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Rodent malaria parasites *Plasmodium chabaudi* and *P. vinckei* do not increase their rates of gametocytogenesis in response to mosquito probing

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Several vector-borne infectious agents facultatively alter their life history strategies in response to local vector densities. Some evidence suggests that malaria parasites invest more heavily in transmission stage production (gametocytogenesis) when vectors are present. Such a strategy could rapidly increase malaria transmission rates, particularly when adult mosquitoes begin to appear after dry seasons. However, in contrast to a recent experiment with a rodent malaria (*Plasmodium chabaudi*), we found no change in gametocytogenesis in either *P. chabaudi* or in another rodent malaria, *P. vinckei*, when their mouse hosts were exposed to mosquitoes. Positive results in the earlier study may have been because mosquito-feeding caused anaemia in hosts, a known promoter of gametocytogenesis. The substantial evidence that malaria and a variety of other parasites facultatively alter transmission strategies in response to a variety of environmental influences makes our results surprising.

Keywords: gametocytogenesis; mosquito-feeding; *Plasmodium chabaudi*; *Plasmodium vinckei*; sex ratio; anaemia

1. INTRODUCTION

Parasites possess a remarkable arsenal of strategies to enhance their transmission and consequent fitness (Bull *et al.* 1991; Moore 2001). One strategy is to respond to environmental fluctuations in transmission opportunities. For instance, even comparatively simple vector-transmitted viruses may increase replication rates facultatively to enhance transmission when their hosts are exposed to vector salivary contents or are fed upon by vectors (Jones *et al.* 1992; Limesand *et al.* 2000). Similarly, the protozoan *Leishmania* increases its infectivity by dramatically increasing its multiplication rate when exposed to salivary extracts from sandfly vectors (Titus & Ribeiro 1988). Here we test for facultative responses of malaria parasites (*Plasmodium*, Phylum Apicomplexa) when their vertebrate hosts are exposed to probing by mosquito vectors. Some direct (Billingsley *et al.* 2005) and indirect (Paul *et al.* 2004) evidence suggests that malaria parasites can respond in this way. Such responses would substantially enhance malaria transmission in areas where vector densities are variable and may explain the rapid onset of transmission in highly seasonal environments.

In vertebrate blood, asexual stages of *Plasmodium* can opt either for persistence within hosts via continued production of asexuals, or for transmission to mosquitoes (and subsequently to new vertebrate hosts) by production of sexual stages called gametocytes (Carter & Graves 1988). Hence, when vertebrate hosts are being fed upon regularly by mosquitoes, *Plasmodium* could take advantage of the increase in transmission opportunities by investing more in gametocytogenesis. Such a strategy might be particularly favoured by natural selection in highly seasonal environments, such as in Sudan, where mosquitoes are absent for 7–9 months of the year. During the dry season, parasites are barely detectable in the blood (Nassir *et al.* 2005) suggesting that it may be costly to produce gametocytes that are lost from a host's blood stream before a transmission season begins. However, parasites would have a substantial fitness advantage if they were able to upregulate gametocytogenesis in response to the appearance of mosquitoes at the onset of the rainy season. *Plasmodium* parasites can facultatively increase their rates of gametocytogenesis in response to environmental factors (reviewed by Carter & Graves 1988; Dyer & Day 2000; Talman *et al.* 2004), many of which probably affect transmission success, such as subcurative drugs (Buckling *et al.* 1997, 1999*a*,*b*) and host anaemia (Trager & Gill 1992; Gautret *et al.* 1996, 1997; Paul *et al.* 2000; Reece *et al.* 2005).

