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TESTING SEX RATIO THEORY WITH THE MALARIA PARASITE *PLASMODIUM MEXICANUM* IN NATURAL AND EXPERIMENTAL INFECTIONS

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The malaria parasite (*Plasmodium*) life history accords well with the assumptions of local mate competition (LMC) of sex ratio theory. Within a single meal of the blood-feeding vector, sexually dimorphic gametocyte cells produce gametes (females produce one, males several) that mate and undergo sexual recombination. The theory posits several factors drive the *Plasmodium* sex ratio: male fecundity (gametes/male gametocyte), number and relative abundance of parasite clones, and gametocyte density. We measured these traits for the lizard malaria parasite, *Plasmodium mexicanum*, with a large sample of natural infections and infections from experiments that manipulated clonal diversity. Sex ratio in single-clone infections was slightly female-biased, but matched predictions of theory for this low-fecundity species. Sex ratio was less female-biased in clonally diverse infections as predicted by LMC for the experimental, but not natural infections. Gametocyte density was not positively related to sex ratio. These results are explained by the *P. mexicanum* life history of naturally low clonal diversity and high gametocyte production. This is the first study of a natural malaria system that examines all traits relevant to LMC in individual vertebrate hosts and suggests a striking example of sex ratio theory having significance for human public health.

KEY WORDS: **Clonal diversity, Fertility Insurance, gametocyte, local mate competition, male fecundity.**

When founding the study of sex ratio evolution, Darwin lamented the paucity of cross-taxa data that are required to reveal general patterns; in short, he found "the materials are scanty" (Darwin 1871). Today there is no shortage of such data, and sex ratio theory has developed into one of the most successful and productive research programs in evolutionary biology (Charnov 1982; West 2009). However, until recent years, research has centered primarily on the sex ratios of large, charismatic organisms, such as plants and animals, with little notice given to sexually reproducing protists, the dioecious single-celled eukaryotes. Ghiselin (1974) long ago noted that these protists, with their diverse life histories, should offer unexpected insights into the generality of life-history theory. We focus here on one example, the malaria parasites (*Plasmodium* and related genera in the phylum Apicomplexa, *sensu* Martinsen et al. 2008).

The life cycle of *Plasmodium*, including the process of sexual recombination by dimorphic cells, was understood by 1910 (Garnham 1966). This was years after Darwin's brief discussion of sex ratio evolution (1871), but also after Düsing (1884) presented a clear mathematical treatment that introduced the modern theory of sex ratios (translation in Edwards 2000). Sex ratio theory, though, was not applied to malaria parasites until many decades later (Schall 1989; Read et al. 1992). At that time, very few data on sex ratios of malaria parasites had been published (Schall 1989); thus, the materials were again scanty. Several studies have now reported on sex ratios of the malaria parasite sex cells and find variation among parasite species, among populations at different sites, among infections at any site, and even within infections over time (reviewed in Schall 2009). The origin of this variation begs an explanation, and that explanation should emerge from sex ratio theory.

EVOLUTION
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Plasmodium parasites replicate asexually in the blood of a vertebrate host (bird, squamate reptile, or mammal) over multiple cycles. An infected host may harbor one to several genetically distinct clones of parasite (Read and Day 1992; Paul and Day 1998; Vardo and Schall 2007). Some cells exit the cycle of asexual replication and develop into male and female sexual cells, the gametocytes, which undergo no further reproduction in the vertebrate's blood. The parasites are transmitted between hosts by blood-feeding insect vectors; within minutes of blood ingestion, male and female gametocytes form gametes that mate, creating an ephemeral diploid zygote (all other stages are haploid). Thus, all mating takes place between the gametocytes within a single blood meal. Female gametocytes produce a single female gamete, and males produce up to eight flagellated male gametes (the maximum based on three rounds of mitosis; Alano and Carter 1990; Lobo and Kumar 1998). We refer to the number of gametes produced by a male gametocyte as male fecundity. Meiosis followed by further asexual replication results in haploid transmission-stage parasites (sporozoites) that migrate to the insect's salivary glands.

