Sex ratio of *Plasmodium falciparum* gametocytes in inhabitants of Dielmo, Senegal

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SUMMARY

An epidemiological survey was conducted during a 4-month period of intense malaria transmission in Dielmo, a holoendemic Senegalese village. Two thick blood smears per inhabitant were collected weekly. The sex ratio of *Plasmodium falciparum* gametocytes (gamete precursors) was studied in 50 gametocyte carriers. All age classes were represented (mean 19·7 years; range: 2 months–75 years); 42 (84%) of them did not receive antimalarial treatment. Overall 668 thick smears were examined until 100 gametocytes had been counted or for 40 min. A total of 11 204 gametocytes were observed with a mean sex ratio of 0·346 (95% CI 0·317–0·374), i.e. 2·89 females per 1 male. Among the 284 thick smears in which at least 10 gametocytes were observed, the mean percentage of male gametocytes was 27·8%, with a range of 0–82%. Great variability was observed between gametocyte carriers and also between thick smears from the same gametocyte carrier. A multivariate analysis was performed which highlighted the fact that only 2 variables had a significant effect on the sex ratio. Anaemia was associated with an increased percentage of males (Prevalence Rate Ratio [PPR] of male gametocytes was multiplied by 1·65 if haematocrit rate $<32\%$) and a wave of gametocytes was associated with an increased percentage of female gametocytes (PRR was multiplied by 0·48 during the peak of gametocytaemia and for the 2 weeks following this peak). The variables without significant effect on sex ratio were: age, sex, clinical status and sickle cell trait status of the gametocyte carrier, density of asexual parasites, quinine treatment, and gametocyte density (when taking account of its waves). These results are discussed in regard of possible differential production, mortality or sequestration of one gametocyte sex and selective advantages for the transmission of parasites.

Key words: *Plasmodium falciparum*, gametocyte, sex ratio, malaria transmission, holoendemy.

INTRODUCTION

In 1880 Laveran observed gametocytes of *Plasmodium falciparum* in a drop of fresh blood from soldiers who presented severe intermittent fever. They were the first malaria parasites of any stage to be seen and recognized. The existence of 2 types of gametocyte, male and female, for the most part female-biased, was also established in the 19th century, like the essential importance of both sexes for the passage of the parasite from man to mosquito (Carter & Graves, 1988). However, the studies with precise counts of male : female gametocytes were scarce and mainly obtained after chemotherapy (James, 1931; Shute & Maryon, 1951; Smalley & Sinden, 1977). Read *et al*. (1992) noted 'as far as we are aware, only two studies have attempted to systematically investigate sex ratio in natural populations in the absence of chemotherapy; both studies were concerned with lizard malaria'. At that time, only 2 distributions of sex ratios for *P. falciparum* gametocytes had been published, in the Madang, Papua New Guinea (Read *et al*. 1992) and the town of Yaounde, Cameroon (Robert *et al*. 1996).

What is the essential background understanding of *P. falciparum* gametocytes which is most relevant for sex ratio studies? Gametocytes emanate from the asexual blood-stage parasites. Direct production of gametocytes from exo-erythrocytic stages has not been observed. Sexual differentiation is not the result of the segregation of sex chromosomes, since cloned lines originating from a single haploid parasite produce both male and female gametocytes (Trager *et al*. 1981). The most male-biased sex ratios demonstrated higher infectivity for the mosquito vector (Boyd, Stratman-Thomas & Kitchen, 1935; Robert *et al*. 1996). The commitment to sexual differentiation occurs prior to schizont maturation (Silvestrini, Alano & Williams, 2000). All the merozoites produced by a single sexually-committed schizont become either all male or all female gametocytes, with an equal gametocyte number for both sexes, indicating that allocation of parasite resources is equal for each sex of gametocyte (Smith *et al*. 2000). A greater percentage of schizonts that produce female gametocytes than those that yield males explains the female-biased sex ratio. Sex ratios of