In addition to enhancing gametocytogenesis in response to vector-probing, *Plasmodium* parasites may also alter their gametocyte sex ratio (proportion of male gametocytes). Within seconds of a mosquito taking a

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blood meal from a *Plasmodium*-infected vertebrate host, (male) microgametocytes become as many as eight microgametes, each of which is capable of fertilizing single macrogametes that arise from each (female) macrogametocyte (Carter & Graves 1988). As Read *et al.* (1992) detail, in the case of single genetic lineages (hereafter, clones), local mate competition (wherein brothers compete to mate with sisters; Hamilton 1967) selects for greater investment in macrogametocytes (more female-biased gametocyte sex ratios) within vertebrate hosts. This prediction has been supported in a variety of apicomplexans (Read *et al.* 1995; West *et al.* 2001, 2002). The level of local mate competition could be reduced when vector activity is increased because there will be more opportunities for male gametes to outbreed, which selects for less female-biased gametocyte sex ratios. In single-clone infections, low gametocyte densities may also select for less female-biased sex ratios due to fertility insurance, wherein sufficient microgametocytes must be present to provide microgametes with sufficient opportunities to find macrogametes within vectors (West *et al.* 2002; Gardner *et al.* 2003). If greater gametocyte densities reduce this requirement for fertility insurance, sex ratios are predicted to become more female-biased. As is the case for gametocytogenesis, facultative alterations in gametocyte sex ratios have been seen in *P. vinckei* and *P. gallinaceum* in response to anaemia (Paul *et al.* 2000; Reece *et al.* 2005).

In a re-interpretation of epidemiological data published in the early and late 1900s, Paul *et al.* (2004) provided strong circumstantial evidence that gametocytogenesis in human malaria (*Plasmodium falciparum*) is enhanced as a consequence of mosquito-feeding. More recently, Billingsley *et al.* (2005) found that in *Plasmodium chabaudi* gametocytogenesis occurred earlier and to a greater extent in mice that were exposed to mosquito probing (as opposed to feeding). Mosquito-feeding was reduced in their experiments by moving mice regularly to reduce the probability of inducing host anaemia, which is known to promote gametocytogenesis in *P. chabaudi* (e.g. Gautret *et al.* 1997; Reece *et al.* 2005). Excessive mosquito-feeding could also substantially reduce the population of parasites in each host. However, Billingsley *et al.* (2005) did not determine whether mosquitoes in their experiments had obtained blood, which could have caused anaemia and altered parasite populations in their mice. To address this potential problem, we repeated Billingsley *et al.*'s experiments while concomitantly measuring host anaemia and evaluating the proportion of mosquitoes that obtained blood. We also used larger sample sizes and extended tests to another rodent malaria species, *P. vinckei*. In addition, we tested whether *Plasmodium* parasites facultatively altered their gametocyte sex ratio when their hosts were probed by mosquitoes.

2. MATERIAL AND METHODS

(a) *General experimental methods*

We maintained 40 female MF1 mice, aged 10–12 weeks (Harlan-Olac, UK), on a 12 h light/12 h dark cycle, *ad libitum* diets (41B, Harlan-Teklad, UK) and *ad libitum* water containing 0.05% para-aminobenzoic acid (PABA) (to enhance parasite growth). Twenty mice were assigned randomly to the *P. chabaudi* experiment and 20 were assigned to the *P. vinckei* experiment. Within each experiment, mice were randomly assigned to cages of five and within each cage randomly assigned to control and experimental (=probing) treatments. Both control and experimental mice were infected via intra-peritoneal inoculation of 10^6 parasitized red blood cells in a 0.1 ml carrier consisting of 47.5% Ringers (27 mM KCl, 27 mM $CaCl_2$, 0.15 M NaCl), 50% heat-inactivated calf serum and 2.5% heparin (200 units ml^{-1}). We infected mice in the *P. chabaudi* experiment with clone DK as this clone was used in Billingsley *et al.* (2005) and we used clone BS in the *P. vinckei* experiment.

On days on which mice were probed (exposed to mosquitoes that were given only 20 s intervals to acquire blood), we lightly anaesthetized both control and experimental mice using one part Rompun and two parts Vetalar, diluted to 50% with distilled water. We gave 0.05 ml to 30 g mice and for mice weighing 2.5 g less or more than 30 g, we decreased or increased doses by 0.01 ml, respectively. We also diluted the 50% anaesthetic by 5% each day to reduce the cumulative effects of anaesthetic build-up. Each experimental mouse was placed on top of a screened container containing between 30 and 50 female *Anopheles stephensi* mosquitoes. Mosquitoes probed mice in the *P. chabaudi* experiment from day 1 to 13 (post-infection) and mice in the *P. vinckei* experiment from day 1 to 8. Because effects of probing may be more obvious when parasites are at very low densities, we also probed mice in the *P. chabaudi* experiment from day 27 to 29.