The first use of sex ratio theory in the study of malaria parasites (Schall 1989) followed Düsing's conclusion that the equilibrium after selection will show equal proportions of males and females. However, Read et al. (1992) recognized that the brief mating in the vector between gametocytes taken from a single vertebrate host matches the population structure assumed for Hamilton's (1967) local mate competition (LMC) model. LMC predicts female-biased sex ratios will be favored by selection when a population is divided into local breeding patches containing the offspring of only one or a few mothers. For malaria parasites, these breeding patches are the blood meals taken by vectors. Testing LMC for malaria parasites requires accurate counts of male and female gametocytes in the vertebrate host's blood, measures of male gametocyte fecundity, assessments of clone numbers within the host and their relative abundance, and measures of factors that may limit fertilization success (below). However, few empirical studies with malaria parasites to date have measured all the relevant factors because gathering the required data has proven challenging. For example, tests using the *Plasmodium* species infecting humans are hindered by a striking feature of the life histories of these parasites: they typically produce few gametocytes in the blood, preventing reliable sex ratio measures for most individual infections (Taylor and Read 1997). Other natural systems in which gametocyte density is higher lack data on fecundity and molecular techniques for detailed comparisons of clone numbers and sex ratio (e.g., *Leucocytozoon spp*. [Read et al. 1995] and *Haemoproteus spp*. [Shutler et al. 1995]). The most detailed and elegant tests of the predictions of the LMC model use a rodent malaria parasite of African thicket rats in laboratory mice (Reece et al. 2008). Ideally, though evaluations of the theory should examine natural parasite–host associations that have coevolved.

Here, we present two studies of gametocyte sex ratio of *Plasmodium mexicanum*, a parasite of the western fence lizard (*Sceloporus occidentalis*) in northern California. This parasite produces large numbers of gametocytes in the lizard host's blood, which allows reliable counts of males and females for most infections. Thus, we were able to gather data on both gametocyte sex ratio and density of the gametocytes in the blood (which likely affects fertilization rates). Using microsatellite markers, we obtained a direct estimate of number of clones present as well as their relative abundance. We also have an estimate of the male fecundity of *P. mexicanum* (Neal 2011). In the first study, we measured sex ratio and clonal diversity for a large series of naturally infected lizards sampled over three warm seasons. In the second study, we used infections initiated for two experiments in which clonal diversity was experimentally manipulated in the natural lizard host, and gathered similar data for all replicate recipient infections. This is therefore the first report that measures all the relevant factors highlighted by sex ratio theory for a natural *Plasmodium* system: accurate measures of gametocyte sex ratio, male fecundity, number of clones and their relative abundance, and gametocyte density.

The Model

The LMC model notes that when only brothers and sisters are present in a patch, a mother can maximize her reproductive output by producing just enough sons to mate with all of her daughters (Hamilton 1967). If more mothers contribute offspring to a patch, then the mothers must balance two competing selective forces: producing more daughters increases the reproductive output of the patch (the number of zygotes), but producing more sons increases fitness relative to others in the patch (as long as males are the less common gender) because on average each son will obtain more mates than each daughter (Taylor and Bulmer 1980; Wilson and Colwell 1981; Frank 1986). Balancing these competing selective pressures yields an expected equilibrium sex ratio of $r = (N - 1)/2N$, where N is the number of mothers depositing equal numbers of offspring in the patch. This relationship can also be expressed in terms of the probability of selfing, s (or, equivalently, the coefficient of inbreeding, F [Dye and Godfray 1993]): $r = (1 - s)/2$ (Read et al. 1992). Note that the critical factor is the likelihood of selfing, which could be high even in multiclone infections if one clone predominates. If this sex ratio does not result in enough males to mate with all females, the minimum $r_{\min} = 1/(1 + c)$, where c is the male fecundity (the number of females each male can mate), is favored instead (Hamilton 1967). Figure 1 presents the equilibrium sex ratio for several values of c and the full range of possible selfing values. For *Plasmodium*, when $c = 8$ (the maximum suspected for these parasites), a single-clone infection

