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The malaria parasite, *Plasmodium falciparum*, increases the frequency of multiple feeding of its mosquito vector, *Anopheles gambiae*

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It has often been suggested that vector-borne parasites alter their vector's feeding behaviour to increase their transmission, but these claims are often based on laboratory studies and lack rigorous testing in a natural situation. We show in this field study that the malaria parasite, *Plasmodium falciparum*, alters the blood-feeding behaviour of its mosquito vector, *Anopheles gambiae s.l.*, in two ways. First, mosquitoes infected with sporozoites, the parasite stage that is transmitted from the mosquito to a human, took up larger blood meals than uninfected mosquitoes. Whereas 72% of the uninfected mosquitoes had obtained a full blood meal, 82% of the infected ones had engorged fully. Second, mosquitoes harbouring sporozoites were more likely to bite several people per night. Twenty-two per cent of the infected mosquitoes, but only 10% of the uninfected mosquitoes, contained blood from at least two people. We conclude that the observed changes in blood-feeding behaviour allow the parasite to spread more rapidly among human hosts, and thus confirm that the parasite manipulates the mosquito to increase its own transmission.

Keywords: *Anopheles gambiae*; *Plasmodium falciparum*; malaria life cycle; blood feeding; behavioural manipulation; transmission success

1. INTRODUCTION

As the rate of transmission of vector-borne parasites is influenced by the biting rate of the vector, evolution should favour parasites with the ability to manipulate the biting behaviour of their vector hosts. Not only will an increase in biting rate bring with it a proportional increase in the parasite's transmission success, it will also have epidemiological consequences. In particular, increasing the biting rate will lead to a decrease in the size of the vector population required to sustain the parasite (Dobson 1988), thus making the parasite more difficult to eradicate.

Several studies support the idea that parasites do indeed increase the biting rate of their vectors. A common mechanism appears to be that the parasite impairs the vector's ability to obtain a full blood meal and therefore induces the vector to bite several times before it is fully engorged. *Leishmania*-infected sandflies, for example, have difficulty obtaining a full blood meal, probably because the blood flow through the foregut is impaired (Jefferies *et al*. 1986). Therefore, infected sandflies probe more often than uninfected flies and, as the probing is infectious, several hosts can be infected by one fly (Beach *et al*. 1985). The plague bacterium *Yersinia pestis*, transmitted by the tropical rat flea *Xenopsylla cheopis*, blocks the proventriculus of the flea and causes regurgitation when the flea is blood feeding (Bacot & Martin 1914). Trypanosomes can affect the mechanoreceptive sensilla of their tsetse fly hosts, so that infected flies probe more frequently than uninfected flies (Jenni *et al*. 1980; Roberts 1981). As parasites are frequently transmitted during probing, transmission is likely to be increased. However, not all experiments have found these behavioural changes (Moloo 1983; Moloo & Dar 1985). The most detailed studies of altered feeding behaviour mediated by parasites have been done with the malaria system, where sporozoites of the parasite *Plasmodium gallinaceum* lower the apyrase activity in the salivary glands of infected *Aedes aegypti*. As a result, an infected mosquito's ability to locate blood is impaired, and it probes for a longer time than an uninfected individual (Rossignol *et al*. 1984). In the laboratory, *P. falciparum* sporozoites in naturally infected *Anopheles gambiae* increase not only the duration of probing, but also the number of probes and the likelihood that the mosquitoes will begin to probe (Wekesa *et al*. 1992). These laboratory studies on altered feeding behaviour are complemented by a study in a natural setting, where *A. punctulatus* infected with *P. falciparum* or *P. vivax* obtained a full blood meal earlier in the night than uninfected mosquitoes (Koella & Packer 1996). Possible interpretations of this observation are that infected mosquitoes are less likely to interrupt their feeding when they are disturbed, or that they are more likely to resume their feeding if they have been interrupted.

