The sex ratio of *Plasmodium* gametocytes

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SUMMARY

Sex ratio theory usually predicts an equilibrium sex ratio and equal proportions of males and females in a population, including the progenitors of the reproductive cells of protozoans. This proposal was tested with three species of malarial parasites of lizards, *Plasmodium mexicanum* of the western fence lizard, and *P. agamae* and *P. giganteum* of the African rainbow lizard, using single samples from naturally infected lizards, repeated samples from free-ranging lizards (*P. mexicanum* only), and repeated samples from laboratory maintained animals. Macrogametocytes were usually more abundant than microgametocytes, and were slightly larger, revealing a typically greater investment of resources by the progenitors of female reproductive cells. However, the proportion of microgametocytes varied among the three species and among infections within each species of *Plasmodium*. The sex ratio of gametocytes often remained constant within infections followed over time even if the absolute number of gametocytes was changing. However, the equilibrium sex ratio of gametocytes varied among those infections that had an unchanging microgametocyte proportion. Thus, although an equilibrium sex ratio apparently occurs for most infections, there appears to be no characteristic proportion of microgametocytes for any of the species. Potential explanations for this conflict with theory are presented.

Key words: *Plasmodium*, gametocytes, sex ratio, lizard malaria.

INTRODUCTION

The theory of sex ratios generally predicts an equilibrium proportion of males and females in a population, usually with the ratio stabilizing at equal investment in the two sexes. Modern theory has its origin in Fisher's (1930) reasoning that in populations with unequal proportions of the sexes, individuals of the rarer sex will produce more offspring on the average than individuals of the more common sex. Thus, a stable condition occurs only when males and females are equally abundant, and consequently have equal fitness. More precisely in this situation, the allocation of resources to male and female offspring should be equal; a 1 : 1 sex ratio of offspring should obtain only when males and females are equally costly to produce. Fisher's theory of sex ratios and the allocation of resources to the sexes has been developed substantially in recent years (Ghiselin, 1974; Trivers & Hare, 1976; Charnov, 1982; Karlin & Lessard, 1986). Modern theory supports Fisher's conclusion, but recognizes that special ecological circumstances can result in extraordinary sex ratios (Hamilton, 1967; Colwell, 1981).

Despite the importance of sex ratio and sex allocation theory in evolutionary ecology, the concepts have not been applied to protozoan parasites. For example, malarial parasites (*Plasmodium* spp.) produce male and female gametocytes (microgametocytes and macrogametocytes) in the blood of vertebrate hosts. Within the insect host, the microgametocyte undergoes cytokinesis that yields several smaller motile gametes that may fuse with female gametocytes. Intuition might suggest the sex ratio should favour macrogametocytes to maximize the number of unions of macro- and microgametocytes within the parasite population (Scudo, 1967). Such a view assumes selection acts here at the group level. In contrast, Fisher's argument based on selection acting on individuals can readily be applied to protozoans (Ghiselin, 1974) and predicts, instead, equal investment in male and female gametocytes.

Most theory predicts a characteristic equilibrium sex ratio. Does such an equilibrium exist in malarial infections, and is the allocation of resources to micro- and macrogametocytes equal as predicted by Fisher's model? Application of sex ratio theory to malarial parasites requires data on the ratio of microgametocytes and macrogametocytes both among infections and within infections over time. The diversity of *Plasmodium* species, perhaps 140 (Kreier, 1977), permits valuable interspecific comparisons once data on gametocyte sex ratios for many species become available. Curiously, published data on sex ratios of malarial gametocytes are scanty. In recent general reviews of *Plasmodium* biology, the issue of gametocyte sex ratios have been entirely ignored (Coatney, Collins, Warren & Contacos, 1971; Killick-Kendrick & Peters, 1978; Kreier, 1980; Bailey, 1982; Bruce-Chwatt, 1985).

Here I report gametocyte studies on natural infections of three species of lizard malaria, *P. mexicanum*, a parasite of the western fence lizard, *Sceloporus occidentalis*, in California, and *P. agamae* and *P. giganteum* of the rainbow lizard, *Agama agama*, in West Africa. I had four goals in this research: (1) to perfect methods for counting the ratio of micro- and macrogametocytes in infections

of several species of malarial parasites of lizards; (2) to estimate the investment of resources in micro- and macrogametocytes within an infection by measuring the size of the cells; (3) to determine the sex ratios of gametocytes among infections and within infections over time; (4) to relate observed sex ratios of gametocytes to the predicted ratio emerging from Fisher's theory.