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clones derived from a single isolate differed significantly and 1 clone maintained through 15 subcultures showed a stable sex ratio, suggesting that the sex ratio may be clone specific (Burkot, Williams & Schneider, 1984). One microgametocyte produces up to 8 male gametes and 1 macrogametocyte produces 1 female gamete. Competitive adaptation selects low values of male : female sex ratios in cases of high selfing rates and, inversely, selects high male : female sex ratios in cases of high rates of outcrossing. As predicted by local mate competition theory, the number of clones per individual is a determinant in this balance (Read *et al*. 1992). The level of anaemia relates to high male : female sex ratio; erythropoietin, the human hormone stimulating the production of erythrocytes, is probably a signal used by the parasite to increase its proportion of males (Paul *et al*. 2000). Two recent papers review the present knowledge on sex ratio of malaria parasites and other Apicomplexa (West, Reece & Read, 2001; Paul, Brey & Robert, 2002); both emphasize the lack of available data.

We present here data on the sex ratios of *P. falciparum* gametocytes in naturally infected people living in a village in a holoendemic area, with special reference to longitudinal observations up to 17 weeks, mostly in asymptomatic and untreated individuals. Our aim was to describe the natural history of gametocyte sex ratio, and to highlight the variables involved in its variations.

MATERIALS AND METHODS

The Dielmo village

The geographical and epidemiological characteristics of the Dielmo village, Senegal, have been previously described in detail (Trape *et al*. 1994). It is a holoendemic zone in which all 247 villagers volunteered to participate in a longitudinal study on malaria which included entomological, parasitological, clinical and immunological surveys. The data presented in this paper were collected from June to September 1990, a period of 17 weeks during which the entomological inoculation rate for *Plasmodium* sp. was estimated at 130 bites by infected anophelines per person resulting in 101 infections due to *P. falciparum*. Twice a week, each villager was visited at home to make a thick blood smear. The smears were left to dry for 24–48 h, the haemoglobin removed, and stained with Giemsa's stain. Two hundred microscopic oil-immersion fields were examined on each thick smear. It was assumed that 0·5 μl of blood were observed in this way. Gametocyte density per μl of blood was calculated as double the number of gametocytes observed. Prevalence in the 2–9 years of *Plasmodium* spp. was 92%. In all blood smears, the slide positivity rate for *P. falciparum* gametocytes was 49·6% in the 0–1 year, 38·4% in the 5–9 years, and 11·3% in the $\geqslant$60 years.

The thick smears for sex ratio estimation

Among the 247 villagers, 206 were present during the whole period or absent for less than 10 days; they had experience typical of residence in a malaria holoendemic area. Among these 206 people, 27 never had detectable gametocytes. In total 179 villagers presented with at least 1 thick smear positive for *P. falciparum* gametocytes, most of them harbouring relatively small gametocyte densities.

The criteria for inclusion of thick smears and individuals in the present study on sex ratios were established as follow. We retained all the thick smears from 50 individuals (23 males and 27 females) with at least 1 gametocyte observed. These comprised 15 individuals with a gametocytaemia $\geqslant 20/\mu$l for at least 4 consecutive thick smears (245 thick smears), 4 individuals with 1 patent gametocyte wave (36 thick smears), 1 individual with permanent gametocytaemia at a density always lower than 20/μl (32 thick smears), and 30 individuals randomly selected from the remaining (179–20 = 159) villagers with lower gametocytaemias (355 thick smears).

These 50 individuals were distributed in the following classes of age: 0–1 year, 6 (12%); 2–4 years, 9 (18%); 5–9 years, 8 (16%); and $\geqslant$10 years, 27 (54%). The mean age was 19·7 years and the range 2 months–75 years. Forty-two individuals did not receive any antimalarials during the study; 8 had between 1 and 6 uncomplicated malaria attacks and were treated with quinine.

Overall 668 thick smears were selected. Each thick smear was microscopically read, focusing on determination of the sex of gametocytes, till 100 gametocytes were observed or for 40 min. Gametocyte sex was determined on morphological features (Carter & Graves, 1988; Robert *et al*. 1996). Throughout we defined sex ratio as the ratio of males to females; otherwise we speak of percentage of gametocytes that were male. The 95% confidence interval for the mean sex ratio was calculated taking into account the autocorrelation between the successive observations made in the same individual.

