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Evolutionary sex allocation theory explains sex ratios in natural *Plasmodium falciparum* infections

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Abstract

Malaria transmission is achieved by sexual stages, called gametocytes, and the proportion of gametocytes that are male versus female (sex ratio) influences transmission success. In malaria model systems, variation in gametocyte sex ratios can be explained by the predictions of evolutionary sex allocation theory. We test these predictions using natural *Plasmodium falciparum* infections. The predicted negative correlation between sex ratio and gametocyte density holds: the sex ratio increases when gametocyte densities decrease, and this is most apparent in single genotype infections and in the dry season. We do not observe higher gametocyte sex ratios in mixed compared with single genotype infections.

Keywords: Fertility insurance, Local mate competition, Sex allocation, Gametocyte density, Seasonal malaria transmission, Competition

Malaria parasites (*Plasmodium* spp.) replicate asexually in their vertebrate hosts, but require a single round of sexual reproduction to achieve between-host transmission via mosquito vectors. Sexual stages, called gametocytes, are formed in the vertebrate host and both the density and sex ratio (defined as the proportion that are male) of gametocytes shape parasite fitness/transmission (Reece et al., 2008; Mitri et al., 2009; Bradley et al., 2018). Gametocyte sex ratios of malaria parasites vary considerably, both during infections and in different hosts. Studies using a variety of model systems (rodent and lizard parasites, and the human parasite *Plasmodium falciparum* in vitro) have demonstrated that variation in gametocyte sex ratios is influenced by parasite genotype, genetic diversity of infections (multiplicity of infection, MOI), transmission-blocking immunity, and the density of red blood cells, reticulocytes and gametocytes (Paul et al., 2000, 2002; West et al., 2001; Reece et al., 2005, 2008; Ramiro et al., 2011).

Evolutionary sex allocation theory can explain why parasite fitness is maximised by adjusting sex ratio in response to the above factors (West et al., 2001; Paul et al., 2002; Gardner et al., 2003; Reece et al., 2008; Neal and Schall, 2014). Evolutionary sex allocation theory predicts that each clone within an infection should adjust its gametocyte sex ratio in response to the number of co-infecting clones within the host, gametocyte density and the number of gametes produced by each male gametocyte (Nee et al., 2002; Gardner et al., 2003). Specifically, in single genotype infections, the number of offspring is maximised (and thus, competition for females is minimised; “local mate competition theory”) when just enough males are produced to fertilise all females. For malaria parasites this means a female-biased gametocyte sex ratio (up to eight females per male) because female and male gametocytes each transform into one female gamete or up to eight male gametes, respectively. However, malaria infections often contain multiple parasite genotypes and males can fertilise females from their own as well as unrelated genotypes. Whereas fitness for parasites in single genotype infections is determined by the total number of offspring, the fittest genotype for outbreeding parasites in mixed genotype infections is that with

the highest genetic representation in the next generation (i.e. that with the highest proportion of matings). Thus, higher sex ratios are favoured in mixed genotype infections because each genotype can take advantage of the females belonging to co-infecting genotypes by producing male gametocytes, the more fecund sex (Supplementary Fig. S1A). However, at low gametocyte densities (e.g. due to low gametocyte conversion or during anaemia) or when male gametocyte survival and/or fecundity is low (e.g. due to immunity or drugs, which both can disproportionately affect males), the probability that a blood meal contains enough males to fertilise all females is reduced (Paul et al., 2002; Ramiro et al., 2011; Delves et al., 2013). Therefore, parasites should compensate by increasing their investment in males (West et al., 2001; Gardner et al., 2003) (Supplementary Fig. S1B).

