Density-dependent impact of the human malaria parasite *Plasmodium falciparum* gametocyte sex ratio on mosquito infection rates

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Malaria parasites produce male and female life cycle stages (gametocytes) that must fertilize to achieve successful colonization of the mosquito. Gametocyte sex ratios have been shown to be under strong selection pressure both as an adaptive response to a worsening blood environment for transmission and according to the number of co-infecting clones in the vertebrate. Evidence for an impact of sex ratio on the transmission success of *Plasmodium falciparum* has, however, been more controversial. Theoretical models of fertilization predict that increasingly male sex ratios will be favoured at low gametocyte densities to ensure fertilization. Here, we analyse *in vitro* transmission studies of *P. falciparum* to *Anopheles gambiae* mosquitoes and test this prediction. We find that there is a discernible effect of sex ratio on transmission but which is dependent upon the gametocyte density. While increasingly male sex ratios do give higher transmission success at low gametocyte densities, they reduce success at higher densities. This therefore provides empirical confirmation that sex ratio has an immediate impact on transmission success and that it is density-dependent. Identifying the signals used by the parasite to alter its sex ratio is essential to determine the success of transmission-blocking vaccines that aim to impede the fertilization process.

Keywords: *Plasmodium falciparum*; *Anopheles gambiae*; gametocyte sex ratio; transmission success

1. INTRODUCTION

The transmission of *Plasmodium* spp. from the vertebrate host to the insect vector is accomplished solely by the sexual stages, the male or female gametocytes, which are produced from proliferating asexual stage parasites and that transform into gametes once inside the insect host's bloodmeal (Carter & Graves 1988). A single haploid parasite clone can produce both male and female gametocytes (Sinden 1983). Fertilization of a female gamete by a male must be achieved for the successful establishment of the parasite in the vector, as evident by the presence of oocysts that will yield the sporozoite stage necessary for infection of the vertebrate host. Whereas each female gametocyte produces one female gamete, each male gametocyte can produce up to eight male gametes (Talman *et al.* 2004). The gametocyte sex ratio in the vertebrate host is generally female-biased, potentially balancing the greater number of male gametes per gametocyte and hence maximizing fertilization and transmission success (Schall 1989). This would be the case if the infections were always comprised of a single genotype and thus would be effectively clonal in their sexual reproduction. However, infections are very often comprised of several genetic clones and evolutionary theory predicts that the sex ratio becomes less female-biased as clone number increases, because of

competition among clones for mating success—local mate competition (LMC) (Hamilton 1967; Read *et al.* 1992; Reece *et al.* 2008; Schall 2009). This is because clones that produce more males will be present at a higher frequency among the mating males and hence obtain a disproportionate share of the mates (West *et al.* 2001). Animal model studies have recently provided evidence that parasites can facultatively alter their sex ratio according to the number of co-infecting clones (Reece *et al.* 2008).

In addition to adaptive sex allocation according to co-infecting clone number, whereby a parasite clone maximizes its transmission success relative to co-infecting clones, parasites are also thought to alter their sex allocation to ensure successful fertilization. Fertility insurance, whereby the parasite must produce enough males to fertilize its females, is predicted to be important when either the actual gametocyte density is very low or when the gametocyte viability and efficacy are poor (Paul *et al.* 1999). Moreover, such fertilization insurance can impact upon the optimal sex ratio as predicted by LMC (West *et al.* 2002; Gardner *et al.* 2003). There is some evidence that the parasite facultatively alters its sex allocation to ensure fertilization. The sex ratio of *Plasmodium falciparum*, the aetiological agent of malignant tertian human malaria, becomes increasingly male during the course of infection (Shute & Maryon 1951; Paul *et al.* 2002; Robert *et al.* 2003) and animal model studies suggest that this is in response to a worsening blood environment that reduces male gamete efficacy (Paul *et al.* 2000).