Statistical analysis

We defined the variations of the gametocyte density as follows. One peak is the highest gametocyte density $\geqslant 25/\mu$l of blood observed during the 2 weeks period, including the 7 previous days and the 7 following days. A gametocytaemia observed during the 7 days before a peak constitutes one upward phase. A gametocytaemia observed during the 28 days after a peak constitutes one downward phase. In the absence of peak, upward phase or downward phase, the phase is basic. When a gametocytaemia was

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observed $\leqslant$28 days after a peak and $\leqslant$7 days before another peak, only the nearest peak was considered in the analysis.

The dependent variable was the prevalence rate of males among gametocytes taking the number of male gametocytes as numerator and the number of gametocytes observed on a thick smear as denominator. The statistical analysis was faced with 2 difficulties; first, the large variability in the number of gametocytes observed in a thick smear; secondly, the special structure of our data set with strong linkages between gametocytes in the same thick smear and between sequential thick smears performed on the same individual. Thus, we used a generalized estimating equation approach for regression analysis for a Binomial distribution (Zeger & Liang, 1986). This approach allows (i) robust analysis for binomial distributed variables with correlated structure and (ii) the number of examined gametocytes to vary across thick smears. We assumed a within-individual autoregressive correlation structure for all the thick smears of each gametocyte carrier. This procedure necessitates equal delays between two consecutive observations of gametocytes; that was formally wrong for our data but judged acceptable considering that 81% of the delays were between 2 and 5 days (median = 4 days, interquartile intervals 25–75% = 3–4 days). For more confidence, a stationary structure and an exchangeable correlation structure were both also tested (results not shown because they were in agreement with those obtained from the autoregressive correlation structure). In addition, in a separate analysis, we used a generalized estimating equation approach for regression analysis for Poisson distribution, assuming that the number of gametocytes examined on thick smears followed a Poisson-stopped binomial distribution (Cameron & Trevedi, 1998) (results not shown because they were in agreement with those obtained from a Binomial distribution). The work was done with the software STATA 7.0 (Stata Corporation, College Station, TX, USA, 2001). This analysis required at least 2 values of sex ratio per individual, a condition satisfied with 39 individuals (ind) and a total of 552 thick smears (th sm). It was carried out in 2 successive steps.

In the first step, the following variables were tested in simple bivariate analysis against the percentage of male gametocytes. (1) Age of gametocyte carrier (2 ind $<$1 year, 8 ind 2–4 years, 8 ind 5–9 years, 8 ind 10–19 years and 13 ind $\geqslant$20 years); (2) sex of gametocyte carrier (18 females *vs* 21 males); (3) haemoglobin type (32 AA, 1 AC, and 6 AS); (4) ethnic group (28 Sereres, 7 Mandingues, 4 others); (5) haemoglobin ratio $\geqslant$9·5 g/dl at the beginning of the survey (6 ind $<$ *vs* 33 ind $\geqslant$9·5 g/dl); (6) haematocrit rate $\geqslant$32·0% at the beginning of the survey (12 ind $<$ *vs* 27 ind $\geqslant$32·0%); (7) number of erythrocytes $\geqslant 4{\cdot}5 \times 10^6/\mu$l of blood at the beginning of the survey (24 ind $<$ *vs* 15 ind $\geqslant 4{\cdot}5 \times 10^6/\mu$l of blood); (8) malaria symptoms within $\pm$3 days (yes for 226 th sm *vs* no for 326 th sm); (9) temperature $\geqslant$37·5 °C (yes for 184 th sm *vs* no for 368 th sm); (10) peak of temperature $\geqslant$37·5 °C within $\pm$3 days (yes for 399 th sm *vs* no for 153 th sm); (11) delay post quinine treatment within the 7 following days (18 th sm), or 7–13 days (15 th sm), or 14–30 days (20 th sm), or $\geqslant$30 days (499 th sm); (12) gametocyte density (13 th sm $<$1 gametocyte/200 microscopic fields, 348 th sm between 1 and 4, and 89 th sm between 5 and 9, or 102 th sm $\geqslant$10); (13) gametocytaemia phase (26 th sm as peaks, 41 th sm as upward phases; 115 th sm as downward phases, and 370 th sm as basic phases); (14) asexual parasite density (171 th sm $<$1 trophozoite/100 leukocytes, 357 th sm between 1 and 199, and 24 th sm $\geqslant$200); (15) asexual parasite phase within $\pm$7 days around a peak (31 th sm as peaks $\geqslant$50 trophozoites/100 leukocytes, 87 th sm as upward phases, 84 th sm as downward phases, and 350 th sm as basic phases).