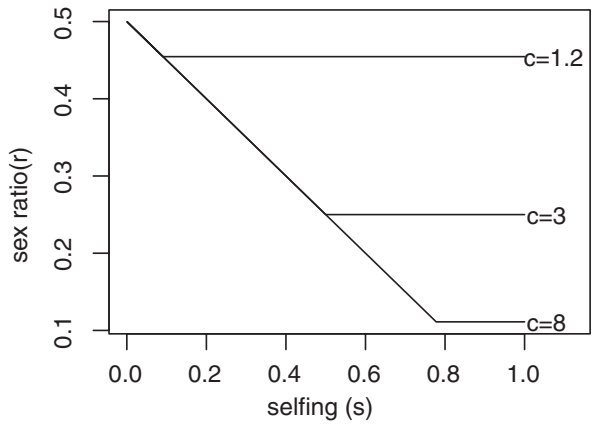

Figure 1. Predicted relationship between selfing (*s*) and sex ratio (*r*). For high selfing rates, the sex ratios predicted are often limited by the fecundity of males (*c*). The three values of *c* shown here are the maximum male fecundity of *Plasmodium* gametocytes ($c = 8$), the median male fecundity of *P. mexicanum* reported by Neal (2011; $c = 3$), and the median male fecundity of *P. mexicanum* if only 40% of gametes are successful at mating ($c = 1.2$). Expressions for the relationships shown are given in the text.

should produce 11% males, with a greater number of males as the number of clones (adjusted for their relative abundance) increases.

The LMC model assumes that all offspring in a patch are able to mate with one another, but West et al. (2001, 2002) recognized that factors such as very low gametocyte density, a strong antigametocyte immune response, or a quickly clotting blood meal could limit the number of gametes able to interact with one another within a patch, creating extremely local mating groups. Small breeding groups and a strongly female-biased sex ratio combined make it likely that there will not be enough males present to mate all females. Under such conditions, more males should be produced to ensure union with female gametes, an extension of LMC referred to as Fertility Insurance. This is particularly true if fecundity is also low, and the interaction of mating group size and male fecundity can further decrease the degree of female bias predicted (Gardner et al. 2003). In summary, the model predicts that three factors influence the sex ratios in malaria parasites: number of clones in infections and their relative proportions (LMC), male fecundity (LMC and Fertility Insurance), and number of gametocytes in a mating group (Fertility Insurance).

Tests of the LMC model have produced mixed results, with some studies showing a good fit (Read et al. 1995; Reece et al. 2008; Sowunmi et al. 2009; Neal 2011), and others failing to support the predictions of the model (Shutler et al. 1995; Osgood and Schall 2004). Indeed, studies reach contrary conclusions for almost every factor likely to influence gametocyte sex ratios. For example, gametocyte density (important for Fertility Insurance) can be negatively related to sex ratio (Robert et al. 2003; Merino et al. 2004; Reece et al. 2008), positively related (Pickering et al. 2000, Schall 2000), or not related (Neal and Schall 2010). The age of the infection, which likely influences the strength of the immune attack, can be related to increase in male gametocytes (Paul et al. 1999; Osgood et al. 2002; Robert et al. 2003), or not (Schall 1989; Osgood and Schall 2004; Neal and Schall 2010). The best support for the model comes from experimental infections of chicken malaria (Paul 2000) or rodent malaria in laboratory mice (Reece et al. 2008). Studies on natural infections need a measure of both gametocyte sex ratio and potential selfing of clones. In most such studies, clonal diversity is not directly measured, but instead surrogates are used, such as prevalence of the parasite in a host population (Read et al. 1995; Shutler et al. 1995), the number of donor infections combined in experimental infections (Osgood and Schall 2002), or parasitemia (assumed higher in multiclone infections due to competition; Pickering et al. 2000; Schall 2000).