Experiments with *Plasmodium chabaudi* in vivo and *P. falciparum* in vitro are consistent with predictions that higher sex ratios are produced in mixed genotype infections, and when fertility insurance is required to avoid male limitation (Paul et al., 2002; Reece et al., 2008; Mitri et al., 2009). Data from natural *P. falciparum* infections are scarce, but higher sex ratios are observed in mixed genotype infections and in anaemic hosts (Read et al., 1992; Robert et al., 2003; Sowunmi et al., 2009a, 2009b). Other studies - generally related to *Plasmodium* spp. infecting lizards - do not support fertility insurance theory but these produce high gametocyte densities and therefore may not require fertility insurance (Neal and Schall, 2014). However, whether sex ratios are adjusted according to variation in the genetic diversity of infections and male limitation have rarely been tested simultaneously in natural infections with parasite species expected to adopt both sex allocation strategies (Neal and Schall, 2014). Here, we examine whether the rules of sex allocation theory apply to the gametocyte sex ratios of *P. falciparum* in natural infections.

Until the recent development of sex-specific reverse-transcriptase quantitative PCR (RT-qPCR) assays (Schneider et al., 2015; Santolamazza et al., 2017; Stone et al., 2017), methods to determine the sex ratio of *P. falciparum* gametocytes were laborious and suffered from low

accuracy, particularly at low densities. We took advantage of sex-specific RT-qPCR assays to test whether gametocyte sex ratios in natural *P. falciparum* infections in Asar village, Eastern Sudan, follow the predictions of theories for local mate competition and fertility insurance. In this region, transmission is distinctly seasonal (September–December), chronic infections may persist asymptomatically throughout the year and mixed genotype infections are common (Gadalla et al., 2016). Ethical clearance was granted by the Ethical Committee of the Sudanese Ministry of Health. A detailed description of the study and methods used has been previously published (Gadalla et al., 2016). Briefly, individuals with symptomatic *P. falciparum* infections were enrolled after informed consent was obtained. Monthly blood samples were collected between November 2001 and October 2002, and another sample in December 2002. Symptomatic infections were treated with antimalarial drugs after samples were taken, according to national treatment guidelines at the time of the study. Therefore, we investigate sex ratios independently of any impact of drugs. Thirty-one individuals had at least three parasite-positive samples during the study and produced gametocytes, resulting in a total of 98 gametocyte-positive samples. Gametocytes, sex ratio and MOI (minimum number of genotypes present) were quantified by sex-specific RT-qPCRs and *P. falciparum* gene Pfg377 genotyping, respectively (Abdel-Wahab et al., 2002; Gadalla et al., 2016) (see Supplementary Methods S1). Using these samples, we tested the predictions (Supplementary Fig. S1) that gametocyte sex ratios are negatively correlated with gametocyte densities (fertility insurance) and are higher in mixed versus single genotype infections (local mate competition).

Plasmodium falciparum gametocyte sex ratios vary considerably between samples, ranging from all male to all female. Overall, as expected, the majority of samples (94%) have a female-biased gametocyte sex ratio (mean $0.07 \pm \text{S.E.M. } 0.02$). The relationship between gametocyte density and sex ratio varies with MOI (interaction between MOI and gametocyte density: $\chi^2_1 = 76.06$, $P < 0.001$) (Fig. 1A, model A in Table 1). Despite low MOI in this area (mean minimum number of genotypes present $1.55 \pm \text{S.E.M. } 0.07$), we observe a difference in sex ratio between

single and mixed genotype infections (Fig. 1A). This is, however, the opposite of the pattern predicted by local mate competition theory: sex ratios are higher in single than in mixed genotype infections. Further, in single infections, the sex ratio decreases with increasing gametocyte densities, following the logistic pattern predicted by fertility insurance (Supplementary Fig. S1B) (effect size $-2.08 \pm \text{S.E.M. } 0.13$; $z=-15.60$, $P<0.001$), but not in mixed genotype infections (effect size $0.33 \pm \text{S.E.M. } 0.39$; $z=0.84$, $P=0.40$) (Fig. 1A). Our data originate from an area with distinct seasonal transmission and parasites are suggested to adopt different transmission strategies in different seasons (Cornet et al., 2014; Gadalla et al., 2016; Pigeault et al., 2018). Accounting for the impact of season on fertility insurance (interaction between season and gametocyte density, $\chi^2_1 = 7.06$, $P=0.008$) and local mate competition (interaction between season and MOI, $\chi^2_1 = 18.43$, $P<0.001$) significantly improves the explanatory power of our model ($\Delta\text{AIC}=62.97$; model B in Table 1). When season is taken into account, fertility insurance is detectable in both single and mixed genotype infections (Fig. 1B and C). Specifically, fertility insurance is more apparent in single compared with mixed genotype infections (interaction between season and MOI, effect size $-0.93 \pm \text{S.E.M. } 0.38$; $z=-2.46$, $P=0.014$), and in the dry season compared with the wet transmission season (interaction between season and gametocyte density, effect size $-0.81 \pm \text{S.E.M. } 0.31$; $z=-2.59$, $P=0.010$). It is possible that the observed impact of MOI may vary with season, but we cannot formally test for this because the model including the interaction between season, gametocyte density and MOI does not converge.

