivity to mosquitoes (BOUDIN *et al.*, 1989; READ *et al.*, 1992; NODEN *et al.*, 1994). BOYD *et al.* (1935) were apparently alone in suggesting that variations in sex ratio, perhaps due to variations in microgametocyte densities, might affect the infectivity of gametocyte carriers.

We studied the effects of the gametocyte sex ratio on the infectivity of naturally infected carriers of *P. falciparum* gametocytes to anophelines by means of experimental infections conducted in the town of Yaoundé, Cameroon.

Materials and Methods

Experimental infections

Thick blood films were prepared from patients with malaria-like complaints and stained with Giemsa's stain. Gametocyte density was based on a count against 1000 leucocytes, assuming an average number of 8000 leucocytes/μL of blood. Gametocyte sex was determined based on the 5 classical criteria (CARTER & GRAVES, 1988): (i) females are larger than males, (ii) the ends of the cells are angular in females and round in males, (iii) the nucleus is smaller in females than in males, (iv) the granules of malaria pigment are centrally located in females and more widely scattered in males, and (v) the cytoplasm stains deep blue in females and pale purple in males. Throughout, we defined sex ratio as the ratio of males to females; but most of the analyses were done using the proportion of gametocytes that were male.

Venous blood was collected and immediately placed in a Parafilm™ membrane feeder and maintained at 37°C. Batches of a local strain of laboratory-reared *Anopheles gambiae s.s.* were allowed to feed for 15 min. Midguts were dissected and examined for oocysts after 7 d as described previously (TCHUINKAM *et al.*, 1993).

Enrolment criteria were: at least 20 mosquitoes dissected for each experimental feed, with volunteers being at least 4 years old, not infected with species of *Plasmodium* other than *P. falciparum* and with a gametocyte density >55 μL (at least 7 gametocytes observed in the thick blood film).

Data analysis

When the dependent variable was a proportion (sex ratio, or proportion of mosquitoes infected), logistic regression models with Williams's correction for over-dispersion (COLLET, 1991) were used. Models were fitted to the data using the GLIM® statistical package (CRAWLEY, 1993). Parameter estimates (with twice the associated standard errors as the approximate 95% confidence intervals [CI]) were determined by maximum likelihood; statistical significance was tested using change in deviance (ΔD), which approximates to a χ^2 distribution with the corresponding degrees of freedom. Other regressions were simple least squares. Data on mean oocyst load (total oocysts/number of dissected mosquitoes) and densities of trophozoites (+1) and gametocytes were log-transformed.

When sex ratio was an independent variable, it was subject to angular transformation. This has the disadvantage that all sex ratios were weighted equally, even though some are based on considerably larger gametocyte counts than others (see below). Consequently, several analyses were repeated, excluding experiments in which sex ratios were based on gametocyte counts of less than 15 or 30.

All the analyses of the effects of sex ratio on infectivity were repeated, controlling statistically for the effects of total gametocyte density (i.e., asking, for a given gametocyte density, whether sex ratio has an effect on infectiv-

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ity); these results (not presented) did not alter the conclusions.

Results

Ninety experiments were included in the analysis; 3921 gametocytes were observed and 3162 mosquitoes were dissected. The mean gametocyte density was 247·4/μL (range 56–1416/μL, median 160/μL). Gametocytes were sexed as 824 males (21%) and 2968 (76%) females, with 129 (3%) of indeterminate sex. The overall mean proportion of males was 0·217 (95% CI 0·204–0·231), which is about 3·6 females for every male. The total number of males plus females ranged between 3 and 288 (mean 42·1, median 23). All but 2 sex ratios were female-biased (i.e., with more females than males); the highest observed proportion of males was 0·54 (95% CI 0·33–0·74) (Fig. 1); there were significant differences

