Transmission success of the malaria parasite *Plasmodium mexicanum* into its vector: role of gametocyte density and sex ratio

J. J. SCHALL*

Department of Biology, University of Vermont, Burlington, Vermont 05405, USA

(*Received 18 February 2000; revised 7 June 2000; accepted 12 June 2000*)

SUMMARY

The life-cycle of *Plasmodium* depends on transmission of the parasite from the vertebrate host into its vector when the insect takes a bloodmeal. Transmission success may depend in part on the parasite's gametocyte density and sex ratio in the blood. *P. mexicanum*, a parasite of fence lizards in California, USA, exploits the sandfly *Lutzomyia vexator* as its vector. In experimental transmissions using naturally infected lizards as donors of blood, transmission success (measured as percentage of vectors infected and number of parasite oocysts on the insect's midgut) was positively related to gametocyte density, although density above 20/1000 erythrocytes did not improve transmission. Sex ratio (proportion of microgametocytes in the infection) was positively correlated with gametocyte density. Transmission improved with higher proportion of microgametocytes, but partial correlations revealed that this was a result only of higher gametocyte densities. These results agree with the theory of virulence and sex ratios because single clone infections should produce a more female-biased sex ratio and grow to the minimum parasitaemia that would maximize clonal transmission, whereas multiple clone infections will be closer to a 1:1 sex ratio and yield a higher parasitaemia when each clone competes for transmission to the vector.

Key words: Plasmodium, malaria, sex ratio, gametocytes, lizard malaria, transmission success.

INTRODUCTION

The *Plasmodium* life-cycle requires the malaria parasite's transmission from its vertebrate host into the vector when the insect takes a bloodmeal. Vector suitability for transmission can vary based on the insect's genotype or age, and the vertebrate host may hinder transfer by transmission blocking immunity (reviewed by Carter & Graves, 1988; Sinden *et al.* 1996). Here I focus on 2 characteristics of the parasite itself that could influence transmission success, the density and sex ratio of gametocytes in the vertebrate host's blood. Natural selection should alter these life-history traits to increase the efficiency of the parasite's transfer from vertebrate to insect. Each trait, though, is subject to other selective pressures that should result in complex adaptive trade-offs.

High gametocyte parasitaemia is likely to favour easy transmission, but comes at a cost to host survival because rapid asexual replication is required to produce large numbers of gametocytes (Mackinnon & Read, 1999*a*; Eisen & Schall, 2000). A trade-off should result between the acute benefit to transmission of high gametocyte numbers and the long-term cost to the durability of the infection (host life-span) (Mackinnon & Read, 1999*a*, *b*). Sex ratio should also influence transmission success because each male gametocyte (microgametocyte) produces K gametes (K varies among species of *Plasmodium*), whereas each female cell (macrogametocyte) produces but 1 gamete. Maximal transmission should obtain when the sex ratio is 1 male:K females (Robert *et al.* 1996). However, evolutionary theory predicts that female-biased sex ratios will be favoured by natural selection only when the genetic diversity of infections is low and inbreeding of the parasite within the vector is substantial (Read *et al.* 1992). Thus, selection on sex ratios may actually decrease the infection's overall transmission success whenever clonal diversity in the infection is high.

I conducted a transmission study with *Plasmodium mexicanum*, a parasite of lizards, and its vector, the sandfly *Lutzomyia vexator* (Ayala, 1971). A lizard malaria parasite was chosen for this study in part because the reptile immune system is likely to mount a weaker and less diverse attack against malaria infection (Schall, 1996). The results of similar previous studies on mammal and bird malaria infections (reviewed in Discussion section) have produced conflicting results that may have been confounded by immune-mediated effects such as transmission blocking immunity. My goals were to test the predictions that higher gametocyte density would increase transmission success, as would a more female-biased sex ratio of gametocytes.

