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GAMETOCYTE SEX RATIO OF A MALARIA PARASITE: EXPERIMENTAL TEST OF HERITABILITY

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ABSTRACT: The gametocyte sex ratio of *Plasmodium mexicanum*, a malaria parasite of western fence lizards, was studied in a modified garden experiment. Each of 6 naturally infected lizards was used to initiate 20 replicate-infections in naive western fence lizards. A significant donor effect was observed for the sex ratios of recipient infections at their maximal parasitemia, and this effect was associated with the sex ratio of the donor infection. In 20 infections in which sex ratio was followed during the course of the infection, 9 revealed constant sex ratios and 11 showed an increase in proportion of males over time. Recipient sex ratio was correlated with another life-history trait, a composite of rate of asexual replication and peak parasitemia, such that higher Rate–Peak scores were associated with infections with less female-biased sex ratios. These results are placed into the context of sex ratio theory that concludes that the degree of selfing of parasite genotypes (number of parasite clones) within the vector will influence the evolution of gametocyte sex ratio. The theory predicts that the sex ratio should be under some genetic control and thus be heritable as observed in the experiment. Clonal diversity should also influence the life-history trait, Rate–Peak, which was found to be correlated with sex ratio.

Sex ratio theory seeks to understand how ecological and evolutionary forces shape the proportion of males to females within populations of dioecious species. The theory has not only been notably successful in understanding sex ratio of large multicellular organisms (Charnov, 1982), but also applies to protozoans that produce dimorphic sex cells (Ghiselin, 1974). For example, malaria parasites (*Plasmodium* spp. and related apicomplexan genera) undergo asexual replication of haploid cells in the vertebrate host, casting male and female gametocytes into blood cells. When these gametocytes are taken up by a blood-feeding insect vector, each male gametocyte yields 1 to several mobile male gametes, each of which can fertilize a single female gamete produced by a female gametocyte (Bruce-Chwatt, 1985). The mechanism of gametocyte sex determination in *Plasmodium* spp. is poorly understood (Paul et al., 2000), but experimental infections initiated with a single parasite cell produce both males and females, demonstrating there is no genetic difference between male and female gametocytes (Lobo and Kumar, 1998). However, the probability of the developing gametocyte becoming male or female could have a genetic or an environmental component or both (Taylor, 1997).

Gametocyte sex ratios in *Plasmodium* spp. infections tend to be female-biased but vary among parasite species and infections within a species (Schall, 1989; Paul et al., 1999). Sex ratio theory provides a possible explanation for the observed variation in gametocyte sex ratios. Malaria parasites occur in subdivided populations (hosts) that may generate substantial population structure, including variation in the genetic diversity, or number of clones, within infections (Day et al., 1992). The theory of local mate competition (LMC) (Hamilton, 1967) predicts that, when only 1 clone of parasite is present in an infection, inbreeding is complete, and the evolutionary stable strategy (ESS) for gametocyte sex ratio would include just enough males to mate with all females (Read et al., 1992). Competition for mates would, therefore, be eliminated for genetically identical male gametes. In contrast, genetically more diverse infections would result in reduced inbreeding of parasites within the vector and a less female-biased gametocyte sex ratio. A selfing rate near 0 would favor a 1:1 sex ratio (Read et al., 1992; Dye and Godfray, 1993). These predictions of sex ratio theory apply only if mating among genotypes in the vector is random and if male and female offspring are equally expensive to produce for the precursor cell. Both appear to be the case for *Plasmodium* spp. Mating is random (Ranford-Cartwright, 1995), and the parasite cell that is fated to yield gametocytes will produce the same number of either all male or all female progeny (Silvestrini et al., 2000; Smith et al., 2000).

Natural selection could solve the ESS for gametocyte sex ratio in 2 ways. First, there could be genetically fixed sex ratios, either a single genotype or some stable polymorphism that matches the prevailing, long-term genetic diversity present in infections at a particular location (Read et al., 1992, 1995). Alternatively, selection could favor a phenotypically plastic response in which each infection alters its sex ratio to reach the local ESS. The parasite cells that yield gametocytes could monitor cues in the environment that indicate the clonal diversity of the infection and then produce the appropriate gender of daughter gametocytes. Some mixed strategy of genetically fixed and phenotypic plasticity is predicted by game theory (Maynard Smith, 1982), so these 2 mechanisms are not mutually exclusive.