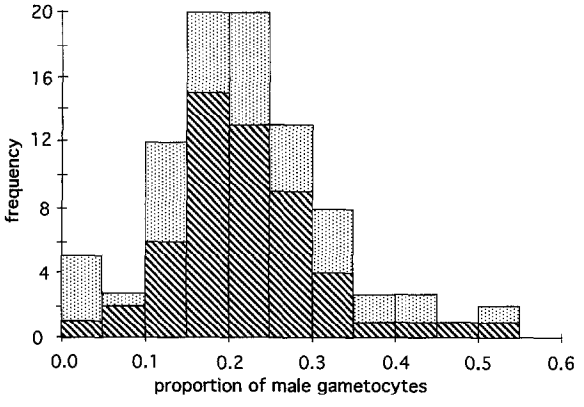

Fig. 1. Frequency distribution of proportion of *Plasmodium falciparum* male gametocytes in 90 gametocyte carriers in Yaoundé, Cameroon. Hatched portions of bars refer to 54 carriers in whom the proportion of males was estimated among 15 or more gametocytes.

between the sex ratios of the 90 gametocyte carriers ($\Delta D=171$, d.f.=89, $P<0·001$). Sex ratio was unrelated to host sex ($\Delta D=0·33$, d.f.=1, not significant [NS]) and age ($\Delta D=0·60$, d.f.=1, NS). Sex ratio was not correlated with the density of trophozoites ($\Delta D=0·11$, d.f.=1, NS) or with the density of gametocytes ($\Delta D=0·06$, d.f.=1, NS). This latter relationship was not improved by fitting an appropriately scaled quadratic term, so there was no indication that sex ratio was maximal at some intermediate gametocyte density.

People with higher gametocyte densities were more likely to be infective (i.e., to infect at least one mosquito; $\Delta D=8·78$, d.f.=1, $P<0·005$). The proportion of infected mosquitoes varied significantly between gametocyte carriers ($\Delta D=796·2$, d.f.=89, $P<0·0001$) around an overall average of 14·9% (95% CI 13·7–16·2%). Sixty-seven gametocyte carriers (74%) gave rise to at least one infected mosquito. The maximum proportion of infected mosquitoes was 72%. The mean oocyst density (number of oocysts per dissected mosquito) was 0·81 (range 0–8·3). Similarly, the percentage of infected mosquitoes was strongly correlated with the density of all gametocytes ($\Delta D=15·14$, d.f.=1, $P<0·001$) and with the density of either sex (males, $\Delta D=14·77$, d.f.=1, $P<0·001$; females, $\Delta D=11·79$, d.f.=1, $P<0·001$). Male and female gametocyte densities were highly correlated with each other ($r=0·70$, $P<0·0001$), and consequently neither sex predicted the proportion of infected mosquitoes significantly better than the other.

Sex ratio and proportion of infected mosquitoes

The probability that a gametocyte carrier was infective (i.e., infected at least one mosquito) was unrelated to the sex ratio ($\Delta D=0·88$, d.f.=1, NS). Gametocyte sex ratio in carriers was unrelated to the proportion of infected mosquitoes ($\Delta D=1·92$, d.f.=1, NS; Fig. 2), even considering only those gametocyte carriers whose sex ratio estimates were based on 15 or more gametocytes ($\Delta D=1·24$, d.f.=1, NS). Spearman's rank correlations on all data, or on sex ratios based on 15 or more gametocytes, led to the same conclusions ($r_S=0·19$, $n=90$, $P=0·08$; $r_S=0·18$, $n=46$, $P=0·20$). However, on theoretical grounds, a linear relationship was not expected: all other things being equal, fertilization rates should reach a peak at the female-biased gametocyte sex ratio which results in a 1:1 gamete sex ratio, and decline at more or less biased ratios as one of the sexes becomes limiting among the gametes. The number of viable gametes per male gametocyte has not been determined; at most, 8 are produced, but morphological evidence suggests that only 4–6 of these are viable (reviewed by READ *et al.*, 1992, p. 391). Given this uncertainty, we examined whether infectivity reached a peak at some sex ratio, as follows. For each gametocyte carrier, we calculated the deviation of the observed gametocyte sex ratio from either (i) the average gametocyte sex ratio observed in the population (1:3·6), (ii) a 1:5 sex ratio, or (iii) a 1:8 sex ratio. Each of these values was fitted to the relevant statistical model as a squared term. Should infectivity reach a peak around one of these values, the parameter estimate for the squared deviation term should be negative and significant, so that smaller and larger values would be less infective. Analysed in this way, there was no evidence that the proportion of mosquitoes infected was greatest at (i) the observed mean gametocyte sex ratio, (ii) the sex ratio of 1:5, or (iii) the sex ratio of 1:8 which would be expected to result in most zygotes if all male gametes were viable ($\Delta D=0·37$, $\Delta D=0·07$, $\Delta D=0·20$, respectively, d.f.=1, NS in each case). This qualitative picture was unaltered when sex ratios based on a count of fewer than 15 or 30 gametocytes were excluded from the analysis.