In the second step, the variables with P value $<0{\cdot}50$ in the bivariate analysis have been considered in the multivariate analysis of the proportion of male gametocytes. The conserved variables were tested altogether then those with P value $\geqslant 0{\cdot}05$ were removed one by one from the model. The remaining variables with $P<0{\cdot}05$ were considered to have a significant effect on sex ratio.

RESULTS

Overall, from the 50 individuals of Dielmo included in the study, among the 668 thick smears microscopically read for sex ratio estimation, 614 had at least 1 gametocyte. In total 11 204 gametocytes were sexed as 25·6% males and 73·8% females, with 0·6% of indeterminate sex. The mean sex ratio was 0·346 (95% CI 0·317–0·374), i.e. 2·89 females for every male.

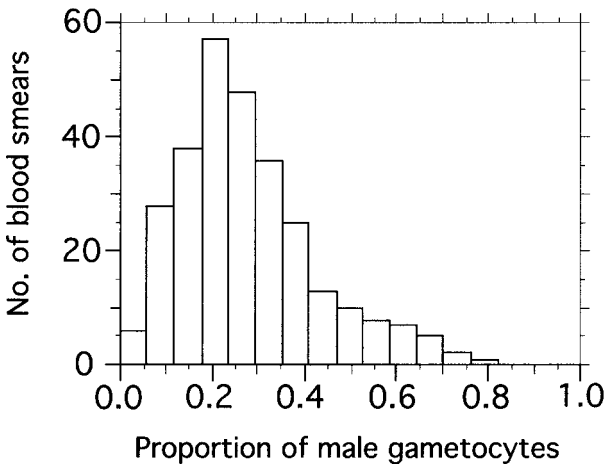

Fig. 1. Distribution of proportion of *Plasmodium falciparum* male gametocytes in 284 thick blood smears with at least 10 observed gametocytes, collected during an epidemiological survey of 28 villagers from Dielmo, Senegal.