However, it is not clear whether the increased biting frequency suggested by these studies translates to increased transmission of the parasites. First, all but Koella & Packer's study were laboratory based, and were conducted partly with unnatural host–parasite systems and anaesthetized hosts (Rossignol *et al*. 1984; Wekesa *et al*. 1992). Second, demonstrating a higher number of bites is not sufficient to conclude enhanced transmission. Increased

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biting on a single host may have little influence on the parasite's rate of transmission, unless the probability that an infection is established after a single bite is low; increased biting rate is more likely to increase transmission, if it is coupled with a larger number of people that are bitten.

We studied, in a natural setting, the malaria parasite's ability to alter its vector's blood-feeding behaviour to its benefit by investigating whether *A. gambiae* infected with *P. falciparum* sporozoites are more likely to bite several people than uninfected mosquitoes.

2. MATERIAL AND METHODS

Our study took place in Namawala, situated 30 km west of Ifakara in the Kilombero district of Tanzania, from 23 May to 1 June 1996. The area has been described in detail by Tanner *et al.* (1987).

We screened 32 male volunteers from the area, all above the age of 15, for genetic differences at the three microsatellite loci HUMF13A1, HUMFES/FPS and HUMVWA31/A (Kimpton *et al.* 1993; method described below). Selecting 19 people, we formed 13 groups of three individuals; three people participated in four groups, one in three groups, nine in two groups and six in one group. Within a group, individuals could be identified by a unique allele at one or more of the loci. Over the duration of the study (ten nights) the groups slept in one of three houses in a random order. Any volunteer showing signs of malaria up to a month after the study was given treatment.

We collected female *A. gambiae* inside the houses between 0700 and 0900. At this time the mosquitoes rest on the walls and ceilings, having fed during the night. The mosquitoes were frozen within 1 h. From each mosquito we obtained the following data: wing length, infection status and blood meal size. In a subsample of the mosquitoes, we determined the number of people from which the individual mosquito had engorged blood.

(a) *Wing length*

We determined mosquito size by measuring the wings from the tip, excluding the fringe, to the distal end of the allula. The wings were fixed onto slides and recorded with a video camera mounted on a dissecting microscope. The picture was copied to a computer and wing lengths measured to the nearest 0.01 mm with the program NIH Image v. 1.60 (`http://rsb.info.nih.gov/nih-image`).

(b) *Infection status*

We assayed parasite infection by testing each head/thorax for the presence of *P. falciparum* circumsporozoite protein using an enzyme-linked immunosorbent assay (ELISA) specific for the (NANP)40 repeat (Campbell *et al.* 1987). To make comparisons among plates more reliable, we standardized ELISA values for each plate by dividing them by the mean of the positive controls and multiplying them by 100. Transformed values higher than the mean of all negative controls plus three times their standard deviation were considered positive.

(c) *Blood meal size*

We assigned the size of the blood meal to one of the following categories: empty, one-quarter full, half full or full, according to the expansion of the abdomen as indicated by distension of the pleural membranes.

(d) *Number of people bitten*

We estimated the number of people bitten by a mosquito by comparing the blood in the mosquito's abdomen with that of the people sleeping in the house. We selected 442 mosquitoes for blood meal analysis as follows: we grouped mosquitoes by infection status and the blood meal sizes (not including the empty mosquitoes), and selected 100 mosquitoes randomly from each of the six groups. In groups with less than 100 mosquitoes we analysed all mosquitoes. We isolated DNA using a modified hexadecyltrimethylammonium bromide (CTAB) isolation method (Doyle & Doyle 1987). Because we isolated DNA from blood, we homogenized each abdomen in 200 µl of distilled water instead of CTAB. After rinsing the DNA in 80% ethanol, we used a polymerase chain reaction (PCR) to amplify three microsatellite loci bordered by the three primers HUMF13A1, HUMFES/FPS and HUMVWA31/A (Kimpton *et al.* 1993). With a kinase reaction, one primer in each set was radioactively labelled with ^{33}P. We amplified the DNA with 31 cycles of PCR in a total volume of 6 µl under the conditions described by Kimpton *et al.* (1993), separated 2.5 µl of the PCR products on polyacrylamide gels, and visualized the DNA profile by placing the dried gel on radioactive sensitive film.