* Tel: +802 656 0448. Fax: +802 656 2914.
E-mail: jschall@zoo.uvm.edu

MATERIALS AND METHODS

Plasmodium mexicanum is an unusual malaria parasite because it exploits a sandfly, *Lutzomyia vexator*, rather than mosquito vector (Ayala, 1971). However, molecular phylogenetic studies show that lizard malaria parasites are true *Plasmodium* (Qari *et al.* 1996), and the life-cycle of *P. mexicanum* is very similar to that of all other *Plasmodium* species (Fialho & Schall, 1995; Eisen & Schall, 2000). The study was conducted at the Hopland Field Station in Mendocino County California, approximately 100 km north of San Francisco. The biology of *P. mexicanum* has been under study at this site since 1978 (reviewed by Schall, 1996). Approximately 25% of adult lizards are infected, with substantial variation in gametocyte parasitaemia and sex ratio among infections (Bromwich & Schall, 1986; Schall, 1989). Gametocytes of *P. mexicanum* are long lived (at least months, Schall, 1989), a high proportion of *L. vexator* become infected after feeding on an infected lizard (Fialho & Schall, 1995), and gametocytes of long-established infections retain the ability to produce gametes (Schall, unpublished observations), all of which suggest a rather meager immune response to gametocytes.

Fence lizards were collected during June–August and held in a large vector-proof outdoor cage. Blood smears were made from a drop of blood from a toe clip and treated with Giemsa's stain. Gametocyte sex ratio typically remains constant for each infection (Schall, 1989); nonetheless, a second blood smear was made if >3 days passed before the lizard was used in transmission experiments. Gametocytes were scored as male or female based on staining colour and distribution of haemozoin pigment (Ayala, 1970). Only large gametocytes were scored because immature gametocytes do not reveal dimorphism and I assumed that immature gametocytes are unable to develop into gametes in the vector. Counts of 50–100 gametocytes provide reliable estimates of true sex ratio (Schall, 1989), so 50–99 (30% of infections), 100 (48%), and 101–223 (22%) gametocytes were counted. For infections with low gametocyte density, several blood smears had to be completely scanned to yield sufficient gametocytes for an accurate estimate of sex ratio. Gametocyte density is expressed as total number of gametocytes seen in 1000 counted erythrocytes.

Lutzomyia vexator sandflies were collected from funnel traps set over the burrow entrances of ground squirrels, *Spermophilus beecheyi*. The traps were set in late afternoon (16.00–18.00 h) and any sandflies in the traps were removed during the night (22.00–24.00 h). Sandflies exiting the rodent burrows are almost exclusively newly eclosed adults (Chaniotis & Anderson, 1968) and were thus assumed for the experiments to be previously unfed vectors. Some experiments used sandflies raised in the laboratory (generation 7–10); no differences in results were observed for wild caught versus laboratory reared vectors, and the data were pooled for analyses. A second species of sandfly, *L. stewarti*, occurs at low density at the site, and therefore the spermathaeca of each vector was examined during dissection to identify the insect to species (Young & Perkins, 1984). The data for the rare species were discarded from the analysis.

The vectors were fed as described by Fiahlo & Schall (1995). Briefly, an infected lizard, with its head covered by a mask to prevent its feeding on the insects, was placed into a cloth cage holding 25–100 female sandflies. Every few hours the cage was inspected and any engorged sandflies were removed to other cloth cages kept in an incubator set at 26 °C and 75% relative humidity. After 9 days, the vectors were removed and dissected to detect and count oocysts under $\times 450$ magnification. The oocysts are always developed by day 9 at 26 °C in *L. vexator* (Fialho & Schall, 1995). Batches of sandflies were fed on lizards captured during June, July, and August when lizards and the sandfly vectors are active at the site.

RESULTS

Variation in gametocyte sex ratio and density

Mean sex ratio (percentage microgametocytes) of the 63 lizards used to feed batches of sandflies was 37·2; the distribution was not skewed, but significantly platykurtic (skew = 0·022; kurtosis = $-0{\cdot}147$). Modal sex ratio of the 63 natural infections was close to the mean (Fig. 1). Of 63 infections, 35% ($N=22$) showed no significant bias to gender, 63% ($N=40$) were female biased, and 2% ($N=1$) male biased (χ^2 tests). Gametocyte density among infections tended to be low (Fig. 2). Gametocyte density and sex ratio were positively correlated (Spearman nonparametric correlation, $\rho = 0{\cdot}397$, $N=63$, $P=0{\cdot}002$); that is, infections tended to be less female biased with higher parasitaemia of gametocytes.