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Although there is now evidence that the gametocyte sex ratio is influenced by natural selection, empirical data relating the gametocyte sex ratio to transmission success have been more controversial. Studies by *Boyd et al.* (1935) and *Boyd* (1949) postulated that the number of males, rather than females, determine infection success. *In vitro* transmission studies using different clones of *P. falciparum* from a single infection isolate showed that the clones with a higher male sex ratio were more infectious to mosquitoes (*Burkot et al.* 1984). However, other *in vitro* studies of mosquito infection rates found no effect of the gametocyte population structure, including sex ratio, on infection success (*Noden et al.* 1994). A cross-sectional study assessing the effect of the sex ratio on the infectiousness of natural *P. falciparum* infections in man revealed the same tendency for highest transmission success to be associated with a higher proportion of male gametocytes (*Robert et al.* 1996). Transmission studies in natural *P. mexicanum* infections in lizards also found transmission improved with a higher proportion of male gametocytes, but this was the result of the higher gametocyte densities (*Schall* 2000). Thus, paradoxically, when sex ratio is shown to have a significant effect on transmission success, males are highlighted as the limiting factor and yet the majority of natural infections are female-biased. Animal model studies demonstrated that experimental manipulation of the sex ratio reduced transmission success (*Paul et al.* 2000), and thus producing the correct sex ratio at the appropriate moment was critical. *Reece et al.* (2008) also provided experimental evidence for an effect of sex ratio on transmission success, controlling for gametocyte density. Crucially, the theoretical models based on observations of sex ratio, gametocyte density and transmission predict that there will be an interaction between sex ratio and gametocyte density and that increasingly male sex ratios would become increasingly important when gametocyte density is low, but should have no effect or even reduce transmission success at higher gametocyte densities (*Paul et al.* 1999; *West et al.* 2002; *Gardner et al.* 2003).

Here we test the prediction that there is an interaction between gametocyte sex ratio and density with respect to transmission success by analysis of controlled *in vitro* transmission studies of *P. falciparum* to its vector mosquito *Anopheles gambiae*, where the impact of the human immune state is excluded.

2. MATERIAL AND METHODS

(a) Gametocyte culture and sex determination

Gametocytes of *P. falciparum* NF54 isolate were produced in an automated large-scale culture system as described previously (*Ponnudurai et al.* 1983). NF54 was grown in 10 ml RPMI 1640 medium (PAA), supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid and L-glutamine, 10 per cent heat-inactivated human serum and sodium bicarbonate (0.2% final concentration) under a constant gas supply (5% CO₂, 1% O₂, 94% N₂). Fresh O+ red blood cells (RBCs) were added for a 7 per cent haematocrit. All cultures were initiated at 0.5 per cent parasitaemia. Fourteen days after initiating the culture, gametocyte maturity was assessed on Giemsa-stained thin blood smears. Differences among the parasite cryostablate and the donor RBCs used can generate considerable

variation in gametocyte phenotypes. The NF54 isolate is composed of several parasite clones and thus the clonal diversity and relative abundance is expected to vary among cryostablates, with potential consequences for observed gametocyte sex ratio and density; clones from a single isolate have been shown to differ in their capacity for gametocyte production (*Graves et al.* 1984) and sex ratio (*Burkot et al.* 1984). Moreover, gametocyte sex ratio and especially density exhibit some clonal variation due to environmental influences (*Burkot et al.* 1984; *Graves et al.* 1984). In particular, the nature of the RBCs and the presence of very young RBCs (reticulocytes) have been shown to have a strong effect on gametocyte production *in vitro* (*Trager et al.* 1999). Mature gametocytes were sexed by morphological criteria as previously described (*Carter & Graves* 1988; *Robert et al.* 1996). Briefly: (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. The gametocyte sex ratio is defined as the proportion of all mature gametocytes that are male. Furthermore, male gametocyte maturity was verified by performing an exflagellation test as previously described (*Bhattacharyya & Kumar* 2001).