Given that sex ratio is unrelated to the probability that a person is infective, gametocyte carriers that are uninfective for other reasons may introduce unnecessary 'noise' into the analyses. We therefore analysed the effects of sex ratio on the proportion of mosquitoes that became infected in feeds only on people who were infective. Again, there was no effect of sex ratio ($r=0·13$, $\Delta D=1·16$, d.f.=1, $n=67$, NS). However, this analysis included people with fewer than 15 gametocytes counted. Excluding these less reliable estimates, gametocyte sex ratio was positively correlated with the proportion of infected mosquitoes ($r=0·31$, $\Delta D=4·69$, d.f.=1, $n=46$, $P<0·05$; Fig. 3). This was also apparent in the further reduced data set involving sex ratios based only on counts of 30 or more gametocytes ($r=0·33$, $\Delta D=4·33$, d.f.=1, $n=37$, $P<0·05$); in this reduced data set, gametocyte density did not correlate with the proportion of infected mosquitoes ($\Delta D=1·86$, d.f.=1, $n=46$, NS), indicating that at gametocytaemia higher than 240/μL,

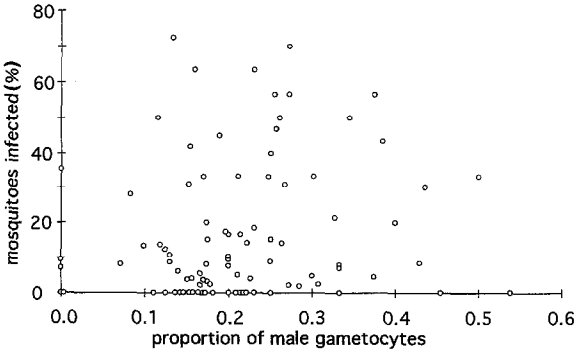

Fig. 2. Relationship between the proportion of male gametocytes and the percentage of infected *Anopheles gambiae* in 90 infections with *Plasmodium falciparum*.