Adjusting the ratio of male versus female gametocytes should enable malaria parasites to optimise gametocyte sex ratios in response to changing in-host conditions in a manner that maximises fitness. Our data suggest that the complex patterns of gametocyte sex ratios for *P. falciparum* parasites in natural infections are consistent with predictions of fertility insurance theory concerning male limitation. In addition, our data suggest that fertility insurance is more apparent in single compared with mixed genotype infections, and in the dry compared with the

wet transmission season. We find no evidence for local mate competition in relation to MOI. Our marker to assess the extent of multiplicity (Pfg377) has at least five alleles in this parasite population (Abdel-Wahab et al., 2002) and it is possible that multiple genotypes within an infection share the same allele. If so, then our approach is conservative; the difference between single and mixed infections is likely greater. However, whilst we found a difference in sex ratios between single and mixed genotype infections, the pattern was the opposite of that predicted: sex ratios are more female-biased in mixed genotype infections. There are several potential explanations for this result. In theory, male gametocytes may be detected less efficiently in mixed genotype infections. However, sex-specific gametocyte assays (Schneider et al., 2015; Santolamazza et al., 2017; Stone et al., 2017) target conserved areas of the genome and there is no evidence that these assays function differently between single and mixed genotype infections. Our study reports a mean sex ratio lower than most field studies (Tadesse et al., 2019). This could be associated with males being detected less efficiently than females (Schneider et al., 2015; Stone et al., 2017). If samples with low gametocyte densities had particularly high sex ratios but were not detected, and therefore not included in our study, the true population mean may be closer to the theoretical minimum of approximately 0.1 (Tadesse et al., 2019). Moreover, any problems introduced by low gametocyte densities cannot explain why local mate competition is not apparent at higher gametocyte densities. Additionally, whereas mixed genotype infections in this seasonal transmission area are common, the genotype ratios within infections may be heavily skewed. If so, rare genotypes may be hard to detect by PCR. However, by definition rare genotypes will not influence the genetic diversity of gametocyte mating groups much and thus sex ratios that are best in single infections remain optimal (or very close to) in this case. Further research is required to investigate how proportional representation of genotypes within mixed genotype infections impacts sex ratios.

Finally, we measured sex ratios of mature gametocytes and thus some, potentially sex-specific, gametocyte death may have occurred. Male gametocytes are more vulnerable to drug treatment and to immune attack (e.g. Ramiro et al., 2011; Delves et al., 2013; Dicko et al., 2018). If such immune responses are stronger in mixed genotype infections, male mortality rates may be elevated substantially beyond that for which fertility insurance can compensate. High male mortality could simultaneously dampen fertility insurance (especially in mixed genotype infections) and local mate competition. Elevated male mortality may also explain observations that: (i) *P. chabaudi* matches sex ratio to the predictions of local mate competition and fertility insurance before infections peak and immune responses develop (Reece et al., 2008); (ii) why parasites in natural, chronic *Plasmodium mexicanum* infections do not match local mate competition - compared with experimental infections of naïve lizards (Neal and Schall, 2014); and (iii) because antimalarial immune responses peak during the wet season, compared with the dry season in Eastern Sudan (e.g. Cavanagh 1998), parasites match fertility insurance better in the dry season. However, explaining why parasites cannot sufficiently increase male production in mixed genotype infections to compensate for high levels of male-specific mortality is a challenge. Non-mutually exclusive hypotheses include that parasites may have an upper limit in their ability to detect male killing (or its proxies); mechanistic constraints on development may limit the production of males; or increasing male production even more may be counterproductive if subsequent killing of these males disproportionately stimulates anti-male immune responses.