Measures of transmission success

Only 2/63 feedings failed to produce any infected vectors, and 71% of 499 fed sandflies developed oocysts on their midguts. Most infected sandflies harboured few oocysts (Fig. 2). The mean number of oocysts was correlated with percentage of sandflies infected (only batches with 10–25 sandflies) (Spearman rank correlation, $\rho = 0{\cdot}704$, $N=28$, $P=0{\cdot}0003$). As only 18/63 batches numbered >10 sandflies, the number of oocysts per fed sandfly was used in most analyses as the measure of transmission success to order to maximize sample size.

Gametocyte density and transmission success

Using only batches of sandflies in which 10 or more

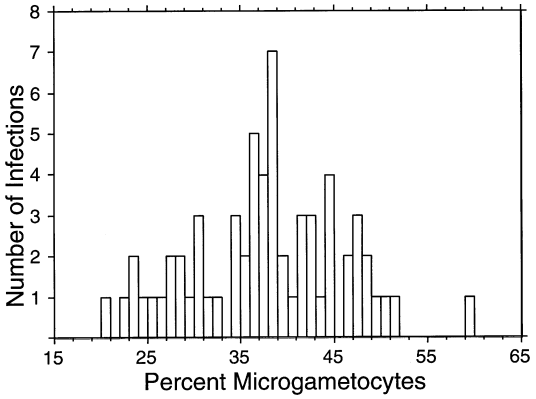

Fig. 1. Distribution of gametocyte sex ratio for 63 infections of *Plasmodium mexicanum* used in an experimental study of transmission.

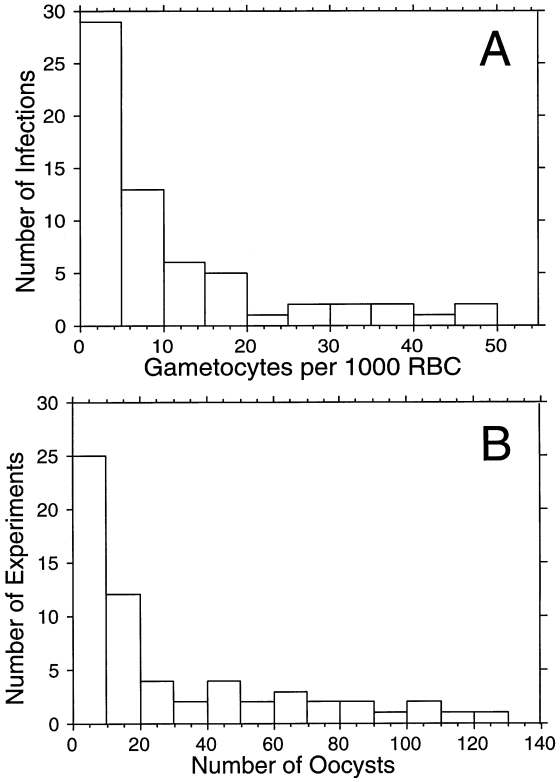

Fig. 2. Distribution of gametocyte parasitaemia in infections of *Plasmodium mexicanum* used in the study (A) and number of oocysts seen on midgut of sandflies (*Lutzomyia vexator*) fed on the infections (B). Two infections did not produce any oocysts in the vectors, so these are not included in the lower figure.

were dissected, gametocyte density was positively correlated with the percentage of sandflies infected (Fig. 3; $\rho = 0{\cdot}525$, $N = 28$, $P < 0{\cdot}006$). Likewise, mean oocysts per sandfly was positively correlated with gametocyte density (Fig. 3; $\rho = 0{\cdot}536$, $N = 63$, $P < 0{\cdot}0001$). Fig. 3 suggests transmission success may improve as gametocyte density increases, but only to some asymptote. Partitioning the data above or below gametocyte density of 20/1000 erythrocytes supports this notion (> 19, $\rho = 0{\cdot}091$, $N = 11$, $P = 0{\cdot}774$, < 20, $\rho = 0{\cdot}390$, $N = 52$, $P = 0{\cdot}005$). This