Reported here are the results from a modified common garden experiment in which western fence lizards (*Sceloporus occidentalis*) naturally infected with the malaria parasite *Plasmodium mexicanum* were used as donors of blood to initiate replicate-infections in lizards previously unexposed to the parasite. The above reasoning suggests 2 predictions for the results of such an experiment. First, if there is some genetic control of gametocyte sex ratio (either genetic diversity of the infection or specific genotypes), there should be a significant donor effect on sex ratio of recipient infections and a correlation between donor and recipient sex ratios. Second, clonal diversity of infections should lead to selective pressure on other life-history traits, especially the rate of parasite replication and parasitemia (Nowak and May, 1994; Frank, 1996; Read and Taylor, 2000). Thus, sex ratio and other life-history traits should be linked. These predictions are explored here and, in light of the finding by Paul et al. (2000) that sex ratio of experimental infections of *P. gallinaceum* change over time, sex ratios of the recipient *P. mexicanum* infections were followed over the history of the infections.

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MATERIALS AND METHODS

The study was conducted at the University of California Hopland Research and Extension Center (HREC) located in southeastern Mendocino County, California, where *P. mexicanum* and its host, the western fence lizard, have been studied for many years (review in Schall, 1996). The experimental design was described previously (Eisen and Schall, 2000). Briefly, 6 naturally infected adult male donor lizards served to initiate infection in uninfected adult male recipient lizards (20 recipients per donor). The donor lizards carried ''mature'' infections of *P. mexicanum* because those infections had been carried over from the previous summer and included both asexual parasite cells (necessary to initiate the recipient infections) and mature gametocytes. Recipient lizards were collected from an area where malaria has not been found in 20 yr (Schall and Marghoob, 1995). Thin blood smears were made from these lizards, stained with Giemsa (pH 7.0, 50 min), and examined to confirm that the recipients were not infected.

Infections were initiated by injecting 2×10^5 asexual parasites into each recipient lizard intraperitoneally. The recipients were then maintained in vector-proof outdoor cages from May to mid-August. Thin blood smears were made weekly and stained with Giemsa. Each smear was examined at ×1,000 magnification for 6 min or until an infected cell was observed. Parasitemia was determined by counting the number of parasites in 1,000 erythrocytes. A previous study of *P. mexicanum* life-history traits (Eisen and Schall, 2000) used principal components analysis and found that asexual growth and peak parasitemia represent a single trait. Infections growing rapidly are those that reach a higher peak parasitemia. Therefore, this principal component score is used here to measure the life-history trait termed Rate–Peak.

The sex ratio of donor infections was determined for a blood smear made on the date when the recipient infections were initiated and for each recipient infection on the date of maximal gametocyte parasitemia. Changes in sex ratio were studied by scoring gametocytes on smears made each week during the experiment for 20 infections chosen for the entire range of final parasitemia observed (8–2,012 parasites/1,000 erythrocytes). Analyses compared the sex ratio of initial and final smears for each infection. For each count, 100 mature gametocytes were scored as male or female on the basis of staining color, size of the nucleus, and the distribution of pigment granules (Schall, 1989). Sex ratio is defined as the percent of male gametocytes observed in the blood sample. Some infections never produced gametocytes, or the lizard died before the infection reached a stable level, thus reducing the total number of infections in the study from 120 to 109. For some low parasitemia infections, <100 gametocytes could be counted (<20 = 6 infections, 21–50 = 3, 50–90 = 8). To determine the repeatability of the sex ratio counts, 17 smears were scanned twice on different days and gametocytes were scored; the counts did not differ for the 2 scans (Paired *t*-tests, $P > 0.05$).

Proportion data, such as sex ratios, have variances that are neither normally distributed nor independent of the means among groups. Also, confidence intervals calculated for proportions depend on sample size (larger sample sizes of gametocytes yield more reliable estimates of sex ratio). Therefore, comparisons among individual infections were done by chi-square contingency tests. Comparisons among recipient groups, as well as sex ratio versus Rate–Peak, were analyzed by fitting a linear model for binomial data using the JMP statistical package (SAS Institute). Gametocytes were entered as nominal data (male or female), and sex was the response variable for a logistic regression analysis using maximum likelihood. Likelihood ratio tests compared the full model with the one lacking the effect term. A significant difference between the 2 models was viewed as a significant ''effect'' term. This analysis corrected for the typically binomial error structure of proportion data and the variation in sample sizes of counted gametocytes.