Summarizing this model, we make several predictions for our study of both natural and experimental infections of *P. mexicanum*. In making these predictions, we assume that *P. mexicanum* is able to facultatively adjust its sex ratio in response to conditions within the host. Our predictions are as follows: first, gametocyte sex ratio for infections with a single genotype will be between 25 and 45% males based on two estimates of *P. mexicanum* male fecundity, which are lower than the maximum of eight. The modal number of exflagellating male gametes seen for *P. mexicanum* is 3 (Neal 2011), which gives a predicted sex ratio of 25% males ($1/1 + 3$). However, not all male *Plasmodium* gametes may mate (up to 60% of gametes may be malformed [Sinden 1983; Sinden et al. 1978]), and Neal (2011) found a good match between the sex ratios predicted by fecundity and those observed for individual single-clone infections if it was assumed that only 40% of gametes were successful, giving an effective male fecundity of 1.2. This estimate yields a sex ratio of 45% males ($1/1 + 1.2$). Second, gametocyte sex ratio should shift from female biased in single clone infections toward more males as clone number and diversity of clones (equal relative proportions) increases. Third, all else being constant, lower gametocyte density will favor a higher proportion of males.

Materials and Methods

STUDY SITE, SAMPLING, AND NATURAL INFECTIONS

Naturally infected and not infected fence lizards were collected at the University of California Hopland Research and Extension Center and two private ranches near the town of Hopland in Mendocino County, California (Schall 1996; Fricke et al. 2010). Lizards were captured using a noose on a fishing pole, and a few drops of blood were taken by toe clip to produce a blood smear for staining (Giemsa) and microscopic examination. Several drops were also dried and frozen for genetic analysis. Infected lizards were identified by scanning slides at 1000× for at least 3 min.

The natural infections surveyed in this study were all sampled from 2007 to 2009.

EXPERIMENTAL MANIPULATION OF CLONAL DIVERSITY

The data on experimental infections reported in this article are taken from two experiments in which infections were generated via intraperitoneal injection of blood from naturally infected donor lizards into not infected recipient lizards. Detailed methods are provided by Osgood and Schall (2004) and Vardo-Zalik and Schall (2009). The total number of asexual stage parasites injected into each lizard was approximately 2×10^5 for all treatments in both experiments, and both experiments included infections initiated with blood from individual donors (single-donor infections) and blood combined from multiple donors (multiple-donor infections). The first experiment (Experiment I, Osgood and Schall 2004) was conducted in May–September 2001 and had 15 total donors. Single-donor infections were initiated for each of the donors in three to 10 recipient lizards per donor, and five groups of multidonor infections were initiated, each combining blood from three donors in 10–18 recipient lizards. This study used seven single-clone and seven multiclone donors. The second experiment (Experiment II, Vardo-Zalik and Schall 2009) was conducted in June–August 2005 and had five total donors. Seven to eight single-donor infections were initiated from each of the first four donors, and eight to 14 multiple-donor infections were initiated with each of four combinations of two to four donors. Experiment II used two single-clone and three multiclone donors.

GAMETOCYTE SEX RATIOS AND GAMETOCYTEMIA

Gametocytemia was determined by counting the number of gametocytes in 1000 erythrocytes from microscope fields sampled over the entire blood smear. Gametocyte sex ratios were estimated for every infection by counting mature male and female gametocytes (with characteristic shape, size, and staining; Schall 1989) until at least 100 were scored or for 1 h of scanning. Most counts reached 100 gametocytes, and none with <50 gametocytes scored were included in the analysis (see results for sample sizes). Sex ratio counts for the experimental infections were all performed by author ATN and the blood smears from which counts were taken were made approximately 11 weeks after the infections were initiated (the latest date available for Experiment II). Sex ratio counts for the 2007 and 2008 natural infections were performed by author ATN, and those for 2009 by author ATN and an assistant. Quality control was tested by both observers counting six smears with very similar results ($P > 0.05$, paired t-test). Counts from infections for which counts were performed by both observers are summed for analysis because counts were made from different parts of the smear.

CLONE NUMBERS AND CLONAL DIVERSITY

DNA was extracted from dried, frozen blood dots made on the same day as the blood smears using the DNeasy kit (Qiagen, Valencia, CA). For the natural infections, we amplified the DNA using PCR at four microsatellite loci (Pmx306, Pmx732, Pmx747, and Pmx839) with the primers and conditions presented by Schall and Vardo (2007). For the experimental infections, each infection was genotyped for the two of these four loci that had the most alleles in the donor infection(s). Previous studies found that these markers, although themselves presumed neutral and noncoding, are associated with variation in sex ratio in single-clone infections (Neal and Schall 2010, 2013), and that single- and multiclone infections (based on these markers) differ in other important life-history traits, including rate of asexual replication and virulence measures (Vardo-Zalik and Schall 2009). These studies show that these microsatellites reveal relevant information about the genetic diversity of *P. mexicanum* infections.