We defined a meal as single if all the identified alleles could have come from the same person. A meal was defined as multiple if at least one locus had more than two alleles or if the alleles at any of the three loci could only have been picked up from biting different people in the group. If none of the identified alleles were found in the people sleeping within the house, the meal was not included in the analysis.

(e) *Statistics*

The effects of infection and mosquito size on the probability that a mosquito had obtained a multiple meal and on the size of blood meal were analysed by a logistic regression. We tested for independence of the mosquito's size and infection status in our sample with a quadratic logistic regression, as a nonlinear association could be expected (Lyimo & Koella 1992). Using several groups of volunteers more than once may have led to a problem of non-independence. We reduced this problem by blocking the analysis with the group of volunteers and by the date of the mosquito collection (nested within the group of volunteers). Following the suggestion of Sokal & Rohlf (1995), we did not include the interaction between the blocking variables and the other dependent variables in the analysis. Because many of the PCRs gave unclear results, we tested whether the likelihood that a PCR failed depended on the size of the mosquito or the blood meal size with a logistic regression.

In all analyses, mosquito size was considered as a continuous variable. However, for graphical presentation of the data the mosquitoes were divided into three size groups of similar number. Similarly, although we analysed blood meal size as the four categories between empty and fully fed, we distinguished only between fully fed and any other blood meal size in our figures. All statistical analyses were done with the statistical package JMP v. 3.1.6 (`http://www.sas.com/otherprods/jmp/home.html`).

3. RESULTS

We collected between 10 and 174 *A. gambiae* per house per night; there was no difference in the numbers of mosquitoes caught from the different groups of volunteers (ANOVA: $F_{12,17}=0.613$, $p=0.804$). Among the total of 1762

Table 1. *Ordinal logistic regression of sporozoite infection and mosquito size, controlling for date and house of capture, on size of blood meal*

(Mosquito size represented by wing length, and size of blood meal estimated as one of four categories between empty and fully fed; $n=1760$.)

source	d.f.	χ^2	p
group of volunteers (random)	12	15.77	0.202
date (group) (random)	17	67.07	<0.001
wing length	1	4.75	0.029
infection	1	4.59	0.032
wing length × infection	1	5.11	0.024

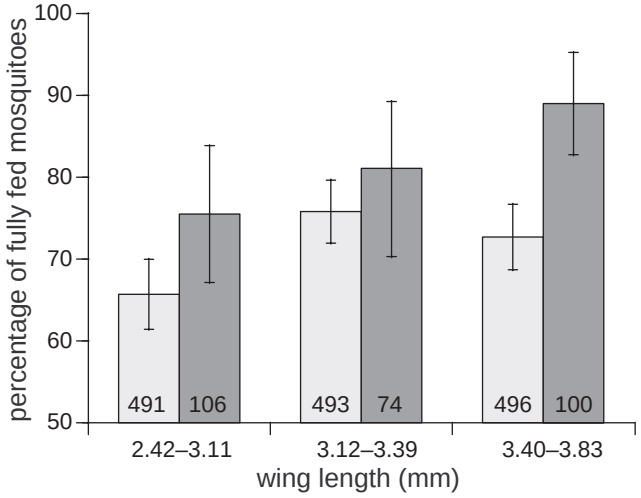

Figure 1. Effect of infection with malaria sporozoites and size of the mosquito on the probability that the mosquito is fully fed. The bars show the percentage of fully fed mosquitoes in each size group and the vertical lines show the 95% confidence intervals of the binomial distribution. The light bars represent the uninfected mosquitoes and the dark bars the infected mosquitoes.

mosquitoes, 280 (15.9%) tested positive for circumsporozoite protein; the wing lengths ranged from 2.42 to 3.83 mm and were normally distributed (Shapiro–Wilks test $W=0.989$, $p=0.81$) with a mean of 3.26 mm and a standard deviation of 0.19 mm. No association between mosquito size and sporozoite prevalence was apparent ($\chi^2=1.26$, $p=0.53$).