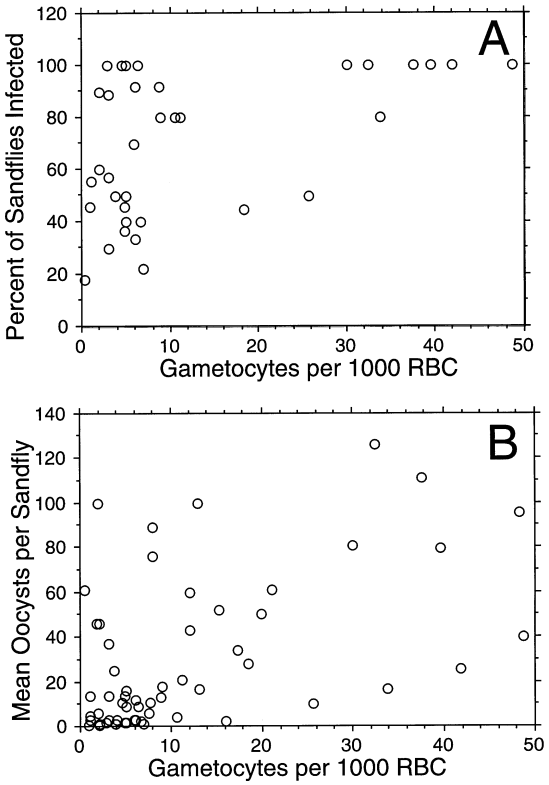

Fig. 3. Percentage of sandflies (*Lutzomyia vexator*) becoming infected with *Plasmodium mexicanum* after feeding on an infected lizard (A) and mean number of oocysts seen per sandfly midgut examined (B) compared with gametocyte parasitaemia. Percentage of sandflies infected is based on experiments with > 9 vectors, and mean oocyst numbers based on all experiments.

analysis could not be duplicated for percentage of vectors infected because of the small sample sizes available.

Gametocyte sex ratio and transmission success

When infections were partitioned into 2 groups, sex ratio biased to females, or no bias (the single male biased infection was not included), no significant difference was observed for percentage of sandflies infected or mean number of oocysts (Mann–Whitney U-tests, $N = 62$, $P > 0{\cdot}05$). The likelihood that individual sandflies became infected was not related to the gametocyte sex ratio found in its bloodmeal (mean and median sex ratio for infected sandflies = 37·5/38·0 and for non-infected sandflies = 37·9/38·0; U test, $N = 354$, $P > 0{\cdot}05$).

If maximum transmission success occurs when the number of male gametes is equal to female gametes, then peak transmission should depend on the number of gametes produced by each male gametocyte ($= K$). Preliminary observations (Schall, unpublished observations) found a mode of 2 flagella (range 1–4) produced by a male gametocyte during exflagellation. At $K = 2$, peak transmission should occur at 33 % males, close to the mean of 37·2 % in the 63 studied infections. Thus, transmission success

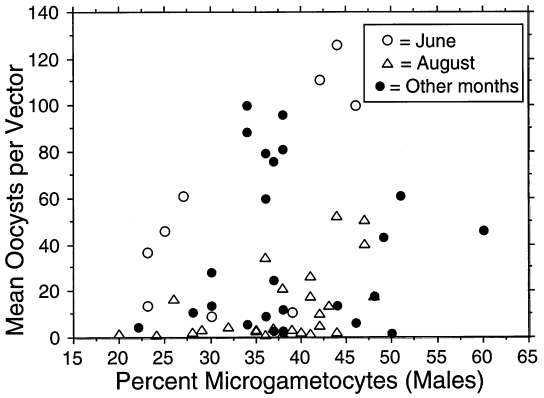

Fig. 4. Mean oocyst numbers per midgut of sandflies (*Lutzomyia vexator*) fed on lizard infected with *Plasmodium mexicanum* based on sex ratio (percentage of microgametocytes). Data for June, August, and other months are indicated.

should rise as sex ratio increases to 33 %, then fall above this value (Robert *et al*. 1996). If $K = 1$, then transmission success should decline from lowest to highest proportion males over the entire range observed in the infections (Fig. 1). If $K = 4$, transmission success should increase over the observed range. Neither a positive relationship (predicted if $K = 1$) nor negative relationship (predicted for $K = 4$) was observed (Fig. 4; Spearman correlations, $P > 0{\cdot}10$). For $K = 2$, the data were examined for the range below 35 % males (predicted positive) and above 35 % males (predicted negative relationship). No relationships were observed (Spearman correlations, $P > 0{\cdot}50$). Using appropriately transformed data (ln of mean oocyst number per vector and ARCSIN of proportion males), product–moment correlations also produced no significant relationships.