Despite the unexpected results for sex ratios in mixed genotype infections, in a seasonal malaria setting in Eastern Sudan we observed fertility insurance in natural human malaria infections. Facultative adjustment of gametocyte sex ratios in natural infections has implications for disease control. For example, parasites that are exposed to interventions with sex-specific effects (e.g. drug treatment disproportionately affecting male gametocytes or transmission-reducing vaccines targeting females only) can adjust both their conversion rates and gametocyte

sex ratio to partially compensate, thus undermining the efficacy of such interventions (Reece et al., 2008; Wu et al., 2008; Sowunmi et al., 2009b; Ramiro et al., 2011; Delves et al., 2013; Dicko et al., 2018). Furthermore, if the cues that parasites use to determine the appropriate level of fertility insurance can be identified, parasites could be tricked into producing suboptimal gametocyte sex ratios, thus reducing transmission (Carter et al., 2014). Finally, we recommend future studies extend our approach to different epidemiological settings, categorise data by season, and gather data on immunity to explore the extent to which male-biased mortality affects the ability of *P. falciparum* to follow the rules of sex allocation theory.

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Figure Legend

Fig. 1. Fertility insurance in natural *Plasmodium falciparum* infections. Fertility insurance is predicted in single, but – at first sight – not mixed genotype infections (A). When season is controlled for, fertility insurance is detected in all infections ((B) dry season in red; (C) wet transmission season in blue). Data shown are mean sex ratios \pm S.E.M. for single (grey circles) and mixed (black triangles) genotype infections. Predicted sex ratios are shown for single (coloured circles, solid line) and mixed genotype infections (coloured triangles, dashed line). Predictions from mixed effect logistic regression models ((A) model A in Table 1; (B and C) model B in Table 1) are at population level, i.e. after controlling inter- individual patient variation to reveal overall patterns. Shaded areas represent 95% prediction intervals, which were estimated by data simulation ($n=1000$; arm package, R v3.2.4, see Supplementary Methods S1). Predictions from mixed effect logistic regression models (model B in Table 1) before inverse logit back transformation are shown for the two season groups in Supplementary Fig. S2A-B.

Supplementary figure legends

Supplementary Fig. S1. Cartoon to illustrate the predictions of evolutionary sex allocation theory, applied to malaria parasites. (A) Local mate competition: the sex ratio (defined as the proportion of gametocytes that are male) is predicted to be positively correlated to the multiplicity of infection (single or mixed genotype infections). (B) Fertility insurance: the sex ratio is predicted to be negatively correlated to gametocyte densities. (C) Combined prediction for sex ratios in single (solid line) and mixed (dashed line) genotype infections.

Supplementary Fig. S2. Fertility insurance in natural *Plasmodium falciparum* infections; untransformed model outcomes. Significant fertility insurance is detected in all infections when season is controlled for ((A) dry season; (B) wet transmission season). Effect sizes \pm S.E.M. and statistical outcomes are: -1.82 ± 0.20 , $z=-8.97$, $P<0.001$ (single infections, wet season); -0.88 ± 0.34 , $z=-2.61$, $P=0.009$ (mixed infections, wet season); -2.63 ± 0.25 , $z=-10.69$, $P<0.001$ (single infections, dry season); -1.69 ± 0.48 , $z=-3.50$, $P=0.0005$ (mixed infections, dry season). Predicted logit sex ratios are shown for single (open circles, solid line) and mixed genotype infections (open triangles, dashed line). Predictions from mixed effect logistic regression models (model B in Table 1 in the main text) are at population level, i.e. after controlling inter- individual patient variation to reveal overall patterns. Shaded areas represent 95% prediction intervals, which were estimated by data simulation ($n=1000$; arm package, R v3.2.4).