RESULTS

Sex ratio of the donor infections varied significantly from 32 to 52% (chi-square contingency table test, $P = 0.017$). However, the donors fell into 2 clusters. Donors 1 and 2 had lower sex ratios (32 and 34%) that differed from 50% males (chi-square test of observed number of males and females vs. expected numbers with a 1:1 ratio; $P < 0.05$). There was no difference in sex ratio for these 2 donors (chi-square contingency table test, $P > 0.05$). Donors 3–6 had higher sex ratios (41–52%) that did not differ significantly from 50% males nor among each other (chi-square, $P > 0.05$). This means that there were 2 clusters of donors (termed here High and Low) with replicates (4 High and 2 Low). Therefore, subsequent analyses look for an effect of all 6 donors, then for the new donor clusters, 1 combining the 2 low sex ratio donors (Low cluster) and the other joining the 4 high sex ratio donors (High cluster).

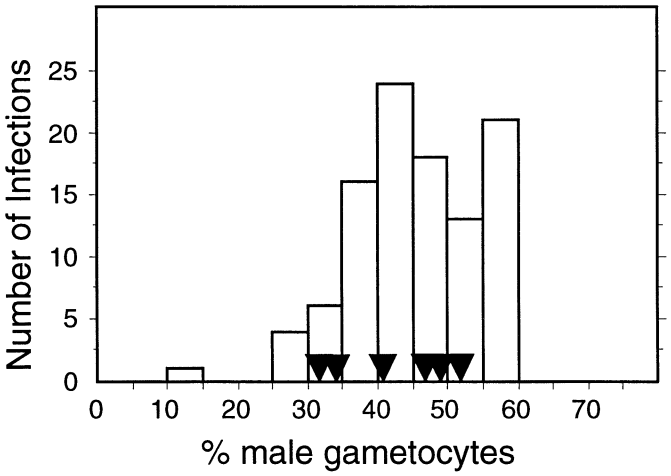

FIGURE 1. Distribution of gametocyte sex ratios for 6 naturally infected donors (indicated with triangle) and experimental recipient infections of *Plasmodium mexicanum* in the western fence lizard *Sceloporus occidentalis*.

Sex ratios of recipient infections varied, and were not normally distributed (Fig. 1; Shapiro–Wilk test, $P = 0.0002$), and favored higher proportions of males. Sex ratio of the donor infections fell within the modal range of those of the recipients (Fig. 1). Incorporation of donor as an effect into the logistic regression resulted in a significant reduction in total deviance (likelihood ratio test, $P < 0.00001$). The analysis was repeated twice, once for donors within the High cluster and once for those in the Low cluster, with no significant effect of donor ($P = 0.32$ and 0.08, respectively). Entering 2 donor clusters, High and Low, revealed a significant donor effect ($P = 0.04$). Therefore, there was a significant donor effect on recipient sex ratio, but this was caused by the difference in donors with high versus low sex ratio. This is visualized in Figure 2, which shows higher mean sex ratios for recipients from the High donor cluster than for those from the Low donor cluster. Although the donor explained a significant portion of the overall deviance in sex ratio of recipient infections, a significant residual deviance remained in all analyses (variation explained by recipient nested within donors) ($P < 0.0001$).

Inspection of the longitudinal data revealed an intriguing trend (Fig. 3). For 11 out of 20 infections, sex ratio became less female-biased over time (a significant difference between initial and final sex ratio; chi-square tests, $P < 0.05$). Thus, about half of the infections revealed a stable sex ratio and the other half a changing proportion of males.

Recipient sex ratio was positively correlated with the principal component score for Rate–Peak (Spearman Rank test, $r_s = 0.224$, $P = 0.026$), but this analysis fails to account for the