(b) Mosquito infection experiments

Gametocyte cultures were centrifuged for 5 min at 1500g and resuspended in fresh RBC (O+) and AB human serum to give a final haematocrit of 40 per cent. This was then deposited in a Parafilm membrane feeder previously warmed at 37°C. For each feed at least 100 nulliparous *A. gambiae* Yaounde strain were left to feed in the dark for 15 min and only fully engorged females were kept for further analysis. Gorged mosquitoes were transferred to small cages and provided with 10 per cent sucrose supplemented with final 0.05 per cent para-amino benzoic acid. Gorged mosquitoes were kept for 8 days at which time a minimum of 30 (when possible) were dissected for the detection of oocysts. A total number of 3501 *A. gambiae* mosquitoes were dissected. The median number per experimental feed was 30 (range 15–50). Midguts were stained with 0.1 per cent mercurio-bromo fluorescein (Fluka) in 1× phosphate buffered saline. The number of females harbouring at least one oocyst and the number of oocysts were counted to determine the prevalence as well as the intensity of infection.

(c) Statistical analyses

All statistical analyses and maximum-likelihood plots were performed using the statistical program GENSTAT v. 7.1. The impact of gametocyte density and sex ratio (arcsine transformed) on the proportion of gorged mosquitoes that had oocysts was analysed by fitting a generalized linear mixed model (GLMM) with a binomial error structure (logistic regression) with experimental replicate nested within the experiment fitted as factors in the random model. This random term takes into account repeated measures (replicates) of feeds on the same culture, as well as variation owing to individual differences among experiments. To examine the impact of sex ratio more rigorously and thus take into account the within-culture sex ratio variation, we then repeated the analysis using only the data falling into the upper and lower quartiles of the observed sex ratios (i.e. the extreme values).

The intensity of infection (i.e. oocyst number per mosquito) was analysed by fitting a GLMM with a Poisson error structure (log-linear regression) and again with replicate nested within the experiment fitted as factors in the random model. Oocyst analyses and calculated means include the uninfected mosquitoes that had taken a blood meal from a feed (i.e. the zeros are not excluded); ungorged female mosquitoes were excluded. In addition to evaluating the effect of gametocyte density and sex ratio on the prevalence and intensity of infection, we considered a third response variable, the transmission efficiency. Transmission efficiency is calculated as the number of gametocytes that are required to generate one oocyst, using mean oocyst number per feed. The effect of gametocyte density and sex ratio on transmission efficiency was again analysed by fitting a GLMM with a Poisson error structure (log-linear regression) and again with replicate nested within the experiment fitted as factors in the random model. In all analyses, because the data were over-dispersed, a dispersion parameter was estimated. Wald statistics, which approximate to a χ^2 distribution, were established in the GLMM.

3. RESULTS

From 2006–2008, 148 mosquito infections were carried out on 95 independent *in vitro* cultures of *P. falciparum* gametocytes. The number of replicate feeds on the same cultures varied from 1 to 5 (mode 1). The median gametocyte density was $23\,250\ \mu\text{l}^{-1}$ (lower quartile 14 400; upper quartile 33 959; range 402–72 450; figure 1a). The median proportion of gametocytes that was male (sex ratio) was 0.46 (lower quartile 0.35; upper quartile 0.52, range 0.04–0.76; figure 1b). Sex ratio did not alter with gametocyte density ($\chi^2 = 0.19$, $p = 0.66$, $n = 95$). The percentage of mosquitoes infected varied from 13 to 100 per cent (mean 57.6%), the number of oocysts per infected mosquito from 1 to 491 and the mean oocyst density per mosquito batch, including uninfected but gorged mosquitoes, ranged from 0.2–66 oocysts per mosquito.

