Malaria parasite growth is stimulated by mosquito probing

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The ability of malaria parasites to respond positively to the presence of feeding mosquito vectors would clearly be advantageous to transmission. In this study, *Anopheles stephensi* mosquitoes probed mice infected with the rodent malaria parasite, *Plasmodium chabaudi*. Growth of asexual stages was accelerated and gametocytes appeared 1–2 days earlier than in controls. This first study, to our knowledge, of the effects of mosquitoes on 'in-host' growth and development of *Plasmodium* has profound implications for malaria epidemiology, suggesting that individuals exposed to high mosquito numbers can contribute disproportionately high numbers of parasites to the transmission pool.

1. INTRODUCTION

Blood-feeding arthropods produce in saliva numerous biologically active, pharmacological agents that improve their feeding success on vertebrate hosts (Ribeiro 1995). Anticoagulants target clotting pathways (Waidhet-Kouadio *et al*. 1998), vasodilators increase blood flow around the bite (Ribeiro *et al*. 1994) and immodulators reduce inflammation responses and host rejection (Ribeiro *et al*. 1984; Wikel *et al*. 1994; Zeidner *et al*. 1999).

Arthropod-borne protozoa and viruses can exploit salivary gland pharmacology to aid transmission (Titus & Ribeiro 1988; Edwards *et al*. 1998; Jones *et al*. 1992). *Anopheles* mosquitoes transmit human malaria parasites, resulting in some 200 million cases per annum. Transmission from the vertebrate host to the mosquito vector requires the formation of gametocytes, a process known as gametocytogenesis (Carter & Graves 1988). Parasites would be expected to optimize infectivity to the mosquito (Sinden 1983); this might be achieved by developmental mechanisms, increasing survival and numbers of the gametocytes, reducing gametocyte numbers if they are harmful in large numbers to the vector, or increasing the probability of gametocyte ingestion by mosquitoes. In this preliminary study, we describe the accelerated growth and modified gametocyte production rate of *Plasmodium chabaudi* resulting from probing by *Anopheles stephensi* mosquitoes on mice. This adaptive response points to hitherto unforeseen plasticity in the regulation of parasite development.

2. MATERIAL AND METHODS

Twelve MF1 mice were infected with avirulent *P. chabaudi* 96DK by serial passage of 100 μl blood at 7% parasitaemia. Mice were lightly anaesthetized with Ropmun-Vetalar (Almeida & Billingsley 1998) daily for 14 days, and exposed for 20 min to 25 or 50 hungry female *A. stephensi*. Control mice were only anaesthetized. Excessive mosquito feeding alters malaria parasitaemia in mice owing to removal of erythrocytes (L. S. Snook and P. F. Billingsley, unpublished observations). Mice were therefore moved across the mosquito cage every few seconds, allowing mosquitoes to probe but not feed. Blood was removed daily from the tail and duplicate bloodsmears were Giemsa stained on blind-coded slides. The percentage of parasitaemia and proportion of parasites at mature gametocyte stage were determined by examination of 100 fields (100× objective) of each duplicate.

The exponential growth model of Buckling *et al*. (1999) was used to compare the observed and expected rates of gametocytogenesis. The maturation period of gametocytes was taken as 2 days and the proportion of parasites producing gametocytes on day t was described by the number of gametocytes at $t+2$ days. We assumed that gametocyte survival was unaffected, gametocyte half life was 12 h, and the asexual cycle took 24 h. Parasitaemias and the proportion of infected cells at the gametocyte stage were used to estimate gametocyte production rate. One mouse in each group demonstrated atypical infections (dotted lines in figure 1a–c) and failed to produce gametocytes, so only three mice per group were included in the analyses.

The mean time to 50% and 80% maximum cumulative parasitaemia and gametocyte rate, respectively, were calculated from figure 2a,b using probit analysis. Life-history traits were estimated using the analysis of Eisen & Schall (2000) and compared using Student's *t*-test.

3. RESULTS

The presence and number of probing mosquitoes positively influenced in-host *P. chabaudi* growth (figure 1). Three control mice showed typical infections, exhibiting peaks on days 8–11 p.i. Mean parasitaemia peaked at 7.3% on day 8 and the proportion of gametocytes at 9.1% of the infected red cells on day 12. The parasite population grew more rapidly to a higher and earlier peak in direct relation to the number of probing mosquitoes (figure 1a–d). Mean parasitaemia peaked at days 7 (8.0%) and 6 (9.9%) post-infection in mice exposed daily to 25 and 50 mosquitoes, respectively. Over the course of infection, mice probed daily by mosquitoes produced significantly more parasites (figure 2a; Wilcoxon signed: rank test-5 mosquitoes, $Z = -2.726$, $p = 0.006$; 50 mosquitoes, $Z = -2.84$, $p = 0.005$). The time until 50% and 80% of the maximum cumulative mean parasitaemia was advanced by 1.20–1.64 days and 0.99–1.57 days, respectively (table 1). The time to peak parasitaemia and the mean duration of parasite increase were increased but not significantly for mice probed by mosquitoes, while the rate of parasite increase was increased only in mice probed by 50 mosquitoes per day (table 2).