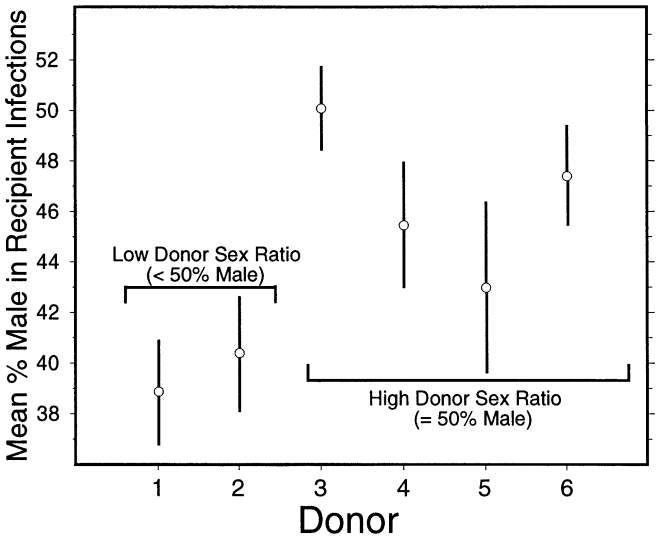

FIGURE 2. Mean and 1 standard error (vertical lines) for gametocyte sex ratio of recipient infections from each of the 6 donor infections of *Plasmodium mexicanum* in the western fence lizard (*Sceloporus occidentalis*). Values are normalized on the basis of a linear model with binomial errors.

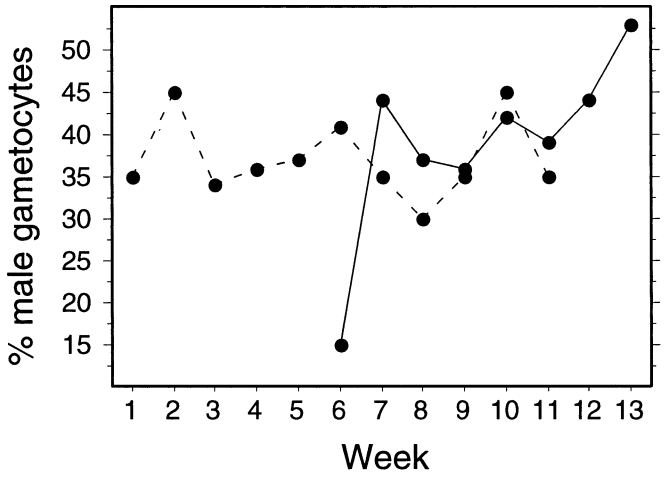

FIGURE 3. History of 2 experimentally induced infections of *Plasmodium mexicanum* in the western fence lizard *Sceloporus occidentalis*. Gametocyte sex ratio is given by week of infection (following patency or the week at which sufficient gametocytes could be scored). The infection shown with solid lines was 1 that revealed a significant change in sex ratio over time, and the infection shown with the dashed line was 1 with no significant change in sex ratio (chi-square tests).

confounding effect of the donor. Therefore, the principal component score for Rate–Peak was nested within donor and used as the effect term and sex as the response in the logistic regression. In this analysis, the life-history trait had a significant effect in the model ($P < 0.0001$) and is thus associated with sex ratio.

DISCUSSION

Several intriguing conclusions emerge from the analysis of experimental infections of *P. mexicanum* in fence lizards. First, gametocyte sex ratio in the recipient infections varied, and some of this variation depended on a donor effect. The underlying basis for this donor effect appears to be the sex ratio of the donor infections. That is, the sex ratio of the donor infections was transmitted to the recipients. This argues that sex ratio in *P. mexicanum* has a genetic basis and is thus heritable. Second, sex ratio of the recipient infections was correlated with another life-history trait, Rate–Peak, such that more rapidly growing infections with higher peak parasitemia were those with less female-biased sex ratios.

These first 2 findings are in accord with expectations of sex ratio theory. The intensity of LMC should vary depending upon the genetic diversity prevailing within infections. This would select for a genetic component controlling sex ratio. Likewise, genetic diversity within infections should influence the rate at which the infection grows and its highest density of cells in the host. It is interesting that a similar relationship between parasitemia and sex ratio was earlier reported for natural infections of *P. mexicanum* (Schall, 2000) and for 2 other lizard malaria parasites from an *Anolis* sp. of Panama (Pickering et al., 2000).

The results cannot distinguish between the 2 possible mechanisms for a malaria parasite to reach its ESS sex ratio, fixed genetic control and facultative adjustment. The distribution of genetic diversities among infections at any site could select for a balanced polymorphism of specialist genotypes, such that each donor contained some mixture of these genotypes that was passed on to the recipients. These same genotypes could also express different Rate–Peak scores. Taylor (1997) provides evidence that clonal isolates of *Plasmodium chabaudi* tend to yield different sex ratios in experimental mouse infections. In contrast, plasticity in sex ratio could also explain the results if donor infections differed in their genetic diversity, and the parasite cells in the recipient infections were able to monitor the local clonal diversity and respond to produce the optimal sex ratio. Clonal diversity itself could influence the rate of parasite increase either because of competition among clones to produce more gametocytes or because mixed clone infections better confound the host's immune response.