We reared mosquitoes from eggs at 27 °C, 70% humidity, 12 h light/12 h dark cycle. We provided them with 10% sucrose dissolved in water until ready for trials, which occurred when they were between 5 and 12 d old. Excessive blood loss to mosquitoes could cause anaemia which may alter asexual population size and provoke gametocytogenesis (Reece *et al.* 2005). Hence, we moved mice every 20 s to dislodge mosquitoes before most had time to feed. To control for effects of moving experimental mice, we also moved control mice every 20 s.

(b) *Data collection*

Daily, following infection, we made thin blood smears from the tail vein to count asexuals, reticulocytes (immature red blood cells) and gametocytes and we measured red blood cell densities (anaemia) using flow cytometry (Coulter Electronics). We stained all smears for 15 min using 10% Giemsa buffered in 90% phosphate solution. We analysed blood smears at 1000× magnification without knowledge of treatment group. Following Crooks (2004), we counted at least 20 reticulocytes, 20 asexuals and 20 gametocytes. However, we stopped counting if we had not reached these targets after searching 100 fields. We sexed *P. vinckei* gametocytes from smears, but *P. chabaudi* gametocytes are not readily sexed from smears using blood straight from the tail vein so we used the method described in Reece *et al.* (2003). As duration of patent parasitemia differs in our study species, we collected these data from days 2 to 15 and days 27 to 31 in the *P. chabaudi* experiment and days 3 to 8 in the *P. vinckei* experiment. Following Buckling *et al.* (1999*b*), we computed the proportion of asexuals that become gametocytes (gametocyte conversion rate).

(c) *Analyses*

Analyses were performed using SAS version 8.2 (SAS Institute 2002). We used repeated measures analyses to control for variation among mice and their infections. Repeated measures ANOVA is robust to deviations from