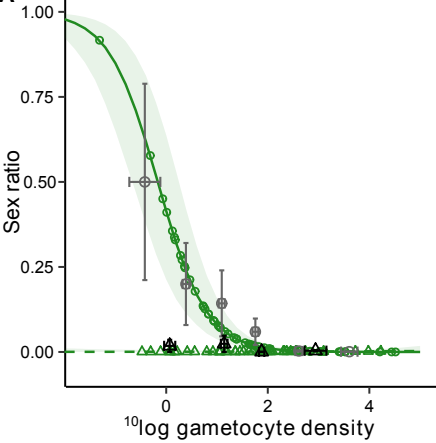
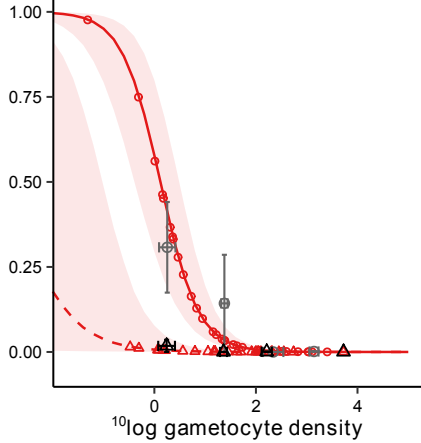
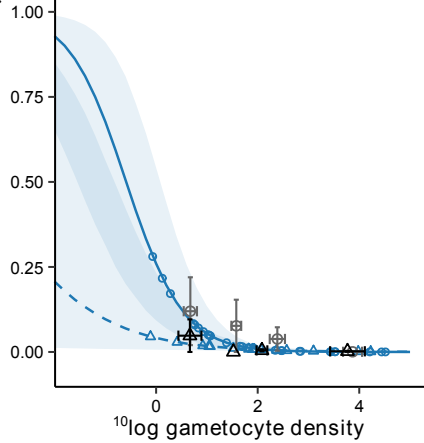
Table 1. Results from mixed effect logistic regression models^a to analyse gametocyte sex ratios^b.

	Chi square	P-value	AIC	r ² marg/cond
Model A: SR~GCT*MOI				
GCT*MOI(mix)	76.06	<0.001	479.24	0.52/0.75
Model B: SR~GCT*MOI+SEAS*GCT+SEAS*MOI				
MOI*SEAS	18.43	<0.001		
GCT:SEAS	7.06	0.008	416.27	0.54/0.77

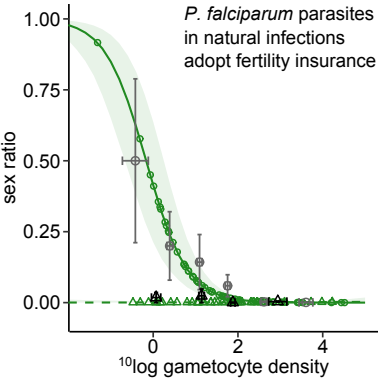
GCT, ¹⁰log gametocyte density/ μ L of blood; MOI, multiplicity of infection (single or mixed); SEAS, season (wet transmission season 1 August – 25 December or dry season 26 December – 31 July); r² marg, marginal; r² cond, conditional (*i.e.* taking into account inter-individual differences between patients).

^a Generalised linear mixed effect models (lme4, R v3.2.4; The R-Foundation, Vienna, Austria, see Supplementary Methods S1) with patient number included as a random effect to account for repeated sampling, and a binomial error structure.

^b Gametocyte sex ratios were analysed as a two-vector response variable (males, females) to enable weighted regression that takes into account sample sizes from which gametocyte sex ratios were estimated.

A**B****C**

P. falciparum parasites
in natural infections
adopt fertility insurance



Highlights

- We apply evolutionary sex allocation theory to *Plasmodium falciparum* in natural infections
- Gametocyte sex ratios and densities are negatively correlated (fertility insurance)
- Fertility insurance is more apparent in the dry compared with the transmission season
- Sex ratios differ between single and mixed genotype infections, but not as expected
- Mixed genotype infections do not have higher sex ratios (no local mate competition)

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