MATERIALS AND METHODS

The study sites were the University of California Hopland Field Station located approximately 160 km north of San Francisco (details given by Bromwich & Schall (1986)), and 23 locations scattered throughout Sierra Leone in West Africa. Lizards were collected either by noosing (in California) or by knocking them from walls, trees and rocks with a long pole (Africa). Blood smears were made from a toe clip, fixed in methanol and then stained in Giemsa at 1:10 at pH 7·2 for 50 min. This staining procedure was chosen after numerous trials with various combinations of pH, stain concentration and staining time, because it best allowed parasites to be seen clearly and micro- and macrogametocytes to be distinguished. Many animals were individually marked with a series of toe clips and later released at the point of capture. A major recapture programme was followed in California that allowed sampling infections of free-ranging lizards repeatedly over time (Bromwich & Schall, 1986); in Africa a mark-recapture study was not possible because the lizards became extremely wary after a single capture. Groups of naturally infected individuals of both lizard species were brought into the laboratory and maintained in enclosures outfitted with rocks, logs, sand, heat lamps and overhead full-spectrum fluorescent lights. Blood smears were made periodically from these laboratory maintained animals, taking care to extract only the smallest quantity of blood with each sampling.

Smears were viewed under 1000×, and parasitaemia determined from counts of 1000–3000 erythrocytes from all areas of the smear. To determine the optimal sample size of gametocytes necessary to assess the ratio of microgametocytes and macrogametocytes, the gametocytes were scored in groups of 25 until the cumulative estimate of sex ratio became relatively constant. Size of gametocytes was determined by measuring their area with a ZIDAS digitizing image analysis system (Carl Zeiss, Inc.) and a drawing tube attached to a microscope.

RESULTS

When stained with Giemsa, microgametocytes and macrogametocytes of all three species differed in ways typical of malarial parasites (Garnham, 1966). Mature male gametocytes stained only slightly with a diffuse pink colour, and no sharp boundaries to the nucleus. Mature females stained pale to dark blue; a well-defined pink nucleus with included darker pink structure were usually obvious. The distribution of malarial pigment typically was more clumped in microgametocytes.

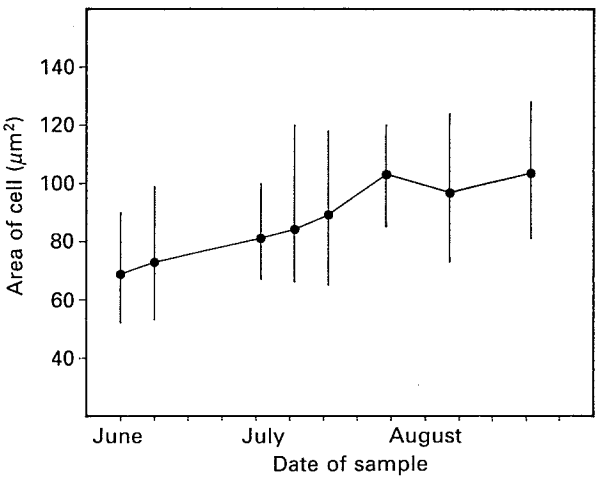

Fig. 1. Area of macrogametocytes during one infection of *Plasmodium mexicanum* in a free-ranging western fence lizard. Gametocyte size reached an asymptote in this infection; in others the size continued to increase throughout the warm season. Each point is the mean of a sample size from 11 to 26; bars represent the range.

Table 1. Proportion of microgametocytes in natural infections of three species of *Plasmodium* in lizards.

(Each sample was taken from a different individual lizard soon after it was collected.)