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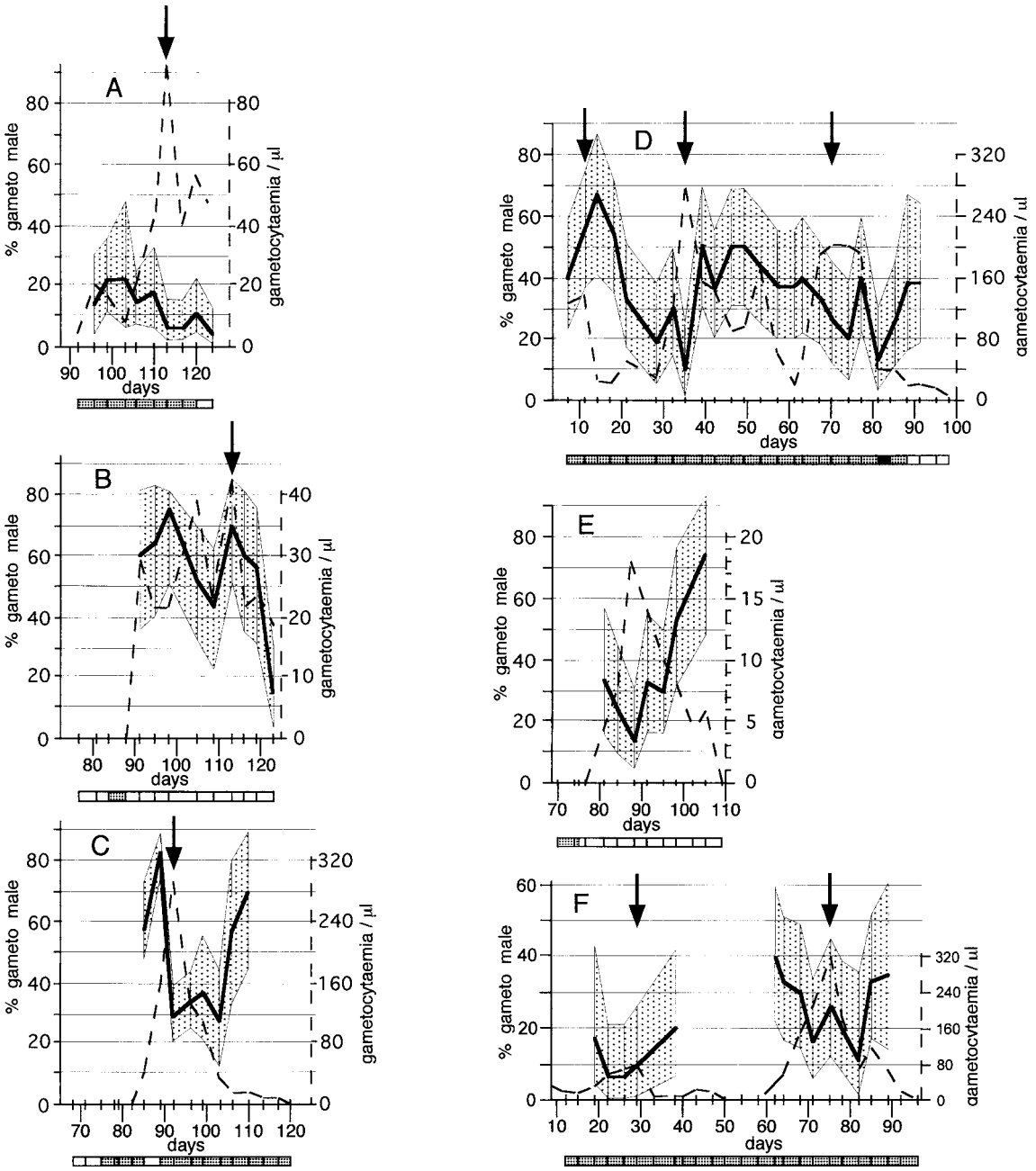

Fig. 2. For legend see opposite.

Among these 614 thick smears, 284 (obtained from 28 individuals) had ⩾10 sexed gametocytes. The mean of their percentages of gametocytes that were male was 27·8% (95% CI 26·4–29·2%), the median 25·0%, with minimum value 0·0% (0 males *vs* 13 females) and maximum value 82·0% (82 males *vs* 18 females). The relative frequencies of these percentages show an asymmetric distribution (Fig. 1).

When considering the 13 villagers who presented at least 9 thick smears with ⩾10 observed gametocytes, the mean percentage of male gametocytes per villager was 26·6% and the median 24·2%. The extreme values of 11·1% and 53·0% were observed respectively in villagers during 29 and 33 consecutive days, each corresponding to a single gametocyte wave (Fig. 2A and B).

The percentage of male gametocytes was negatively correlated with the gametocyte density but not with the trophozoite density ($r=-0{\cdot}130$, $P=0{\cdot}028$ and $r=-0{\cdot}069$, $P=0{\cdot}25$, respectively, with $n=284$ in both cases).