Gametocytes were detected and peaked 2 days earlier at 3 days p.i. in mice probed by mosquitoes (figure 1e–h), compared with controls. Peak gametocyte rates occurred on day 10 in the experimental groups, 2 days earlier than in controls. A higher proportion of mature gametocytes in the parasite population (12.1%) was seen in mice probed by 50 mosquitoes at the peak on day 10, and the cumulative proportion of gametocytes produced increased in direct relation to the number of probing mosquitoes (control = 32.7%; 25 mosquitoes = 37.1%; 50 mosquitoes = 39.9%; $Z = -3.059$, $p = 0.002$ for both groups, Wilcoxon signed rank test; figure 2b). The estimated
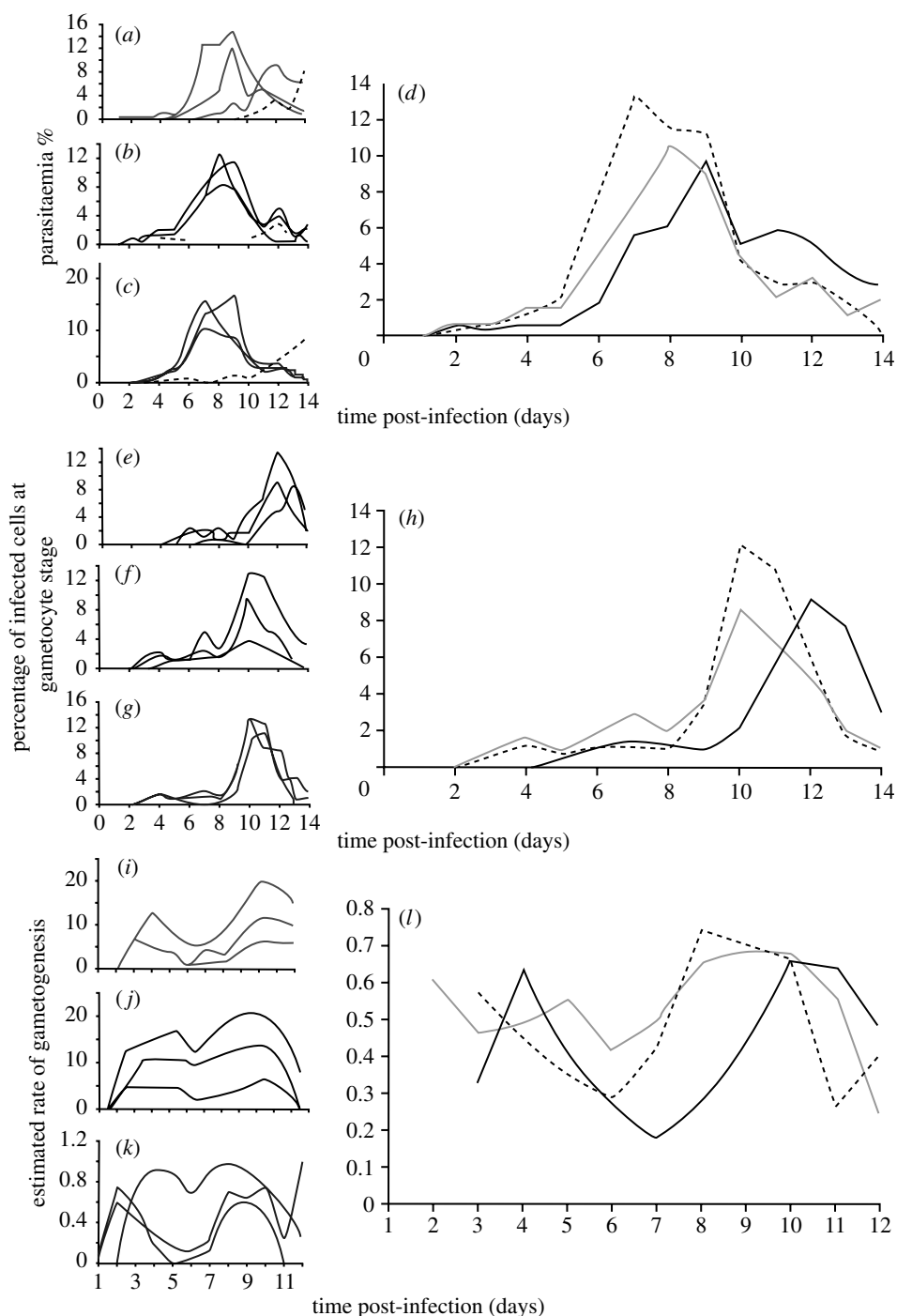
Figure 1. Effect of probing by 25 or 50 female *Anopheles stephensi* on parasite (*a–d*) and gametocyte (*e–h*) populations of *Plasmodium chabaudi* 96DK within MF1 mice. Parasite (*a–c*) and gametocyte (*e–g*) populations in individual hosts are averaged to show the mean parasitaemia (*d*) and gametocyte rate (*h*). Individual (*i–k*) and mean (*l*) estimated rates of gametogenesis are calculated using the model of Buckling *et al.* (1999). In the small panels each line represents the characteristics of infection in an individual mouse. In the large panels, geometric mean values for control (black) mice, or mice probed by 25 (grey) or 50 (dashed line) were calculated from four (*d*) or three (*h,l*) mice.