	P. mexicanum	*P. agamae*	*P. giganteum*
Mean	0·474	0·401	0·371
Range	0·25–0·73	0·29–0·63	0·22–0·51
N	54	30	30

Immature gametocytes of *P. mexicanum* and *P. giganteum* differed from mature cells by their small size and spindle shape. Mature gametocytes of these species ranged from elongated ovals to round cells. Immature *P. agamae* were sometimes spindle shaped, but are often amoeboid, with an irregular cell margin. These observations agree with those of Ayala (1970) on *P. mexicanum*, but contrast with the description of immature gametocytes of the African species given by Garnham (1966). Measurements of gametocytes ($N = 1972$) in 7 infections of *P. mexicanum* followed over time in free-ranging lizards revealed that both macro- and microgametocytes increased in size over the warm season (Kruskal-Wallis tests for all 7 infections, $P < 0{\cdot}001$; Fig. 1). In 4 of these infections, there was no clear asymptote in gametocyte size, whereas in three others the cells appeared to reach a final size (Fig. 1). A second analysis examined changes in the size of the parasite

Table 2. Natural infections of *Plasmodium agamae* (A), *P. giganteum* (G), and *P. mexicanum* (M) followed in rainbow lizards (first two species) and western fence lizards (last species) maintained in the laboratory.

(Animal number indicates malarial species followed. Parasitaemia and change in parasitaemia, if any, given. Parasitaemia is ranked as high (H = > 250 parasites/10000 erythrocytes), intermediate (I = 30–250 parasites/10000 erythrocytes), and low (L = < 30/10000 erythrocytes). Also given are length of time over which the infection was observed in days, number of samples taken, the high and low proportion of microgametocytes observed as a percentage, the range in the proportion of microgametocytes, and the high and low numbers of gametocytes observed/1000 erythrocytes.)

Animal no.	Parasitaemia	Days	Samples	Low–high (%)	Range (%)	No. of gametocytes /1000 erythrocytes
A 81	I constant	20	4	38–54	16	2–5
A 84	H constant	13	3	38–53	15	22–40
A 82	H to I	34	6	32–44	12	2–7
A 99	H constant	49	10	31–41	10	3–7
A 79	I constant	27	5	29–42	13	1–7
A 77	H to I	49	8	30–40	10	3–10
A 74	I to H	53	10	40–48	8	1–31
A 72	I constant	42	6	42–57	15	2–5
A 98	I constant	45	7	38–41	3	3–7
A 87	H constant	27	5	45–50	5	11–24
A 86	H constant	27	5	44–54	10	20–60
G 80	I to H	39	7	33–45	12	< 1–27
G 68	I constant	20	4	42–50	8	1–4
G 02	I to L	27	5	43–51	8	3–13
G 98	L constant	45	7	28–45	17	< 1
G 87	L constant	27	5	27–33	6	< 1–1
G 86	I to H to L	27	5	36–50	14	2–16
S 28	W constant	62	7	34–64	30	< 1
S 40	W constant	87	7	42–60	18	< 1
S 47	W constant	62	6	43–50	7	< 1
S 01	W constant	46	5	44–64	20	< 1
S 03	W constant	46	6	46–54	8	< 1
S 25	W constant	45	6	52–82	30	< 1
S 22	I constant	62	6	47–53	6	2–10
S 36	I constant	70	6	49–56	7	1–17
S 41	W constant	46	6	37–55	18	1–2
S 42	I constant	60	6	34–45	11	7–20
S 60	I to L	55	4	47–56	9	1–7
S 80	H constant	55	7	40–50	10	12–44
S 31	I constant	62	6	39–63	24	1–14

relative to the entire infected cell (parasite area/total cell area). Once again, for all 7 infections studied, the relative size of the parasite increased over time (Kruskal–Wallis tests, $P < 0{\cdot}05$). This analysis eliminated the possibility that changing absolute parasite size over time was a result of smear processing techniques that might have differed over time. In summary, both cell shape and size needed to be examined to determine gametocyte maturity.

Small immature gametocytes of all three species usually appeared to be macrogametocytes; gametocytes that stained in the characteristic male pattern were medium to large cells and usually displayed the typical mature cell form. In all counts used to determine sex ratio of gametocytes, only large mature cells were included. Thus, possible inflation of the numbers of macrogametocytes with immature microgametocytes was greatly reduced, but probably not eliminated.