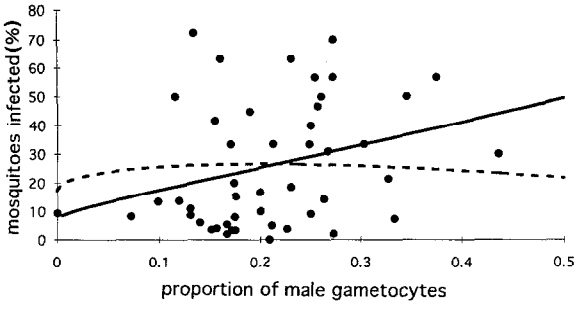

Fig. 3. Proportion of male gametocytes (n=46) and prevalence of oocysts in mosquitoes fed on infective gametocyte carriers with the proportion of males estimated from counts of 15 or more gametocytes. The probability of a mosquito being infected increased with the proportion of male gametocytes in its blood meal (solid line, $P<0{\cdot}05$). A model assuming that infectivity reached a peak at the population mean proportion of males (broken line) did not provide a significant fit to the data. The solid line is the fitted logistic probability curve, with parameters estimated by maximum likelihood, given by $\ln[(\text{mosquitoes infected})/(\text{mosquitoes uninfected})]=\alpha_1+\beta_1\,[\sin^{-1}(\sqrt{\text{proportion of males}})]$; $\Delta D=4{\cdot}69$, d.f.=1, n=46, $P<0{\cdot}05$. The broken line is the best fit logistic probability curve assuming infectivity reached a peak at the population mean proportion of males (which in this data set was 0·20), given by $\ln[(\text{mosquitoes infected})/(\text{mosquitoes uninfected})]=\alpha_2+\beta_2\,[\sin^{-1}(\sqrt{\text{proportion of males}})-0{\cdot}4624]^2$; $\Delta D=0{\cdot}21$, d.f.=1, n=46, not significant. Parameter estimates ± standard error: $\alpha_1=-2{\cdot}61\pm0{\cdot}78$, $\beta_1=3{\cdot}29\pm1{\cdot}59$, $\alpha_2=-1{\cdot}02\pm0{\cdot}18$, $\beta_2=-2{\cdot}53\pm5{\cdot}77$.

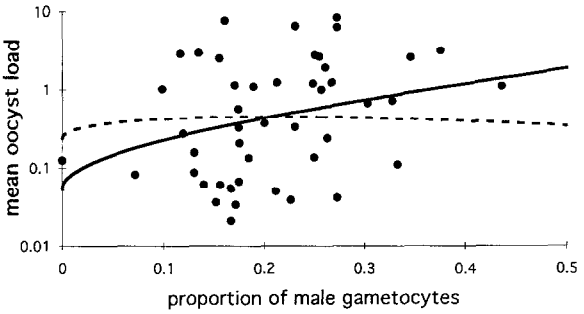

Fig. 4. Proportion of male gametocytes (n=46) and mean oocyst load among the infective gametocyte carriers with the proportion of males estimated from counts of 15 or more gametocytes. Mean oocyst load increased with increasing proportion of males (solid line, $P<0{\cdot}05$). A model assuming that infectivity reached a peak at the population mean proportion of males (broken line) did not provide a significant fit to the data. The solid line is the fitted logistic probability curve, with parameters estimated by maximum likelihood, given by $\ln[(\text{total oocysts})/(\text{mosquitoes dissected})] = \alpha_1+\beta_1\,[\sin^{-1}(\sqrt{\text{proportion of males}})]$; $F_{1,44}=4{\cdot}58$, $P<0{\cdot}05$. The broken line is the best fit logistic probability curve assuming infectivity reached a peak at the population mean proportion of males, given by $\ln[(\text{total oocysts})/(\text{mosquitoes dissected})] = \alpha_2+\beta_2\,[\sin^{-1}(\sqrt{\text{proportion of males}})]-0{\cdot}4719]^2$; $F_{1,44}=0{\cdot}14$, not significant. Parameter estimates±standard error: $\alpha_1=-2{\cdot}95\pm1{\cdot}02$, $\beta_1=4{\cdot}55\pm2{\cdot}13$, $\alpha_2=-0{\cdot}80\pm0{\cdot}28$, $\beta_2=-2{\cdot}89\pm7{\cdot}67$.

gametocyte density did not predict the proportion of infected mosquitoes, but gametocyte sex ratio did. There was no indication that infectivity reached a peak at either the population mean sex ratio (Fig. 3) or ratios of 1:5 or 1:8 ($P>0{\cdot}5$ in all cases). Thus, amongst infective people whose sex ratio estimates were based on a reasonable number of gametocytes, sex ratio significantly predicted the proportion of infected mosquitoes, with infectivity continuously rising as sex ratios increased.