time until 50% and 80% of the maximum cumulative mean gametocyte rate was advanced by 2–2.47 days and 2.15–2.44 days, respectively (table 1). Mean times to first gametocyte and peak gametocyte rate were advanced significantly by approximately 3 days in both experimental groups (table 2).

The estimated rate of gametocytogenesis varied over the infection period. Two peaks were evident, associated with early infection around day 4 and later infection around days 8–10 (figure 1d,i). For mice probed by mosquitoes, the second peak was shifted

forward by 1–2 days and the cumulative estimated rate of gametocytogenesis was increased in both experimental groups (figure 2c).

4. DISCUSSION

Vector saliva can enhance parasite transmission from vector to vertebrate host. Serially passaged *Leishmania* are more likely to establish in mice in the presence of sandfly saliva (Titus & Ribeiro 1988) and transmission of subpatent viral infections between ticks

Table 1. Time until 50 and 80% *Plasmodium chabaudi* cumulative parasitaemia and gametocyte rate in mice probed by *Anopheles stephensi*.

		time (days)		
		control	25 mosquitoes	50 mosquitoes
cumulative parasitaemia	50%[a]	9.26 (8.30–0.33)	7.62 (6.62–8.76)	8.06 (7.03–9.25)
	80%[a]	11.80 (10.58–13.17)	10.23 (8.89–11.77)	10.81 (9.43–12.41)
cumulative gametocyte rate	50%[a]	11.84 (10.83–13.11)	9.37 (8.19–10.70)	9.84 (8.68–11.15)
	80%[a]	14.96 (13.46–16.63)	12.52 (10.95–14.31)	12.81 (11.30–14.52)

[a] Times to 50% and 80% maximum of the cumulative parasitaemia and proportion of infected cells at the gametocyte stage were calculated using probit analysis of the cumulative curves.
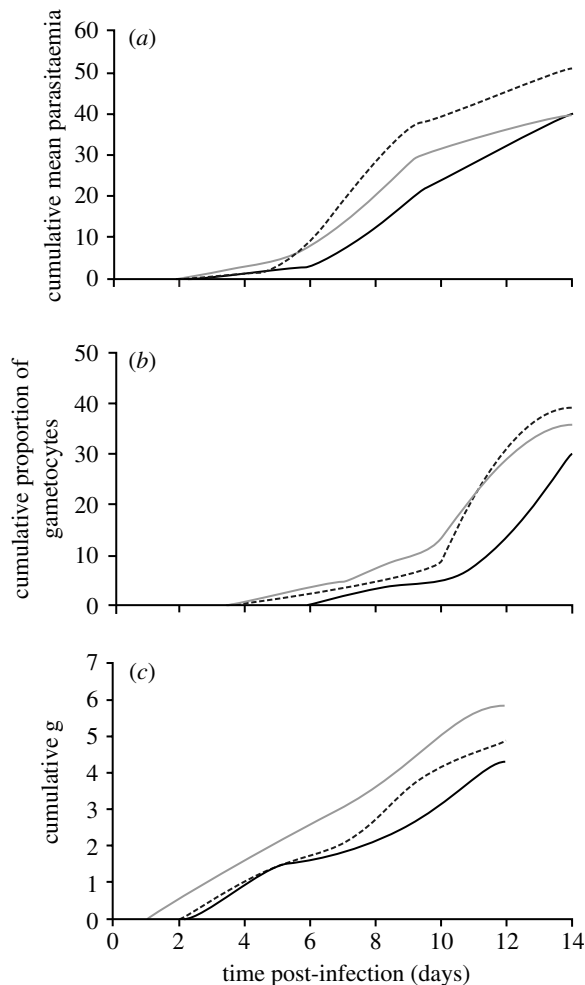