Of the 1762 mosquitoes collected, 128 (7.3%) contained no blood, 185 (10.5%) were one-quarter full, 162 (9.2%) were half full and 1287 (73.0%) were fully fed. Among the infected mosquitoes, 229 (81.8%) had obtained a full blood meal, whereas among the uninfected ones 1058 (71.5%) had done so ($\chi^2=12.7$, $p=0.0004$). The analysis controlling for the group of volunteers and for the date of collection (table 1) showed that the effect of infection was particularly pronounced for large mosquitoes (figure 1).

Of the 442 randomly selected mosquitoes, 145 gave no or unclear PCR results. The reasons for the low success of the PCR are not clear. However, the success was not associated with either the amount of blood found in the mosquito or the mosquito's size (table 2), so that the missing mosquitoes were unlikely to bias the results.

Table 2. *Logistic regression of mosquito size and size of blood meal on the success of the PCR to detect multiple meals*

(Mosquito size represented by wing length, and size of blood meal estimated as one of three categories between one-quarter fed and fully fed; $n=442$.)

source	d.f.	χ^2	p
wing length	1	0.552	0.458
blood meal	2	0.657	0.720
wing length × blood meal	2	0.610	0.737

Among the remaining mosquitoes, 35 contained alleles that were not found within the group of volunteers; these were omitted from the analysis, leaving 262 for the analysis of multiple meals. Of these, 37 (14%) had taken multiple meals and 225 (86%) had taken single meals. Ten per cent (17 out of 173) of the uninfected and 22% (20 out of 89) of the infected mosquitoes had taken multiple blood meals ($\chi^2=7.36$, $p=0.007$). After controlling for variability due to the group of volunteers and the date of catching, the difference remained significant (table 3*a*) and was particularly pronounced for large mosquitoes (figure 2). When only those mosquitoes that had engorged fully were analysed, the same pattern emerged (table 3*b*). A similar pattern also emerged when we used the quantitative ELISA reading as a measure of the number of sporozoites contained in the salivary glands (table 3*c*).

4. DISCUSSION

In our field study, *Anopheles gambiae* infected with *Plasmodium falciparum* sporozoites fed on more hosts than uninfected mosquitoes. Thus, we could confirm previous laboratory-based suggestions that malaria sporozoites manipulate their vector's behaviour in ways that are likely to increase the parasite's transmission.

The parasite's effect on the mosquito's feeding behaviour may have its physiological basis in two aspects of feeding mechanisms. First, in our study, sporozoite infection increased the likelihood that a mosquito would complete its feeding and obtain a full blood meal. When uninfected mosquitoes are disturbed during feeding, they will usually not return to complete their feed if they have already consumed more than a threshold volume of blood (Klowden & Lea 1978). This inhibition of host-seeking behaviour appears to be triggered either by abdominal stretch receptors (Klowden & Lea 1979) or by the release of neurohormones as a result of abdominal stretching (as demonstrated for the blood-sucking bug *Rhodnius prolixus* (Lange *et al*. 1989)). It thus appears that malaria parasites may interfere with this control system by increasing the threshold blood volume at which host-seeking behaviour is inhibited. Therefore, the parasites make their mosquito vector become more persistent, as suggested for *A. punctulatus* (Koella & Packer 1996), induce it to take up more blood, and thus increase the number of host contacts and transmission.

Second, laboratory studies on the mosquito *Aedes aegypti* have shown that sporozoite infection of the salivary glands makes blood feeding less efficient by decreasing the activity of apyrase (Rossignol *et al*. 1984), an enzyme

Table 3. *Logistic regressions of sporozoite infection and mosquito size, controlling for the group of volunteers and the date of capture, on frequency of multiple feeding*

(Mosquito size represented by wing length. In (*a*) and (*b*) sporozoite infection was a binary trait; in (*c*) the ELISA reading was used as a quantitative measure of the number of sporozoites contained in the salivary glands. In (*a*) and (*c*) mosquitoes were at least one-quarter full; in (*b*) only fully fed mosquitoes were analysed. Sample sizes: (*a*) $n=262$; (*b*) $n=123$; (*c*) $n=262$.)