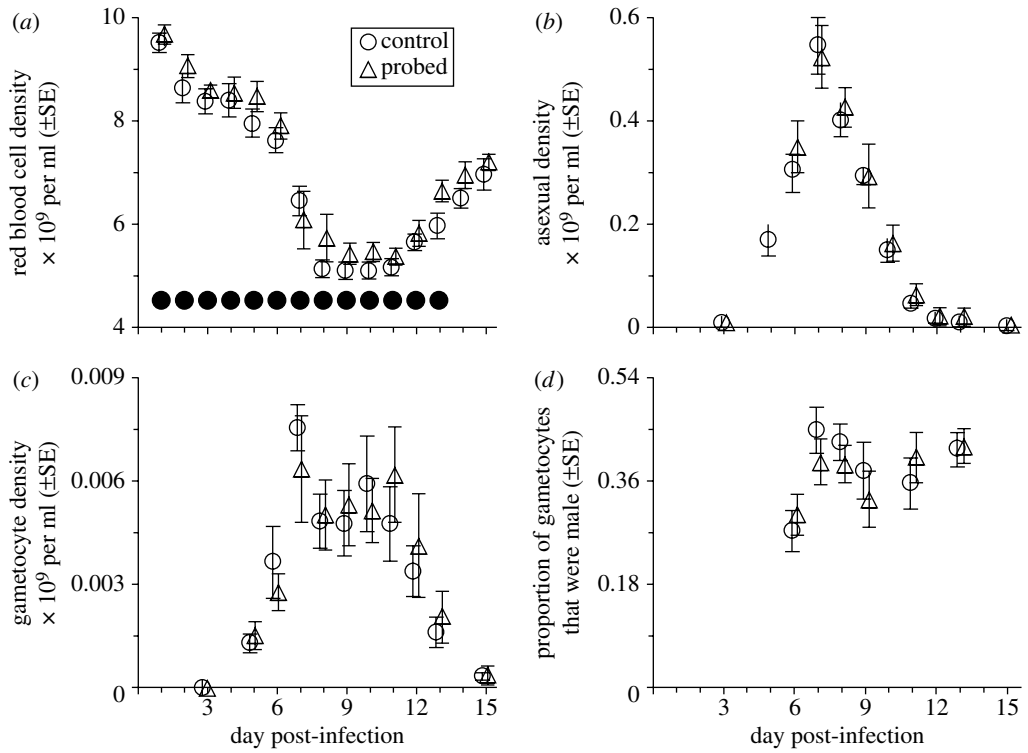

Figure 1. Progress of *Plasmodium chabaudi* infections in control mice and mice exposed to mosquito probing between initial infection and day 13. (*a*) Anaemia (density of red blood cells). Filled circles denote days on which probing occurred. (*b*) Density of asexuals. (*c*) Density of gametocytes. (*d*) Gametocyte sex ratio.

multivariate normality and homogeneity of covariance matrices, although transformations to improve normality (evaluated using Shapiro–Wilk tests) nonetheless reduce the probability of obtaining a Type 1 error. Square root transformations were used on density estimates (for asexuals, gametocytes and reticulocytes). Sex ratios were arcsine square-root transformed.

Our response variables were infection parameters (mass, red blood cell density (per millilitre of blood)), density of asexuals, density of gametocytes, proportion of infected cells that carried gametocytes, gametocyte conversion rate and proportion of gametocytes that were male) and our explanatory variable was treatment (control or probed).

Table 1. Effects of probing (main effects from repeated measures general linear models) by mosquitoes on host and *Plasmodium chabaudi* responses (see also figure 1).

response variable		*p*
mass	$F_{1,15}=4.8$	0.04
red blood cells (per ml)	$F_{1,15}=2.1$	0.17
reticulocyte density	$F_{1,15}=0.3$	0.62
asexual density	$F_{1,16}=0.1$	0.76
gametocyte density	$F_{1,15}=0.1$	0.73
proportion of infected cells that carried gametocytes	$F_{1,15}=0.1$	0.77
gametocyte conversion rate	$F_{1,15}=0.1$	0.77
sex ratio	$F_{1,14}=0.1$	0.76

3. RESULTS

(a) *General observations*

Of 874 mosquitoes that we inspected at irregular intervals from both experiments, 23.8% had blood in their midguts that was visible under a dissecting scope. One control and one experimental mouse died in the first 3 days of the *P. chabaudi* experiment, possibly as a consequence of anaesthesia. A third experimental mouse developed a bruise on its tail at day 12, at which point we stopped collecting samples from it. Before *Plasmodium vinckei* infections were established, a single control mouse became ill from unknown causes and was removed from the experiment. Hence, sample sizes were less than 20 in both experiments.

(b) *P. chabaudi*

Estimates of asexual ($r_S=0.83$, $p<0.0001$) and gametocyte density ($r_S=0.63$, $p=0.004$) for this species were highly repeatable between observers. Effects of *P. chabaudi* infections on red blood cell density were evident by day 2, but asexuals were not detectable in thin blood smears until day 5 and were at very low densities after day 11 (figure 1). Gametocytes were similarly not detected until day 5, but were still detectable at day 13 (figure 1). We thus confined analyses on asexuals to the period between days 5 and 11 and on gametocytes to days 5–13. Gametocyte conversion rates were calculated from day 5 to 11. No asexuals or gametocytes were found between days 27 and 31 in a subsample of smears from both control and probed mice, so we excluded these days from analysis. Sex ratios were only estimated from smears on days 6 through 9 and days 11 and 13.

Mice in the probing treatment were approximately 2 g heavier than the controls; there were no other significant associations between mosquito probing treatment and infection parameters (treatment main effects, figure 1, table 1). The interaction between day and treatment did not significantly affect any infection parameters ($p>0.29$ in all cases). The only infection parameter that did not