Partitioning the data by month revealed only one significant correlation between sex ratio and mean number of oocysts per vector; in August, transmission success was higher for less female-biased gametocyte sex ratio (Fig. 4; $\rho = 0{\cdot}558$, $N = 27$, $P = 0{\cdot}004$). Using transformed data and parametric correlations revealed a positive relationship for the June ($r = 0{\cdot}692$, $N = 9$, $P = 0{\cdot}037$) and August sample ($r = 0{\cdot}500$, $P = 0{\cdot}007$). However, in both June and August, gametocyte density was positively correlated with proportion of males (June $\rho = 0{\cdot}500$, $P = 0{\cdot}23$, August $\rho = 0{\cdot}581$, $P = 0{\cdot}003$). To separate the confounding effects of sex ratio and gametocyte density on transmission success, a partial correlation (transformed data) revealed that for the total data set, gametocyte density was still correlated with transmission success ($r_{\text{partial}} = 0{\cdot}469$; $N = 63$, $P < 0{\cdot}01$), but sex ratio was not correlated with transmission success ($r_{\text{partial}} = 0{\cdot}08$; $P > 0{\cdot}05$). Repeating the analysis for the June and August samples revealed similar results (r_{partial} for sex ratio versus transmission success, $P > 0{\cdot}05$).

Alternative analyses

All the above analyses were repeated using mean oocyst burden calculated using only infected vectors, then all analyses were repeated using data for individual sandflies (rather than means for the groups), both complete data and using only vectors with > 0 oocysts. All results were qualitatively similar to those reported above.

DISCUSSION

Transmission success of *Plasmodium mexicanum* from its lizard host into the sandfly vector was positively related to the gametocyte density, but levelled off at about 20 gametocytes/1000 erythrocytes. A similar non-linear pattern has been observed for *P. falciparum* and *Anopheles* by Noden *et al*. (1994), Rutledge, Gould & Tanticharoen (1969) and Jeffery & Eyles (1955). In contrast, monotonically rising transmission success with an increase in gametocyte density has been reported for *P. gallinaceum* and *Aedes* (Eyles, 1952) and *P. chabaudi* and *Anopheles* (Mackinnon & Read, 1999*a*). More typical, though, are reports of no relationship between gametocyte parasitaemia and transmission success (Muirhead-Thomson & Mercier, 1952; Noden *et al*. 1994; Haji *et al*. 1996 and reviews by Carter & Graves, 1988; Sinden *et al*. 1996).

These conflicting results may have derived from a trade-off between acute transmission success and virulence to the host (Ewald, 1994) which could differ among *Plasmodium*–vector species pairs. Gametocyte density in the vertebrate host's blood depends on the growth rate of asexually replicating cells. More rapidly growing infections tend to produce more gametocytes (Mackinnon & Read, 1999*a*; Eisen & Schall, 2000), but are also likely to yield higher costs to the host (Mackinnon & Read, 1999*b*). For malaria parasites any trade-off must be complex because of the lack of a clear positive relationship between parasitaemia and transmission success. In the case of *P. mexicanum*, why do any infections produce parasite density above the maximum necessary for highest transmission? Although few of the infections studied here revealed gametocyte parasitaemia above the threshold of 20/1000 erythrocytes, larger studies find infections with higher gametocyte parasitaemia are common, some even rising to 150–350 gametocytes/1000 erythrocytes (Schall, 1996). Perhaps infections with a 'surplus' of gametocytes are those that contain many genetically distinct clones, each competing for transmission to the vector and thus resulting in higher total number of gametocytes. Recall that infections with higher gametocyte parasitaemia are also those with a less female-biased sex ratio. Sex ratio theory predicts a less female-biased ratio of

gametocytes when genetic diversity of an infection is high (Read *et al*. 1992).