source	d.f.	χ^2	p
(*a*)			
group of volunteers (random)	12	0.50	1.000
date (group) (random)	17	13.74	0.686
wing length	1	0.08	0.773
infection	1	6.91	0.009
wing length × infection	1	7.87	0.005
(*b*)			
group of volunteers (random)	11	3.43	0.984
date (group) (random)	15	2.04	1.000
wing length	1	0.89	0.347
infection	1	7.03	0.008
wing length × infection	1	7.73	0.005
(*c*)			
group of volunteers (random)	12	0.50	1.000
date (group) (random)	17	10.51	0.881
wing length	1	3.91	0.048
infection	1	6.00	0.014
wing length × infection	1	6.39	0.012

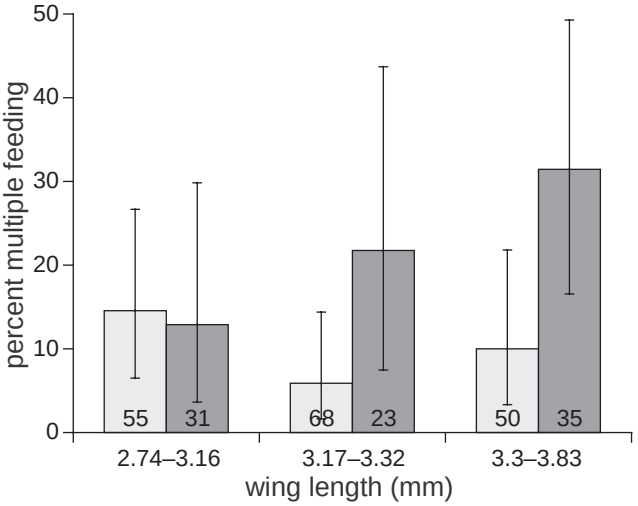

Figure 2. Effect of infection with malaria sporozoites and size of the mosquito on the probability of having a multiple meal. The bars show the percentage of multiple meals in each size group and the vertical lines show the 95% confidence intervals of the binomial distribution. The light bars represent the uninfected mosquitoes and the dark bars the infected mosquitoes.

required by mosquitoes to locate blood when they are probing their host (Ribeiro *et al*. 1985). As a consequence of this feeding impairment, infected mosquitoes probe more often than uninfected ones (Wekesa *et al*. 1992) and they must feed more times in order to obtain a given amount of blood (Rossignol *et al*. 1986).

These two mechanisms—enhanced probing and increased volume of blood meal—act together to increase the biting frequency and the number of host contacts of mosquitoes infected with malaria sporozoites.

This will lead to increased transmission only if parasites are injected into the host during successive bites. A number of laboratory studies make this condition plausible. Salivary glands are not depleted of sporozoites even in vectors that feed up to 15 times (Shute 1945). More direct evidence is the demonstration that *Aedes aegypti* mosquitoes that are allowed to probe on chicks for 10 s at a time can transmit the avian malaria *P. gallinaceum* to at least three birds within a short period of time (Kelly & Edman 1992). Similarly, successive probes deliver a similar number of *P. falciparum* sporozoites in the blood or membrane of a membrane feeder (Ponnudurai *et al*. 1991) and *P. berghei* sporozoites are ejected into mineral oil even after the mosquito has probed extensively on a rodent host (Li *et al*. 1992). Only in one study, where the mosquitoes were attached to a glass slide and then forced to probe into mineral oil, were sporozoites ejected mainly at the beginning of salivation (Rosenberg *et al*. 1990).

Thus, in the laboratory, successive probes generally lead to transmission of sporozoites. These findings are supported by the result of a field study in the Gambia, where 50% of pairs of children sleeping in the same house had identical genotypes (assessed at three loci) of *P. falciparum* parasites, whereas only 1% of pairs picked at random between houses shared the same malaria genotypes (Conway & McBride 1991). The explanation was that individual, infected mosquitoes had bitten (or at least probed) several people within a house and had transmitted the parasite during several of these bites.