Repeated counts of gametocytes from the same slide demonstrated that sex ratio estimates could be seriously biased if counts were made from only a small area of the smear. That is, gametocytes occasionally appeared to clump by sex. To eliminate possible counting errors, all areas of each smear were scanned randomly. Cumulative counts of gametocytes demonstrated that sex ratio estimates stabilized at a sample size of 50–75. Therefore, to assure accuracy, a count of 100 gametocytes was used in this study for each ratio estimate.

Summary statistics are presented in Table 1 for the sex ratio of gametocytes in natural infections sampled at a single time soon after lizards were collected. The ratio of microgametocytes to macrogametocytes varied considerably among infections for all three *Plasmodium* species. There was no correlation between proportion of microgametocytes and parasitaemia for any species (Spearman rank correlation, all $r_s > 0{\cdot}10$). No significant interspecific difference existed in the proportion of microgametocytes for the two parasites of *Agama agama* in Africa, but *P. mexicanum* has a significantly higher proportion of microgametocytes than either African species (Table 1; Mann–Whitney tests, $P = 0{\cdot}228$ and $P < 0{\cdot}001$, respectively).

Infections followed in the laboratory fell into three classes: the proportion of microgametocytes stable over time (range of proportion of microgametocytes $< 10\%$), the proportion changing in a fairly regular fashion (rising or falling), and the proportion varying widely (Table 2, and Fig. 2). Constant sex ratios were fairly common in these laboratory infections (6 of 11 *P. agamae* infections studied, 3 of 6 *P. giganteum*, and 5 of 13 *P. mexicanum*), and the percentage of microgametocytes in some infections varied only slightly over several weeks. Similarly, natural infections of *P. mexicanum* followed in free-ranging fence lizards were predominantly constant in the proportion of microgametocytes (17 of 22 infections; Table 3 and Fig. 3). The range of the percentage of microgametocytes was similar for all three species followed in the laboratory (mean range: *P. mexicanum* = 15·2%; *P. agamae* = 10·6%; *P. giganteum* = 10·8%; Kruskal–Wallis test, $P > 0{\cdot}05$). However, *P. mexicanum* infections followed in the field displayed significantly less variation over time in their gametocyte sex ratios (mean range = 7·6%; U-tests, all $P < 0{\cdot}05$).

The figures also illustrate that, in infections with a fairly constant proportion of microgametocytes, the equilibrium gametocyte sex ratio varied among those infections (Kruskal–Wallis test for all three malarial species, $P < 0{\cdot}05$). Thus, although these three *Plasmodium* species appeared often to reach a constant, or equilibrium, ratio of microgametocytes and macrogametocytes, the infections differed in their final sex ratio.

Perhaps the ratio of male and female gametocytes simply varies over time as new gametocytes are added to the infection, and reaches a constant condition only when the infection ceases to grow. To test this possibility, I used data from Tables 2 and 3 and cast each infection into a 2 × 2 contingency table based on whether gametocyte numbers were constant or changing, and whether the range in the percentage of microgametocytes was 0–10 or > 10. Three tables resulted for infections followed in the laboratory and one for *P. mexicanum* infections followed in the field. These tables revealed no significant relationship between the change in gametocyte numbers and the variation in sex ratio (Fisher exact tests, $P > 0{\cdot}05$). Thus, the constant sex ratios observed in many infections appear to represent true equilibrium ratios