Huge variability was observed during the longitudinal survey of villagers. Sex ratios presented in Fig. 2 were selected with the aim of documenting the heterogeneity of sex ratio over time among some gametocyte carriers. A density-dependent relationship was suspected, with peaks of gametocytes sometimes associated with a minimum in sex ratio (Fig. 2C, D and E). During the downward period of a gametocyte wave, the sex ratio often increased (Fig. 2C and F). Sometimes, frequent variations were observed over a few days (for instance, from

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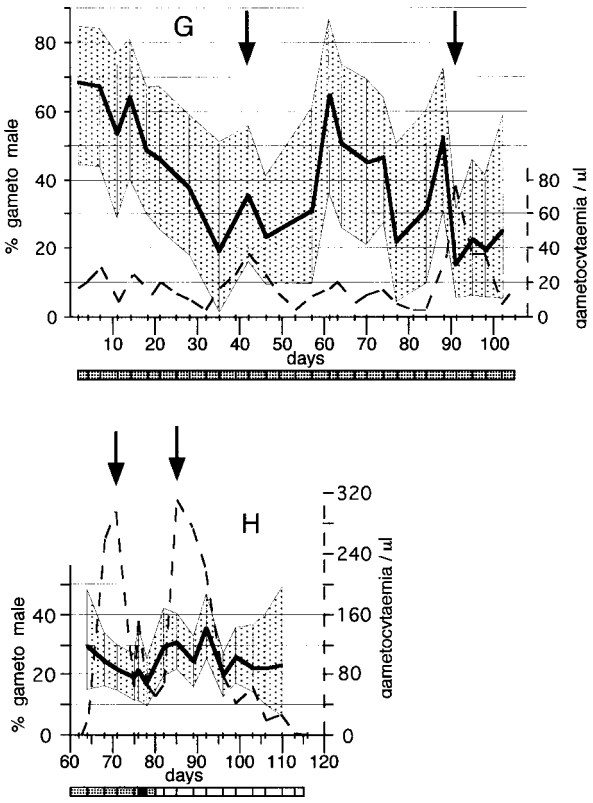

Fig. 2. (A–H) *Plasmodium falciparum* gametocyte sex ratios of 8 individuals to illustrate the range of sex ratio variations over time. Sex ratios are presented for mean (bold line) and exact 95% confidence intervals for binomial distribution (interval in grey between the 2 thin lines). The sex ratio is not indicated if not calculated on at least 10 gametocytes. Arrows indicate the gametocytaemia (dashed line) considered as peak. Abscises indicate the day of follow up, with day 0 as 29 May 1990. Headband under the figure indicate the 3 main levels of *P. falciparum* asexual parasitaemia: no parasites observed on the thick smear (white), parasites (grey), or parasites at very high density and associated with a malaria attack and treatment by quinine (black). Note that the scales of gametocytaemia are not the same in the 5 diagrams. (For information A, B, C, D, E, F, G and H concern respectively the individuals coded 16/11, 05/01, 23/05, 28/13, 04/08, 22/05, 07/05, and 23/06.)

82·0% of male gametocytes at day 89 of the follow up to 29·0% at day 92; Fig. 2C); or slow and continuous variations were observed over 1 month (for instance, from 68·0% at day 2 to 18·2% at day 35; Fig. 2G); or subconstant sex ratio was observed during months (for instance, from 29·0% at day 64 to 23·5% at day 110 with only 1 value among 15 not ranged within 17% and 31%; Fig. 2H). From the simple observation of these curves, it was difficult to extract general trends. Faced with this puzzling data set, the statistical analysis was carried out.

After the first step of the statistical analysis (bivariate analysis) that takes into account the autocorrelation between the successive observations in the same individuals, the following variables had a P value $<0{\cdot}50$: age, haemoglobin type, haemoglobin ratio, haematocrit rate, symptoms at ± 3 days, delay post-quinine treatment, gametocytaemia phase, asexual parasite density, and number of gametocytes observed. These variables were conserved for the second step of the analysis (multivariate). Finally, only the two following variables had significant effect on the gametocyte sex ratio (Table 1). First, a wave of gametocytes was associated with a drop in the proportion of males. This proportion was multiplied by a factor ranging from 0·47 to 0·72 from 7 days before a gametocyte peak to 28 days after the peak (Table 1). It was multiplied by 0·48 (95% CI 0·35–0·66) over the 14 days following a peak. Secondly, a low haematocrit rate was associated with a multiplication by 1·65 (95% CI 1·10–2·47) of the proportion of males. In other words, a wave of gametocytes had female-biased effect, and anaemia had male-biased effect. All the other variables tested were without significant effect on sex ratio: age, sex, sickle cell traits status and clinical status of the gametocyte carrier, quinine treatment, asexual parasite density, variation of the asexual parasite density, and gametocyte density (when taking account of its phases).