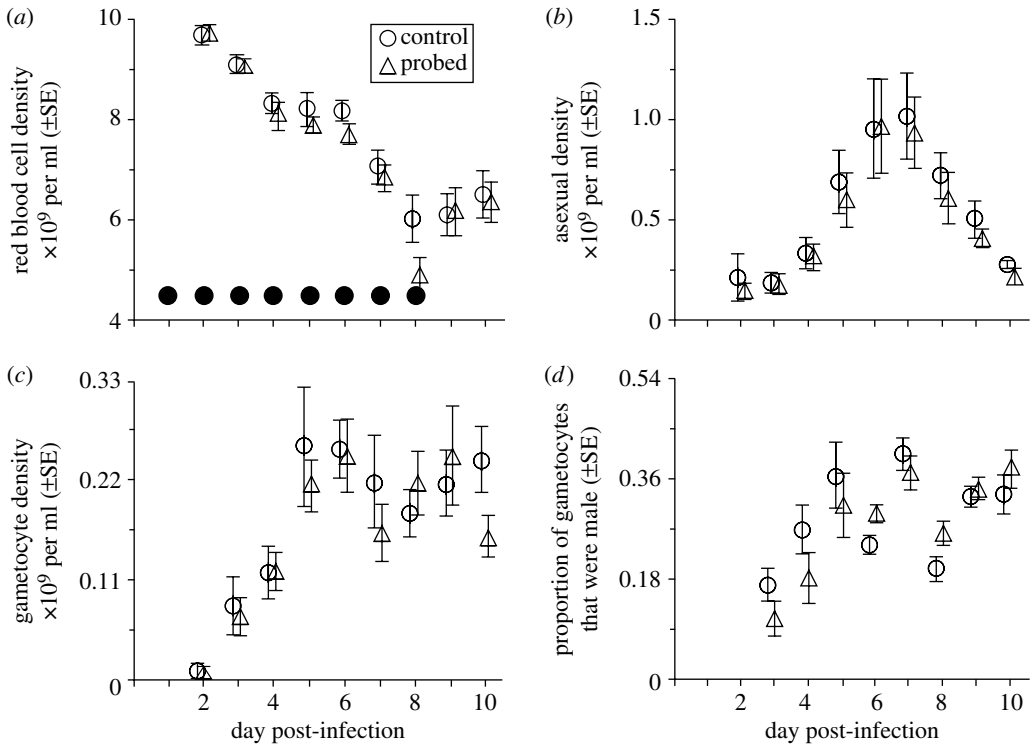

Figure 2. Progress of *Plasmodium vinckei* infections in control mice and mice exposed to mosquito probing between initial infection and day 8. Filled circles denote days on which probing occurred. (*a*) Anaemia (density of red blood cells). (*b*) Density of asexuals. (*c*) Density of gametocytes. (*d*) Gametocyte sex ratio.

change significantly during the course of infection was mass (interaction between day and mass, Wilks' $\lambda = 0.1$, $F_{14,2} = 0.9$, $p = 0.65$); remaining infection parameters changed over the course of monitoring ($p < 0.05$ in all cases).

(c) *P. vinckei*

Infection characteristics (figure 2) were similar to those reported elsewhere for *P. vinckei* (Carter & Walliker 1975; Reece *et al.* 2005). However, two control and two experimental mice did not develop patent parasitemias until most mice had passed peak parasitemias. Analyses including or excluding these mice gave quantitatively similar results, so we present data for all mice. Effects of infection on red blood cell densities were evident by day 3. Asexuals were detectable from day 2 to 10, whereas gametocytes were detectable from day 3 to 10 (figure 2), so we analysed data between these dates.

We found no significant effects of mosquito probing on infection parameters (treatment main effects, figure 2, table 2). There was one single interaction between day and treatment infection parameters; reticulocyte density was significantly higher for probed than for control mice on some days ($F_{8,4} = 0.02$). Remaining interactions between day and treatment were not significantly associated with any infection parameters ($p > 0.15$ in all cases). All infection parameters changed significantly during the course of infection ($p < 0.03$ in all cases).