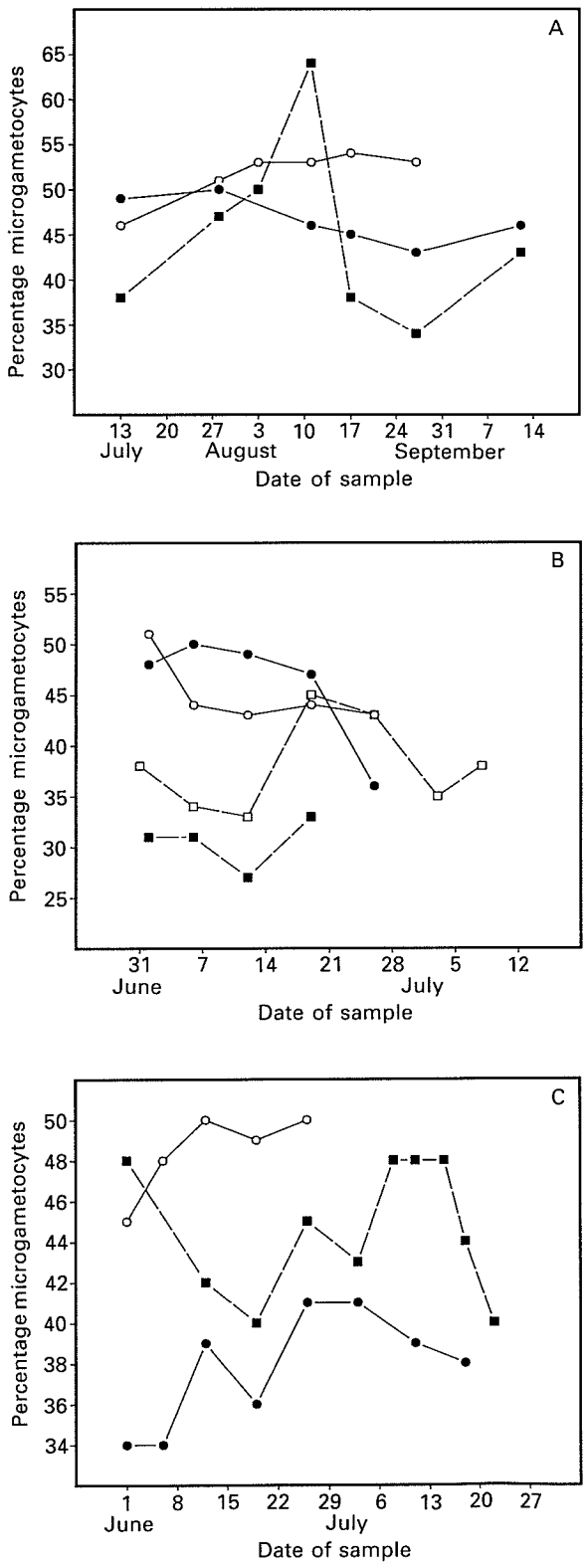

Fig. 2. Examples of natural infections of lizard malaria in laboratory maintained lizard hosts. The percentage of microgametocytes is plotted against time for infections of (A) *Plasmodium mexicanum* in western fence lizards, (B) *P. giganteum* and (C) *P. agamae* in rainbow lizards.

Table 3. Natural infections of *Plasmodium mexicanum* followed in free-ranging western fence lizards.

(Explanation of headings as in Table 2.)

Animal no.	Parasitaemia	Days	Samples	Low-high (%)	Range (%)	No. of gametocytes/ 1000 erythrocytes
3108	H constant	87	8	37–49	12	10–72
3295	H constant	99	6	46–50	4	58–137
3339	H constant	98	7	36–39	3	65–214
3396	I going H	62	4	41–49	8	25–46
3277	H constant	30	2	43–46	3	32–40
3052	L constant	52	6	32–41	9	3–8
3176	I constant	13	2	48–53	5	2–3
3014	L going H	24	3	34–36	2	10–15
3125	H constant	71	7	42–49	7	31–107
3107	L constant	72	5	45–53	8	1–3
3449	I constant	62	5	42–48	6	2–4
3443	I constant	74	7	45–55	10	10–28
3355	L-I constant	97	9	0–53	53	1–13
3334	I to H	56	6	0–54	54	11–30
3319	H to I	76	5	44–63	19	2–33
3306	H+ constant	31	3	43–45	2	88–156
3406	H+ constant	17	3	38–47	9	31–59
3307	I to H	87	4	34–41	5	1–102
3105	L constant	108	10	40–55	15	< 1–4
3008	L to I	9	2	37–44	7	13–14
3510	H constant	28	3	34–40	6	6–26
3162	H to very H	34	3	39–48	9	9–47

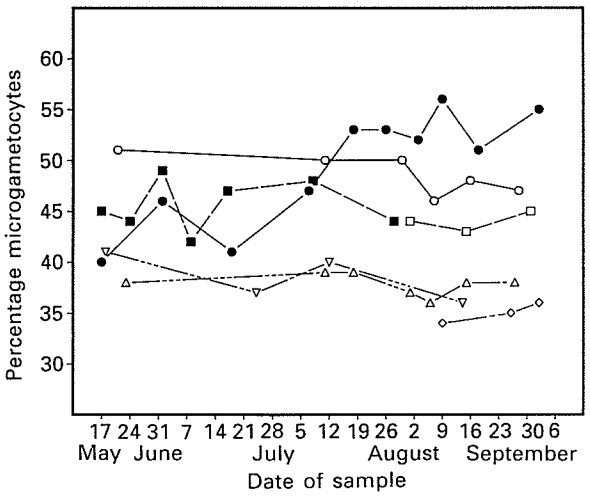

Fig. 3. Examples of natural infections of *Plasmodium mexicanum* in free-ranging lizards.

that are maintained even as the number of gametocytes changes.