It is puzzling that, in our study, the effect of the parasite on the biting behaviour was most apparent among the largest mosquitoes. The obvious explanation—that large mosquitoes generally suffer intense infections at the oocyst stage (Kitthawee *et al*. 1990; Lyimo & Koella 1992; Hogg & Hurd 1997) and that the parasite's ability to manipulate the host increases with its density in the salivary glands—must be ruled out, as the intensity of infection at the sporozoite stage (estimated as the ELISA reading) showed only a weak (and negative) trend with size ($r^2=0.009$, 1761 observations, $p<0.001$). It should also be noted that in the one previous study (Koella & Packer 1996), the opposite trend was apparent: the blood-feeding behaviour of large mosquitoes was less affected by the parasite than that of small mosquitoes.

There are several caveats for the interpretation of our results. First, a number of factors are likely to bias our estimate of multiple feeding. Some factors lead to an underestimate of the number of multiple blood meals. Some of the single meals are based on results for one or two loci, so that the crucial locus to detect a multiple meal could have been missed. Furthermore, the DNA in blood meals taken early in the night is degraded to a higher degree and might therefore not be detected with the PCR technique, although the importance of this is probably negligible (Coulson *et al*. 1990). Alternatively, an

overestimate of multiple meals is possible where two or more people are distinguished by one or two alleles at different loci, because a single person outside the house could have just that allelic combination. However, it seems unlikely that the first or third of these biases is linked to infection. The second bias, on the other hand, may be linked to infection if infected mosquitoes bite earlier in the night (Koella & Packer 1996). If anything, however, this would lessen the difference in proportion of multiple meals between infected and uninfected mosquitoes because, if DNA degradation is important, fewer multiple meals would be detected in infected mosquitoes.

Second, as the stage of parasite infection is associated with the mosquito's age, the results could reflect the effect of age rather than infection. Studies on this issue show contrasting results. In *Ae. aegypti*, for example, 10-day-old mosquitoes bite more frequently in a laboratory setting than 5-day-old ones (Xue *et al*. 1995). However, the experiment allowed no means of distinguishing between the effects of age and those of parity. Other experiments have found that age does not affect blood-feeding behaviour. Thus, the host-seeking behaviour and the amount of blood taken up are similar for 5-day-old and 21-day-old *Ae. aegypti* (Klowden & Lea 1980). In particular, age does not affect the number of probes or the probing time among colonized *A. gambiae* (Wekesa *et al*. 1992), the mosquito of this study.

Third, an alternative interpretation of our results could be that mosquitoes vary in their tendency to switch hosts during a feed. Restless mosquitoes are predisposed to multiple feeding and they might therefore be more likely to pick up an infection. Thus, multiple feeding would be the cause of higher rates of infection, rather than the result. This interpretation, however, seems unlikely. It predicts that infection at every stage of the parasite's development would be associated with an increased biting rate. However, in a laboratory-based study on *P. yoelii nigeriensis* and *A. stephensi*, persistence of the mosquito differed according to the stage of parasite development. In particular, persistence decreased when oocysts were present (Anderson & Hurd 1998), in contrast to the prediction of the alternative interpretation.

Finally, the observed pattern could be a by-product of infection, rather than a behaviour manipulated by the parasite to increase its transmission. Thus, increased biting rate could be an incidental side effect of sporozoites blocking salivary ducts, making blood feeding less efficient. However, if this were the case, one would expect that infected mosquitoes take up less, rather than more blood. Thus, the combination of the two mechanisms is likely to have evolved in order to increase the frequency of multiple feeding.

In conclusion, our observations from a natural setting confirm laboratory-based observations that a vector-borne parasite can manipulate the biting behaviour of its vector to increase its transmission. As biting rate is one of the principal entomological factors determining the rate of transmission (Macdonald 1957), increased multiple feeding by infected mosquitoes has profound implications for the epidemiology of malaria (Dobson 1988). It will, for example, increase the rate at which humans are infected with multiple clones, and thus increase the rate of outcrossing; this is likely to affect the spread of drug resistance (Dye & Williams 1997). At least, any prediction of transmission based on the biting rate of uninfected females is likely to underestimate the rate of malaria transmission and thus mislead an otherwise rational planning of control programmes.

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