DISCUSSION

As far as we know these results on sex ratio of gametocytes of *P. falciparum* are the first published with respect for the natural history of the parasite and obtained from a longitudinal follow up of populations living in a holoendemic village. The following aspects of our results are commented on.

Average sex ratio

In our data set the average sex ratio was 0·346. This value is much higher than the 2 values published for *P. falciparum* in contexts not related to chemotherapy: 0·22 in the town of Yaounde, Cameroon, and 0·18 in villages of Papua New Guinea (Robert *et al.* 1996; Paul *et al.* 1995). These 3 values can be confronted with the level of annual entomological inoculation rate (the number of bites by anophelines with sporozoites in their salivary glands, received per person and per year) estimated to be 173 for *P. falciparum* alone in Dielmo (mean of 5 years, Trape *et al.* 1994; Fontenille *et al.* 1997), 15 in Yaounde (mean of 4 quarters, Fondjo *et al.* 1992; Manga *et al.* 1992) and ranged from 44 to 234 in villages of Papua New Guinea (Burkot *et al.* 1988). In other words, 173, 15 and 44–234 do not constitute a decreasing series as the sex ratio do, and that is surprising: the sex ratio is expected to covary with inoculation rates because it influences the level of selfing (Read *et al.* 1992). We have no clear explanation of these observations.

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Table 1. Prevalence rate ratio (PRR) of male gametocytes, i.e. the chance for one gametocyte to be male, obtained from the multivariate analysis performed with 39 individuals and 552 thick blood smears

	No. of blood smears	PRR	95% confidence interval	*P*
Gametocytaemia phase				$<10^{-4}$
Basic	370	1·00		
1–7 days before a peak of gametocytaemia	41	0·72	0·53–0·96	
Peak	26	0·49	0·34–0·71	
1–7 days after a peak of gametocytaemia	45	0·49	0·36–0·66	
8–14 days after a peak of gametocytaemia	35	0·47	0·33–0·66	
15–21 days after a peak of gametocytaemia	23	0·50	0·38–0·67	
22–28 days after a peak of gametocytaemia	12	0·67	0·33–1·33	
Haematocrit rate				<0·015
⩾32%	462	1·00		
<32%	90	1·65	1·10–2·47	

Variability of sex ratio

Numerous authors had previously noted dramatic variations of sex ratio (Paul *et al*. 1995; Schall, 2000). They concerned inter-populations, inter-individuals, and intra-individuals. The observation of some individuals harbouring an unusual sex ratio implies unexpected host–parasite relationships. Variability over time in the same individual looks erratic in some cases or inversely, looks as regular increase or decrease for weeks. All that is puzzling. Are there independent mechanisms, finely balanced and regulated, that produce male or female gametocytes? Is there selective mortality related to sex of gametocytes? Is their special cytoadherence phenomenon, dependent upon the sex of gametocytes, with consequences for the balance of sexes in the peripheral blood?