Relative allocation of resources to male and female gametocytes was estimated by measuring areas of stained cells. Because gametocytes increase in size over time (Fig. 1), comparisons between males and females were done individually by sample. Measurements from 42 samples of *P. mexicanum* revealed 29 significant differences between male and female gametocyte size, with macrogametocytes averaging 1·24× the size of microgametocytes. None of 4 samples of *P. agamae* revealed significant differences in size between the sexes (U-tests, $P > 0{\cdot}05$), but macrogametocytes were larger in all of 3 samples of *P. giganteum*, averaging 1·18× the size of microgametocytes (U-tests, $P < 0{\cdot}05$). These ambiguous results could be a result of an error in measuring microgametocytes. As a consequence of their tendency to stain only slightly with Giemsa, the precise boundaries of microgametocytes were often very difficult to determine, perhaps frequently leading to an under-estimate of the cells' size. The figures from Table 1 can be adjusted by the relative size of male and female cells to estimate the proportion of resources allocated by microgametocytes versus macrogametocytes: 0·421 for *P. mexicanum*, 0·401 for *P. agamae*, and 0·333 for *P. giganteum*.

DISCUSSION

Sex ratio theory generally predicts an equilibrium proportion of males and females, with resources equally allocated to each sex. Although this theory has not been applied previously in studies of malarial parasites, the concepts appear appropriate for the biology of protozoans that produce dissimilar gametocytes (Ghiselin, 1974). Following this argument the expectation of an equal proportion of males and females does not depend on the number of

gametes produced by each microgametocyte, nor the proportion of these gametes that is viable. This is because the developmental decision to become either a male or female gametocyte is made by the bipotential pre-gametocyte cell. Once its fate is determined, a gametocyte cannot produce both male and female gametes. Therefore, a pre-gametocyte's ultimate success at producing young will depend on the relative proportions of viable microgametocytes and macrogametocytes in the population. The average reproductive sucess of microgametocytes, for example, is simply determined by dividing the number of zygotes produced by the number of viable microgametocytes in the blood taken by the insect host. An undifferentiated gametocyte that is fated to develop into the rarer sex will produce, on the average, more zygotes than will a cell that develops into the more common sex.

The mechanism that drives sexual differentiation of gametocytes is unknown, but some studies suggest gametocyte sex may not be heritable (Walliker, 1976; but see Inselburg, 1983). Environmental sex determination is common in animals and plants, and appears adaptive in varying environments, including those experienced by parasites (Karlin & Lessard, 1986). In this situation a pre-gametocyte cell might monitor the prevailing sex ratio of gametocytes, then alter its development to that of the rarer sex. Once again, a 1:1 gametocyte sex ratio would emerge.

Literature data on sex ratios of malarial gametocytes in natural infections unfortunately are scant, and available primarily for those species infecting humans. This paucity of reliable data on the ratio of micro- and macrogametocytes derives from several important difficulties. In some species gametocytes appear similar to mature schizonts under the light microscope (Taliaferro, 1925; Gambrell, 1937), or may be rare in the peripheral blood (Collins, Contacos, Guinn & Held, 1967). Also, immature gametocytes often appear to be macrogametocytes, which differentiate into both sexes only as they reach maturity (Gambrell, 1937), sometimes making reliable counts difficult. For example, Hawking, Wilson & Gammage (1971) presented gametocyte sex ratio counts for 4 individual hosts followed for 3 days, but combined female and immature male gametocytes in their data. Most important, the majority of *Plasmodium* species have been studied in the laboratory in unnatural hosts. Considering the long-known tendency of laboratory strains of *Plasmodium* to evolve alterations in their biology, including production of gametocytes (Vanderberg & Gwadz, 1980; Bhasin & Trager, 1984; Mons, Boorsma & Van Der Kaay, 1985), any data on sex ratios of gametocytes of laboratory strains may be of only marginal interest. In contrast with these difficulties, the relative ease in differentiating male and female mature gametocytes, and making counts in natural lizard malaria infections, make lizard malaria species useful for the study of the sex ratio of *Plasmodium* gametocytes.