Neither sex ratio ($\chi^2 = 0.54$, $p = 0.46$) nor gametocyte density ($\chi^2 = 1.06$, $p = 0.30$) had an effect on the proportion of mosquitoes infected when each was considered independently. However, there was a significant interaction between the gametocyte density and the sex ratio on the proportion of mosquitoes infected ($\chi^2 = 6.16$, $p = 0.013$, $n = 148$), explaining 3.6 per cent of the observed variation (i.e. adjusted r^2). Using only sex ratios falling into the upper and lower quartiles, the impact of the interaction between gametocyte density and sex ratio on the proportion of mosquitoes infected accounted for a greater proportion of the observed variation ($\chi^2 = 7.69$, $p = 0.006$, $n = 79$, adjusted $r^2 = 4.0\%$). The proportion of mosquitoes infected decreased with male-biased sex ratios as gametocyte density increased and vice versa for female-biased sex ratios. Increasing gametocyte density significantly increased oocyst number per mosquito ($\chi^2 = 8.92$, $p = 0.003$, $n = 3501$), although explained very little of the observed variation in oocyst number (adjusted $r^2 = 0.001$); there was no effect of sex ratio ($\chi^2 = 0.17$, $p = 0.68$) (or any interaction, $\chi^2 = 0.13$, $p = 0.72$). However, transmission efficiency (i.e. the number of gametocytes required to generate one oocyst) was affected by both gametocyte

Figure 1. (a) Distribution of gametocyte densities obtained in the 95 independent *in vitro* cultures of *P. falciparum*, NF54 isolate. (b) Distribution of gametocyte sex ratios obtained in the 95 independent *in vitro* cultures of *P. falciparum*, NF54 isolate.

density ($\chi^2 = 8.94$, $p = 0.003$, $n = 148$), and the interaction between density and sex ratio ($\chi^2 = 11.3$, $p < 0.001$) explaining, respectively, 9.1 and 8.8 per cent of the observed variation (i.e. adjusted r^2). As previously shown, transmission efficiency decreases with increasing gametocyte density, because oocyst burden plateaus at high gametocyte densities (Rutledge *et al.* 1969; Paul *et al.* 2007). Plotting the impact of sex ratio on the transmission efficiency between arbitrarily (above versus below the observed median density) defined high ($>24\,000\ \mu\text{l}^{-1}$) versus low ($<24\,000\ \mu\text{l}^{-1}$) gametocyte densities shows the density-dependent impact of sex ratio: at low gametocyte densities, the number of gametocytes required to generate an oocyst decreases with increasingly male sex ratios, whereas at high densities increasingly female sex ratios are more fertile (per gametocyte) (figure 2).

4. DISCUSSION

In this study, we provide evidence from controlled *in vitro* conditions that gametocyte sex ratio has a significant impact on parasite transmission success and that the effect is dependent upon the gametocyte density. This concurs with predictions from theoretical models (Paul *et al.* 1999; West *et al.* 2002; Gardner *et al.* 2003) and observations of induced infection studies (Paul *et al.* 2000, 2002) that predict an effect of sex ratio, and most especially maleness, when gametocyte density is low. The increased significance of the effect of the interaction when using high versus low sex ratios further confirms this. Despite the confounding factors that must influence transmission success among experiments, the percentage

Figure 2. The relationship between the transmission efficiency (number of gametocytes per mean oocyst number) and the gametocyte sex ratio (arcsine transformed) according to high (grey circles) or low (filled triangles) gametocyte densities. Lines (dotted, high gametocyte density; solid, low) shown are the best-fit maximum-likelihood models established by fitting a GLMM with gametocyte density (a factor of two levels less than or greater than 24 000 gametocytes μL^{-1}) and sex ratio (arcsine transformed) fitted as interacting terms.

of the observed variation in transmission success explained by gametocyte density–sex ratio interaction was significant whether considering the proportion of mosquitoes infected or the transmission efficiency. The lack of main effects for sex ratio and gametocyte density on transmission success contrasts with the previous findings (e.g. Paul *et al.* 2007; Reece *et al.* 2008), but probably reflect the asymptotic relationship between density and mosquito infection rates (Paul *et al.* 2007): at high gametocyte densities, additional increases in density yield no significant increases in infection rates. It is therefore all the more striking that when sex ratio is skewed, density clearly can affect the proportion of mosquitoes infected, as shown by the all-important interaction term. The small influence of gametocyte density and the absence of an effect of sex ratio on the intensity of infection may be the result of the very large differences in oocyst number per mosquito even for the same feed. Such an aggregated distribution is well known in natural systems (Billingsley *et al.* 1994), resulting from natural variation in mosquito susceptibility (Riehle *et al.* 2006) and parasite apoptosis when at high densities within the mosquito (Al-Olayan *et al.* 2002; Hurd *et al.* 2005). High levels of parasite death occurring during the early stages of development in the mosquito (Vaughan 2007) probably smother any discernible direct effects of gametocyte phenotypes (density and sex ratio) on oocyst number.