Table 2. Effects of probing (main effects from repeated measures general linear models) by mosquitoes on host and *Plasmodium vinckei* responses (see also figure 2).

response variable		*p*
mass	$F_{1,17} = 0.3$	0.60
red blood cells (per ml)	$F_{1,17} = 1.7$	0.21
reticulocyte density	$F_{1,11} = 0.4$	0.54
asexual density	$F_{1,12} = 0.1$	0.78
gametocyte density	$F_{1,10} = 1.5$	0.25
proportion of infected cells that carried gametocytes	$F_{1,10} = 0.4$	0.54
gametocyte conversion rate	$F_{1,10} = 1.3$	0.28
sex ratio	$F_{1,10} = 0.8$	0.40

4. DISCUSSION

In contrast to Billingsley *et al.* (2005), we found no evidence of facultative alteration in the timing or level of investment in gametocytogenesis in *P. chabaudi* as a consequence of probing by mosquito vectors (note that they present data on proportion of infected cells at the gametocyte stage, whereas we present gametocyte density). We were similarly unable to detect these responses in another rodent malaria species, *P. vinckei*. In addition, we found no evidence of facultative changes in gametocyte sex ratios in either species to mosquito probing. Our results are thus surprising given the substantial evidence of facultative responses by malaria to a variety of environmental cues in both gametocytogenesis and sex ratio adjustment (e.g. Trager & Gill 1992; Gautret *et al.* 1996, 1997; Buckling *et al.* 1997; Paul *et al.* 2000, 2004; Reece *et al.* 2005).

Although we replicated as closely as possible the methods in Billingsley *et al.* (2005), one possible explanation for higher gametocytogenesis reported by Billingsley *et al.* (2005) is that in their experiments, more mosquitoes obtained partial blood meals and provided more salivary products to their mice. Other vector-borne parasites have been proposed to respond to

components of vector saliva, such as apyrases or maxadilan (Titus & Ribeiro 1988; Jones *et al.* 1992; Limesand *et al.* 2000). This could have caused anaemia, which may signal to parasites that a host's demise is imminent and investment in transmission should thus be expedited. Anaemia is a known trigger of gametocytogenesis (Trager & Gill 1992; Gautret *et al.* 1996, 1997; Paul *et al.* 2000; Reece *et al.* 2005). Another possibility is that moving lightly anaesthetized mice during probing caused stress in Billingsley *et al.*'s experiments and host stress responses promoted gametocytogenesis or interfered with host immune responsiveness, which in turn enabled increased gametocytogenesis. It is plausible that parasites could detect stress responses and be selected to increase rates of gametocytogenesis. The only obvious additional variable between the two studies is the presence (this study) or absence (Billingsley *et al.* 2005) of PABA in the water (0.05%) and solid diet offered *ad libitum* to the infected mice. PABA is included as a supplement to enhance parasite growth and may have counteracted any stress-related responses by the parasite. Billingsley *et al.*'s (2005) significant results may also be due to small sample sizes (Type I error). In addition, their small sample sizes (three mice per treatment group compared to our 7+ mice per treatment group) meant that their analytical methods could not be as robust as those employed here.

On the other hand, we may have failed to detect significant effects of probing because of uncontrolled sources of variation (leading to Type II error). These included the number of mosquitoes in the probing cages, variation in mosquitoes that affected their interest in feeding and so on. While such sources of variation should not have prevented us from detecting any pattern, our data did not even reveal trends in the predicted directions.

If we accept that *P. chabaudi* and *P. vinckei* will generally not respond facultatively to transmission opportunities arising from mosquito probing, we cannot necessarily extrapolate these results to natural circumstances because the system we worked with was artificial and the natural vectors for *P. chabaudi* are still unknown. However, there is a substantial literature documenting facultative responses in gametocytogenesis and sex ratio of *Plasmodium* under similar artificial conditions (Carter & Graves 1988; Buckling *et al.* 1997, 1999*a*,*b*; Dyer & Day 2000; Paul *et al.* 2000, 2004; Reece *et al.* 2005). Moreover, a lack of response is at odds with observations under artificial conditions for viruses and *Leishmania* exposed to their respective vectors and for *Plasmodium* exposed to drugs or anaemic hosts. Another possibility is that these *Plasmodium* species are not exposed naturally to the rate of short-term mosquito probing to which their hosts were exposed in these experiments. Thus, there may not have been selection to favour mechanisms to capitalize on the opportunities that a flush of vector-feeding could provide in the acute phase of parasitemia. In addition, there are likely tradeoffs for parasites that are responsive to mosquito probing, such as investment in quantifying salivary products and, in the case of *Plasmodium*, these may outweigh the potential benefits of responding. The reasons that this trade-off may differ for *Plasmodium* compared to other parasites are worth investigating.

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