Density-dependent relationship

The sex ratio in villagers with the highest gametocyte densities was significantly lower than those observed in the villagers with low density. This observation questions the validity of sex ratio measures performed only in highest gametocyte density, which are, for obvious reasons, easier to obtain. In relation to transmission, when the phase of gametocytaemia is basic (i.e. not during a wave), the gametocyte density in the blood is low and the number of male gametocytes in a bloodmeal may become the limiting factor for the success of mosquito infection. In this occurrence where female gametes may not encounter any male gametes, natural selection favours a high proportion of males (West *et al*. 2002). But during a wave of gametocytaemia, the density of gametocytes is higher and does not constitute a limiting factor anymore. At this time a higher proportion of female gametocytes acts as a selective advantage that favours mosquito infection. To decrease the sex ratio can clearly constitute a selective advantage in increasing the probability of mosquitoes becoming infected (Robert *et al*. 1996; Paul *et al*. 2002). In contrast to this, in *P. maxicanum* and *P. tropiduri*, two parasites of lizards, and in the rodent malaria *P. chabaudi*, sex ratio is positively correlated with gametocyte density (Schall, 2000; Pickering *et al*. 2000; Taylor, 1997); but the basic gametocyte density appears to be much higher than in our data set. Achieving a more male-biased sex ratio is more important for transmitting to mosquitoes when gametocyte numbers are low. With induced *P. falciparum* infections the general trend for the sex ratio is to increase with time and, more specifically, during a single wave of gametocytes (James, 1931; Shute & Maryon, 1951); this observation is not opposed to what was seen in our endemic area with chronic infections, at least in the second part of a wave (i.e. after the peak) when the sex ratio re-increases, up to its basic level.

Population genetics aspects

From a general point of view, selection pressure on sex ratio acts according to the level of isolation of the individuals concerned. In the case of complete isolation leading to obligatory selfing, the gamete sex ratio will tend to be 1 because of higher fitness value. But in a population in which outcrossing is the rule, the production of males will be favoured just because 1 male gametocyte produces several male gametes, in contrast to females that produce only 1 (Read *et al*. 1992; Paul *et al*. 1995). It is now well documented that in high transmission areas, people are infected with a mix of different genotypes and thus different phenotypes (Babiker, Ranford-Cartwright & Walliker, 1999). Each clone of this population follows its own dynamics of multiplication leading to changes of genetic profiles from day to day (Daubersies *et al*. 1996; Farnert *et al*. 1997). Clinical attacks constitute particular population genetics events due to the incoming of a new strain in the peripheral bloodstream, which becomes rapidly dominant (Contamin *et al*. 1995). The *P. falciparum* populations in Dielmo

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are typical of those from a holoendemic area i.e. mixed populations with ancient episodes of isolation (Konaté *et al*. 1999). We can thus speculate that this situation leads to various sex ratios from clone to clone. It is conceivable that a gametocyte wave is not due to the whole parasite population of the infected people but is due to the quantitative increase of a single parasite strain. Moreover, a parasite with a long history of seasonal transmission will be selected to produce many gametocytes, in a short period of time, with a gamete sex ratio equal to 1. Thus the reduced sex ratio observed during gametocyte waves, could result from a single clone with a lower sex ratio invading a population of clones with higher sex ratio value. Unfortunately we are unable to identify the specific genotypes of the gametocytes at the origin of one wave. A PCR *in situ* approach could be of use in order to confirm or deny this assertion.

Anaemia relationship

The low haematocrit rate was clearly identified to be associated with a high proportion of male gametocytes. This expected finding is in line with previous ones made on *P. gallinaceum* and on patients with uncomplicated *P. falciparum* attacks in a hypoendemic area (Paul *et al*. 2000, 2002). Interestingly it had been observed that the blood feeding success of anophelines was reduced when taken on anaemic mice (Taylor & Hurd, 2001). In this context a possible relationship between lower gametocyte intake and higher proportion of males can be hypothesized as a parasite response, via the signal of high erythropoietin level, to increase the mating success of the parasite and thus to maintain an equal transmission rate to the mosquito.

Finally, we described here the natural history of gametocyte sex ratio of *P. falciparum* gametocytes in a holoendemic village. The huge variability in sex ratio was highlighted. Variables implicated in its variations were identified. As a concluding remark we would point to the basic difficulty of estimating the sex ratio on classical thick smears, in the context of low gametocyte densities. In the future, other methods of estimation permitting the examination of larger numbers of gametocytes will increase the power of statistical analysis.

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