Sex ratio and oocyst loads

To determine whether sex ratio had an effect on the number of oocysts per mosquito, we considered only infective gametocyte carriers (i.e., those who infected at least one mosquito) because we know, from the results reported above, that (i) the sex ratio did not affect the probability that a gametocyte carrier was infective, and (ii) gametocyte carriers not infective for some reason could introduce unnecessary 'noise' into the analysis. Among infective gametocyte carriers, sex ratio was unrelated to mean oocyst load ($r=0{\cdot}11$, NS, n=67). The number of oocysts per mosquito was positively correlated with total gametocyte density ($r=0{\cdot}37$, $P<0{\cdot}001$) and to the density of male ($r=0{\cdot}34$, $P<0{\cdot}01$) and female ($r=0{\cdot}32$, $P<0{\cdot}01$) gametocytes; neither sex predicted oocyst load significantly better than the other. However, as with oocyst prevalence, there was a positive correlation between oocyst load and sex ratio when sex ratios were based on counts of 15 or more gametocytes ($r=0{\cdot}31$, $P<0{\cdot}05$, n=46; Fig. 4) and 30 or more gametocytes ($r=0{\cdot}36$, $P<0{\cdot}05$, n=37). Thus, as sex ratios became less female-biased, mean oocyst loads rose. In order to determine whether oocyst load reached a peak at some sex ratio, we used the approach described above; there was no evidence that loads were reduced at any one of the 3 sex ratios (i.e., the average population sex ratio of 1:3·6 (Fig. 4), 1:5 and 1:8), even when analysing only sex ratios based on 15 or more gametocytes, or forcing the curve through the origin ($P>0{\cdot}5$ in all cases).

Discussion

From our results there is no evidence that variation in sex ratio of *P. falciparum* gametocytes contributes to heterogeneity in the probability of a gametocyte carrier being infective. However, we believe this is the first report of a weak but significant relationship between sex ratio and infectivity. Among infective gametocyte carriers, sex ratio was positively linked with both oocyst prevalence and oocyst load. Sex ratio explained around 10% of the variance in mean oocyst load, about the same or more as is (independently) explained by gametocyte density in the same data set. This sex ratio effect was not detectable among all gametocyte carriers in this study (i.e., the infective and uninfective people), presumably because the sex ratio estimates based on very low gametocyte densities were necessarily less reliable, and carriers not infective for some reason obscured the pattern. These reasons could include incompetence or inefficiency of gametocytes, or the presence of transmission-blocking immunity.

Why should oocyst loads continue to increase as sex ratios increase? If this relationship is causal, it is puzzling for 2 reasons. First, in theory, all other things being equal, maximum infectivity should occur at the sex ratio at which there are just sufficient male gametes to fertilize all the female gametes. At least across the range of gametocyte sex ratios present in our study, models assuming a gamete sex ratio of 1:1 conspicuously failed to fit the data (Figs 3 and 4). One possible implication is that more male gametes are required for successful fertilization than would be expected from random mating of gametes, at least given what is currently believed to be the number of viable gametes released per male gametocyte. Second, the most common sex ratios in *P. falciparum* isolates from Cameroon were associated with lower transmission success. This is somewhat paradoxical: variants producing less female-biased sex ratios, as are evidently found in Cameroon, should be favoured by natural selection since these would maximize the probability of infecting a mosquito. One possibility is that some unrecognized selection pressure may maintain more female-biased sex ratios. Perhaps the benefit of increased infectivity is lessened if the associated higher oocyst loads reduce vectorial capacity (survival, activity, etc.); evidence for that is well established for various *Plasmodium* species (MAIER *et al.*, 1987), but remains

controversial for *P. falciparum* (see CHEGE & BEIER, 1990; ROBERT *et al.*, 1990). Gametocyte carriers with gametocyte sex ratios very close to 1 may shed light on these issues, especially if they show reduced infectivity; unfortunately, such carriers were rare among our subjects (Fig. 1).

Our results were obtained from gametocyte carriers presenting 2 characteristics: (i) they harboured high gametocytaemia (>55/μL of blood), well known to represent a small part of the general population of the gametocyte carriers in endemic zones, and (ii) many of them were recruited at the beginning of a simple malaria attack. GARNHAM (1966) suggested that the gametocyte sex ratio decreased during the course of the infection, though we know of no supporting data. It would be interesting to know whether the patterns reported here are found in representative samples of gametocyte carriers and/or in other areas.

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