If each microgametocyte produces >1 viable gametes, infections biased toward macrogametocytes should show a higher transmission success into the vector. Although a positive relationship between proportion of male gametocytes and transmission success was observed for 2 of the 3 months when natural infections were tested, this was a result of higher gametocyte densities. That is, sex ratio itself was unrelated to transmission success for *P. mexicanum*. Few other studies have examined the impact of gametocyte sex ratio on transmission success of *Plasmodium*, but the results generally do not show higher transmission with a more female biased gametocyte sex ratio as predicted by the biology of fertilization in *Plasmodium*.

Boyd, Stratman-Thomas & Kitchen (1935) appear to have been the first to study the probability of a vector becoming infected associated with variation in gametocyte sex ratio. Their data show no effect of sex ratio on transmission success of *P. vivax* or *P. falciparum* (although they draw another conclusion that does not follow from their presented results). Boudin *et al*. (1989) and Noden *et al*. (1994) also found no relationship between sex ratio of *P. falciparum* and transmission success into mosquitoes, but these two studies tested only for a simple correlation between sex ratio and transmission success, rather than for the expected relationship of highest transmission success at 1 male:K females. Robert *et al*. (1996) performed the proper analysis on data for *P. falciparum* but with paradoxical results. Sex ratio and gametocyte density were not correlated and both proportion of mosquitoes infected and oocyst load were maximal for infections closest to 1:1 sex ratio (although the proportion of variance in transmission explained by sex ratio was low, only about 13%). Transmission success was highest when there were presumably more male than female gametes in the mosquito. These results would be expected if only 1 viable gamete is produced per male gametocyte, but in such cases the theory predicts a 1:1 ratio of gametocytes rather than the mostly female-biased ratio observed in the natural infections they examined. Finally, Paul *et al*. (2000) found that sex ratio of *P. gallinaceum* switches from predominantly female early in an infection in chickens to 1:1 later in the infection and transmission success was highest when infections were female biased. Thus, Paul *et al*. (2000) present the only data that show the expected higher transmission with a female-biased ratio.

In summary, the basic biology of *Plasmodium* suggested 2 predictions, that highest transmission success will occur when gametocytes are most common in the blood and when their sex ratio is female biased. The results presented here, and those in previous studies, do not support this simplistic view. For *P. mexicanum*, however, the results can be explained by merging the theory of virulence and sex ratio. If each clone within an infection grows to the optimal density required for its transmission, then multiple clone infections would produce high gametocyte parasitaemia, but also shift sex ratio toward more males. Thus, the study of transmission success of *Plasmodium* cannot consider only proximate mechanisms, but the overall ecology and genetical structure of the parasite.

I thank the staff of the Hopland Field Station for their usual welcome and assistance. Helping in the field were R. Fialho, D. Whitaker, R. Eisen and S. Perkins. The research was funded by a grant from the US-NSF.

REFERENCES

AYALA, S. C. (1970). Lizard malaria in California; description of a strain of *Plasmodium mexicanum*, and biogeography of lizard malaria in North America. *Journal of Parasitology* **56**, 417–425.

AYALA, S. C. (1971). Sporogony and experimental transmission of *Plasmodium mexicanum*. *Journal of Parasitology* **57**, 598–602.

BOUDIN, C., LYANNAZ, J., BOSSENO, M. F., GHAIZE, J. & CARNEVALE, P. (1989). Production de sporozoites de *Plasmodium* humains a Bobo-Dioulasso, Burkina Fasso. *Annales de la Société Belge de Médecine Tropicale* **69**, 3–23.

BOYD, M. F., STRATMAN-THOMAS, W. K. & KITCHEN, S. F. (1935). On the relative susceptibility of *Anopheles quadrimaculatus* to *Plasmodium vivax* and *Plasmodium falciparum*. *Journal of Tropical Medicine and Hygiene* **15**, 485–493.

BROMWICH, C. R. & SCHALL, J. J. (1986). Infection dynamics of *Plasmodium mexicanum*, malarial parasite of lizards. *Ecology* **67**, 1227–1235.

CARTER, R. & GRAVES, P. M. (1988). Gametocytes. In *Principles and Practice of Malariology*, (ed. Wernsdorfer, W. H. & McGregor, I.), pp. 253–305. Churchill Livingstone, Edinburgh.

CHANIOTIS, B. N. & ANDERSON, J. (1968). Age structure, population dynamics and vector potential of *Phlebotomus* in northern California. II. Field population dynamics and natural flagellate infection in parous females. *Journal of Medical Entomology* **5**, 273–292.