Figure 2. Cumulative parasitaemia (*a*), proportion of infected cells at the gametocyte stage (*b*) and estimated rate of gametocytogenesis of *Plasmodium chabaudi* in control (solid) mice and mice probed daily by 25 (grey) or 50 (dashed line) *Anopheles stephensi* mosquitoes.

on the same host results from salivary-mediated co-feeding transmission (Jones *et al.* 1992). In *Plasmodium*, while experiments suggest that sporozoites from the salivary glands are more infectious than those from the oocyst (Touray *et al.* 1992), differential infectivity may be owing more to sporozoite maturation than interaction with salivary factors (Al-Olayan *et al.* 2002). Vector feeding can also affect pathogens within the mouse host; New Jersey virus titres increased in mice fed upon by mosquitoes and LaCrosse virus infections were of longer duration,

higher titre and more pathogenic when delivered by mosquito bite rather than injection (Osorio *et al.* 1996; Limesand *et al.* 2000). Here, we similarly demonstrate that in-host dynamics of a more complex organism, *Plasmodium*, can be modulated by mosquitoes.

It is interesting that both asexual growth and gametocytogenesis are modified by mosquito probing. Both of these will of course ultimately increase gametocyte numbers. The reasons for increased growth are unknown. In the same parasite, gametocyte formation was enhanced following drug-induced increases in reticulocytes (Gautret *et al.* 1996) and perhaps a similar haemopoiesis is stimulated by mosquito salivary factors. We have no evidence from this preliminary study whether reticulocyte function is affected in any way. We also cannot rule out that the limited amount of blood removal that occurred during these experiments could also be a determining factor in the altered parasite growth.

The shift in rate of gametocytogenesis represents an additional response to the presence of vector mosquitoes, and identifies a hitherto unknown plasticity in developmental modulation of the *Plasmodium* life cycle. Gametocytes are subject to finely tuned density-dependent processes, such that maximum transmissibility to the mosquito may not correlate with the maximum gametocytaemia (Fleck *et al.* 1994). The forward shift of gametocyte production may counteract down-regulation of transmission at high parasite densities later in the infection by increasing gametocyte numbers before non-specific inhibitory factors are stimulated. The logical next step in these studies will be to examine infectivity of gametocytes under different mosquito exposure conditions. A similar pattern of increased gametocyte densities in response to mosquito exposure and feeding has been observed for *Plasmodium praecox*-infecting sparrows (Missiroli 1939), but neither the numbers of mosquitoes used nor the prevention of blood removal were controlled.

Although the mechanisms underlying the parasite response are unknown, the results raise some interesting epidemiological considerations. Some individuals are exposed to greater numbers of mosquito bites (Charlwood *et al.* 1995) and such individuals will receive and transmit disproportionately high numbers of parasites (Woolhouse *et al.* 1997), thereby driving heterogeneity in parasite populations and maintaining parasite populations at low

Table 2. Analysis of parasite and gametocyte growth traits of *Plasmodium chabaudi* in mice probed by *Anopheles stephensi*. (Growth characteristics were analysed according to Eisen & Schall (2000).)

	control	25 mosquitoes	50 mosquitoes
mean[a] time to first parasite (days)	1.33 ± 0.33	0.67 ± 0.33	1.33 ± 0.33
mean time to peak parasitaemia (days)	10.00 ± 1.00	8.33 ± 0.33	7.67 ± 0.67
mean duration of parasite increase (days)	8.67 ± 1.20	6.67 ± 0.33	6.00 ± 1.00
mean rate of parasite increase (percentage per day)	$1.35^{b} \pm 0.26$	1.55 ± 0.23	$2.39^{b} \pm 0.34$
mean time to first gametocyte (days)	$6.00^{a,b} \pm 0.58$	$3.33^{a} \pm 0.33$	$3.33^{b} \pm 0.33$
mean time to peak gametocyte rate (days)	$12.33^{a,b} \pm 0.33$	$10.00^{a} \pm 0.00$	$10.33^{b} \pm 0.33$
mean duration of gametocyte increase (days)	6.33 ± 0.33	6.67 ± 0.33	7.00 ± 0.58
mean rate of gametocyte increase (proportion per day)	1.41 ± 0.21	1.10 ± 0.31	1.70 ± 0.21

[a] Means = arithmetic mean $\pm$ s.d.
[b] Numbers with the same letters in the same rows are significantly different from one another, Student's *t*-test, $p < 0.05$.

transmission intensities (Dye & Hasibender 1986). Detailed examination of gametocyte-to-mosquito ratios could provide information essential to our understanding of transmission dynamics (Killeen *et al.* 2000). While 25 or 50 per mouse represents a high number of mosquitoes relative to the body size of a human, mosquito biting rates can reach several hundred per person per night (Charlwood *et al.* 1995) and there is clearly potential for feeding by uninfected mosquitoes to influence parasite infections within the human host.

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