Most published reports simply state that macrogametocytes are usually more abundant than microgametocytes without presenting any quantification of this phenomenon (Laird, 1951; Collins & Contacos, 1969; Collins *et al.* 1969). Some reports, although not providing systematic longitudinal counts on many infections, do provide some data that suggest that the sex ratio of malarial gametocytes varies greatly both among *Plasmodium* species and during an individual infection. Some examples follow. In an infection of *P. ovale* observed for 4 days the proportion of microgametocytes varied from 11–37% (James, Nicol & Shute, 1932). Another infection of *P. ovale* showed a range of 11–38% over 4 days (James *et al.* 1932). Talliaferro (1925) followed 8 infections of *P. cathemerium* in canaries and reported the mean of combined data of 48% microgametocytes. *P. nucleophilum* was reported to display a proportion of microgametocytes of 57% (Manwell & Voter, 1939). In 6 infections of *P. falciparum* followed from 5 to 74 days, the sex ratio varied greatly, but the results were compromised by drug therapy given to the patients and the data given appear to be estimates of sex ratios rather than actual counts (Shute & Maryon, 1951). Finally, an infection of *P. falciparum* was examined by James (1931) who observed a gradual increase in the proportion of microgametocytes over a 13 day period from 24 to 50%.

The three species of *Plasmodium* studied here also demonstrated significant variation in gametocyte sex ratio among species, among infections within a species, and sometimes even within infections over time. The sex ratio, and the ratio of allocation of resources to the sexes (based on sizes of male and female gametocytes), are often far from unity. In most infections, macrogametocytes are more abundant, but male gametocytes sometimes predominate (Tables 2 and 3). A surprising additional result is that many infections appear to reach a constant sex ratio, even when the density of gametocytes continues to increase, but this apparent equilibrium sex ratio differs among infections. Thus, in these infections new gametocytes are added in the sex ratio already present in the infection.

The basic theory leading to a prediction of an equilibrium sex ratio that is uniform among infections or equal investment in male and female cells seems to contrast with the results of this study and with the apparent variation in gametocyte sex ratios seen in other species of *Plasmodium*. Several explanations could account for the apparent discrepancy between basic theory and the outcome of this study. (1) Estimated sex ratios were biased by frequent inclusion of immature male gametocytes in the female count. Only large gametocytes, with the characteristic mature morphology, were counted

here, presumably reducing this source of error. Also, results from the longitudinal study of infections would be improbable if immature males were frequently misidentified as females. For example, in some infections with changing sex ratio over time, the proportion of females increased, which would not be expected if such counting errors had occurred. (2) The relative viabilities of macro- and microgametocytes might vary among infections, but remain constant within an infection over time. If so, the observed variation in sex ratios among infections might be spurious and sex ratios actually would be similar among infections if only viable gametocytes were counted. Viability of gametocytes can change as the cells age (Landau *et al.* 1979; Birago *et al.* 1982), but why viabilities of the two sexes should vary among infections, yet remain constant within an infection, would be difficult to explain. (3) Population structure, including genetic relatedness among individuals in a breeding group, and local mate competition can be important in altering equilibrium sex ratio away from 1:1 (Hamilton, 1967; Colwell, 1967; Charnov, 1982). Such reasoning has been applied only to social insects; its applicability to malarial parasites is uncertain. However, differing population structures caused by changes in the clonal composition of infections could lead to a diversity of selective environments among malarial infections that would lead to locally adaptive sex ratios. (4) Perhaps the mechanism of sexual differentiation is not under selective control, leading to no predictable sex ratio in infections. The timing of gametocyte production in *P. mexicanum* does not seem to be adaptive for a seasonable environment (Bromwich & Schall, 1986), so perhaps sex ratios in gametocytes are also not adaptive.

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