The gametocyte density-dependent impact of sex ratio observed in our study suggests an explanation for observations on natural infections of *P. falciparum*, where female-biased sex ratios dominate despite a general tendency for increased transmission success with maleness (Boyd 1949; Burkot *et al.* 1984; Robert *et al.* 1996).

Transmission success would be expected to increase with more male sex ratios when effective gametocyte density is lower. This will be the case not only when actual observed densities are low, but also if there is any interference in fertilization success resulting from the host immune response. Antibody-dependent transmission-blocking immunity increases with concurrent gametocyte densities (Boudin *et al.* 2005) and fever, induced by the asexual parasitaemia, also reduces infectivity (Naotunne *et al.* 1991; Gouagna *et al.* 2004). Therefore, although measures of gametocyte density, sex ratio and transmission success in natural *P. falciparum* infections provide invaluable information, there is a need to consider gametocyte viability to reveal the impact of sex ratio on transmission success. In the *in vitro* system, the impact of immunological factors can be controlled for, but the sex-specific gametocyte viability is unquantifiable. The gametocyte densities observed here were generally much greater than those in natural infections, where mean densities range from 2 to 60 μL^{-1} in asymptomatic infections, but can reach over 1000 μL^{-1} in symptomatic infections (see references in Paul *et al.* 2007). The viability of gametocytes from a long-term *in vitro* isolate, however, is almost certainly lower than that of field clones continually selected for transmission, thus limiting over-extrapolation from the *in vitro* system to the field setting. Nevertheless, the density-dependent impact of sex ratio on transmission will play a role in determining transmission success and shaping parasite sex ratios.

Animal model studies have shown how malaria parasites modulate sex ratio according to both environmental conditions (Paul *et al.* 2000) and co-infecting clone number (Reece *et al.* 2008). The mechanisms by which the parasite achieves such adaptive sex allocation remain elusive, but probably depend on key host/parasite factors enabling it to monitor the permissiveness of the environment for transmission and to detect other clones. If parasites are capable of facultatively altering their sex allocation according to gametocyte density and if male sex ratios are more critical at low gametocyte densities, there should be a negative correlation between sex ratio and (viable) gametocyte density. Strong correlations between gametocyte density and sex ratio have been previously observed in animal models (Paul *et al.* 2000; Reece *et al.* 2008). They were not observed here, however, suggesting that whatever cues the parasite might use to assess gametocyte density are not present in the *in vitro* system and may thus also be host-derived. Clones derived from a single isolate have previously been shown to have different *in vitro* sex ratios (Burkot *et al.* 1984), which would contribute considerable noise to any straightforward relationship with gametocyte density *in vitro*. Interestingly, the co-occurrence of clones expressing different ‘stable state’ sex ratios suggests that parasite may coexist by adopting sex-allocation strategies that are differentially beneficial according to the current infection environment.

Currently, several transmission-blocking vaccines, which target gamete proteins and parasite fertilization, are being tested (Carter *et al.* 2000; Targett & Greenwood 2008). The extent to which such vaccines circumvent the parasite’s sex-allocation plasticity will be fundamental to their success. That a single parasite clone is capable of producing a large range of sex ratios (i.e. exhibits

considerable phenotypic plasticity) and able to facultatively alter its sex ratio according to the co-infecting clone number (Reece *et al.* 2008) and environmental permissiveness (Paul *et al.* 2000), suggests that the parasite might adaptively alter its sex ratio to respond to the immediate environmental consequences of vaccination. This crucially depends on the signals used by the parasite. Identification of the major environmental factors governing sex determination clearly necessitates more attention.

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