EISEN, R. J. & SCHALL, J. J. (2000). Life history of a malaria parasite (*Plasmodium mexicanum*): Assessment of independent traits and origin of variation. *Proceedings of the Royal Society of London, B* **267**, 793–799.

EWALD, P. W. (1994). *Evolution of Infectious Disease*. Oxford University Press, New York.

EYLES, D. E. (1952). Studies on *Plasmodium gallinaceum*. II. Factors in the blood of vertebrate host influencing mosquito infection. *American Journal of Hygiene* **55**, 276–290.

FIALHO, R. F. & SCHALL, J. J. (1995). Thermal ecology of a malarial parasite and its insect vector: consequences for the parasite's transmission success. *Journal of Animal Ecology* **64**, 553–562.

HAJI, H., SMITH, T., CHARLWOOD, J. D. & MEUWISSEN, J. H. (1996). Absence of relationships between selected human factors and natural infectivity of *Plasmodium falciparum* to mosquitoes in an area of high transmission. *Parasitology* **113**, 425–431.

JEFFERY, G. M. & EYLES, D. E. (1955). Infectivity to mosquitoes of *Plasmodium falciparum* as related to gametocyte density and duration of infection. *American Journal of Tropical Medicine and Hygiene* **4**, 781–789.

MACKINNON, M. J. & READ, A. F. (1999*a*). Genetic relationships between parasite virulence and transmission in the rodent malaria *Plasmodium chabaudi*. *Evolution* **53**, (in the Press).

MACKINNON, M. J. & READ, A. F. (1999*b*). Selection for high and low virulence in the malaria parasite *Plasmodium chabaudi*. *Proceedings of the Royal Society of London, B* **266**, 741–748.

MUIRHEAD-THOMSON, R. C. & MERCIER, E. C. (1952). Factors in malaria transmission by *Anopheles albimanus* in Jamaica, Part I. *Annals of Tropical Medicine and Parasitology* **46**, 103–106.

NODEN, B. H., BEADLE, P. S., VAUGHAN, J. A., PUMPUNI, C. B., KENT, M. D. & BEIER, J. C. (1994). *Plasmodium falciparum*: the population structure of mature gametocyte cultures has little effect on their innate fertility. *Acta Tropica* **58**, 13–19.

PAUL, R. E. L., COULSON, T. N., RAIBAUD, A. & BREY, P. T. (2000). Sex determination in malaria parasites. *Science* **287**, 128–131.

QARI, S. H., SHI, Y. P., PIENIAZEK, N. J., COLLINS, W. E. & LAL, A. A. (1996). Phylogenetic relationship among malaria parasites based on small subunit rRNA gene sequences: monophyletic nature of the human malaria parasite, *Plasmodium falciparum*. *Molecular Phylogenetics and Evolution* **6**, 157–165.

READ, A. F., NARARA, A., NEE, S., KEYMER, A. E. & DAY, K. (1992). Gametocyte sex ratios as indirect measures of outcrossing rates in malaria. *Parasitology* **104**, 387–395.

ROBERT, V., READ, A. F., ESSONG, J., TCHUINKAM, T., MULDER, B., VERHAVE, J. P. & CARNEVALE, P. (1996). Effect of gametocyte sex ratio on infectivity of *Plasmodium falciparum* to *Anopheles gambiae*. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **90**, 621–624.

RUTLEDGE, L. C., GOULD, D. J. & TANTICHAREON, B. (1969). Factors affecting the infection of anophelines with human malaria in Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **63**, 613–619.

SCHALL, J. J. (1989). The sex ratio of *Plasmodium* gametocytes. *Parasitology* **98**, 343–350.

SCHALL, J. J. (1996). Malarial parasites of lizards: diversity and ecology. *Advances in Parasitology* **37**, 255–333.

SINDEN, R. E., BUTCHER, G. A., BILLKER, O. & FLECK, S. L. (1996). Regulation of infectivity of *Plasmodium* to the mosquito vector. *Advances in Parasitology* **38**, 53–117.

YOUNG, D. G. & PERKINS, P. V. (1984). Phlebotomine sand flies of North America (Diptera: Psychodidae). *Mosquito News* **44**, 263–304.