The third finding was that the sex ratio changed during the course of the infection in about half of the studied infections, becoming less female-biased over time. An earlier study of natural infections of *P. mexicanum* followed over time (Schall, 1989) found that sex ratio of most infections remained constant over time, but varied substantially among infections. The current findings do not conflict with those of Schall (1989) if most of those natural infections had already reached their mature, stable sex ratios. Paul et al. (2000) noted similar changing sex ratios in experimental infections of *P. gallinaceum* in chickens. Sex ratio was strongly female-biased early in the infection and later switched to more equal proportions of males and females. The proximate cue for this switch was an elevated level of erythropoietin produced when erythrocytes were destroyed by the growing infection. Paul et al. (2000) proposed that over time the host immune system mounts a potent attack that hinders male gametes as they emerge from the male gametocyte in the vector (the ''transmission blocking immunity'' of Carter and Graves [1988]). This would lead to a reduction in the fecundity of each male gametocyte (number of females mated); the LMC model predicts a higher proportion of males when male fecundity declines. For example, even when an infection is genetically uniform, the optimal sex ratio will be an equal number of

males and females if each male gametocyte only produces 1 viable gamete. This explanation may not apply to the *P. mexicanum* system. The immune response of lizards to malaria infection appears far less effective than that of birds or mammals (for example, infections with very high parasitemia often last for months in lizards with no sign of any reduction from an effective immune response; Bromwich and Schall, 1986; Eisen, 2000). Perhaps the change in sex ratio often seen in *P. mexicanum* infections is driven by how the parasite detects the genetic diversity in the infection. Cues may be absent when infections are weak, so the parasite cannot detect other genotypes present and thus the resulting sex ratio is strongly female-biased. As parasitemia increases, the cues may become more informative, and the sex ratio would begin to shift toward equal number of males and females in those infections in which genetic diversity is high. This hypothesis should be testable by experimentally altering the genetic diversity in *P. mexicanum* infections, which should lead to a shift in sex ratio in high-diversity infections and no such shift in infections with few parasite genotypes.

A last finding is that a significant, and large, residual recipient effect was observed, such that the donor sex ratio accounted for only a small part of the overall deviance in sex ratio among recipient infections. Two shortcomings in the experiment could have obscured the predicted donor effect: (1) the narrow range in the sex ratio of the chosen donor infections, and (2) a possible failure to replicate exactly the genetic structure of the donor infections (as required by a common garden experiment). Sex ratios of natural infections of *P. mexicanum* at the site vary from about 20% males to equal proportions of males and females, but most infections fall in the range of those used as donors (Schall, 1989, 2000). The experiment was originally designed to monitor changes in various life-history traits of the infections but not sex ratio (Eisen and Schall, 2000). Thus, the donor infections were chosen at random relative to their sex ratios, and a narrow range in donor sex ratios was thus not surprising. Inoculation of 2×10^5 parasite cells most likely brought all of the genotypes of parasites into the recipients, but the relative proportion of different genotypes may have been altered. Inbreeding experienced in any population is influenced by the relative proportions of the mating genotypes (Stubblefield and Seger, 1990). Applying that idea to the experiment with *P. mexicanum*, if a recipient infection harbors many clones, but one predominates, then the common clones experience high inbreeding, and a strongly female-biased sex ratio is expected, provided there is phenotypic plasticity in production of male and female gametocytes. A last possibility is that there are host effects that are not reflected in sex ratio theory, and differing host environmental conditions influence production of male versus female gametocytes in unexpected ways.

The findings of a likely genetic influence over gametocyte sex ratio, a change in sex ratio in many infections over time, and a correlation of sex ratio with another important life-history trait, Rate–Peak, argue that studies on sex ratio open a novel window into understanding the population biology of malaria parasites. Light could be cast on such issues as the parasite's transmission biology, interaction with the host immune system, and virulence, as well as the influence of genetic diversity on the evolution of other traits such as drug resistance.

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