After amplification, the product was run on an ABI Prism genetic analyzer (Cornell Core Labs, Ithaca, NY) and electropherograms were viewed using GeneMapper 3.5 software and PeakScanner v1.0 software (both by Applied Biosystems, Foster City, CA). The software allowed visualization of the size of each fragment (length of microsatellite allele based on number of ATT repeats) as well as the strength of the fluorescent signal. The peak height (i.e., strength of fluorescence) on electropherograms corresponds to the relative abundance of the allele in the sample (method verified in Vardo-Zalik et al. 2009; Ford et al. 2010; Ford and Schall 2011). Because all stages of the malaria parasite in a vertebrate host are haploid, each allele at a locus indicates a separate clone.

In the published derivations of sex ratio predictions based on LMC, sex ratio is predicted based on either the number of equally abundant clones or the selfing rate. The minimum number of clones in an infection was estimated as the maximum number of alleles at any one locus. However, not all clones are equally abundant, so we needed a measure of selfing as a predictor of sex ratio. Because selfing depends not only on the relative proportions of clones within an infection, but also on the sex ratios produced by each clone, we were not able to estimate it directly from our data. Instead, we used a diversity measure (Hurlbert's PIE $= 1 - \Sigma p_i^2$; $p_i =$ [height of peak i]/[sum of all peak heights]; no bias correction factor because genetic analysis samples so many parasites that bias correction has almost no effect, Gotelli 2008) to identify infections that had high and low clonal diversity. Diversity takes into account both clone numbers and their relative abundances. With this measure, an infection with a single clone has a diversity of zero, and an infection in which each individual parasite was a different clone would have a diversity of one. Infections with higher diversity therefore have more clones in more equal proportions, and should therefore have less female-biased sex ratios.

We calculated clonal diversity for each locus independently and used the maximum diversity at any one locus in the analysis.

ANALYSIS

Wilson and Hardy (2002) recommend analysis of sex ratio data by generalized linear models (GLM) with binomial error structure. However, the sex ratio data from all three datasets showed significant deviation from a binomial distribution (fit to rounded sex ratio $\times$ 100, $X^2 > 240$, $P < 0.0001$), but not from a normal distribution ($X^2 < 1$, $P > 0.13$). Additionally, for P close to 0.5, the binomial distribution is very similar to a normal distribution with mean np and variance $np(p-1)$, and the average sex ratios observed for *P. mexicanum* are generally between 0.4 and 0.5 (Schall 1989; Osgood et al. 2002, 2003; Osgood and Schall 2004; Neal and Schall 2010; Neal 2011). Therefore, linear models were applied. All analysis on sex ratio was weighted by the number of mature gametocytes counted because we have more confidence in ratios with more gametocytes sampled.

The goodness of fit tests mentioned above were performed in JMP 9.0.0 (SAS Institute, Inc., Cary, NC), and all remaining analysis was performed in R version 2.12.2 (R Development Core Team, Vienna, Austria). We used multiple regression analysis to examine the relationship of the variables expected to influence sex ratio on the sex ratios observed. These variables include number of clones, gametocytemia, and number of donors (one vs. multiple). The analysis was performed separately for each dataset (natural infections, Experiment I, Experiment II). Number of clones is not predicted to be linearly related to sex ratio, but the inverse of clone number is over the range where sex ratio is not constrained by male fecundity (see Fig. 1; $1/N = s$ for equally abundant, unrelated clones), so clone number was transformed to its inverse for use in the multiple regression analysis. Gametocytemia was cube root transformed prior to analysis to reduce the degree of skewness in the data. We therefore fit multiple regression models to sex ratio with all combinations of 1/clone number, gametocytemia$^{1/3}$, 1/clone number $\times$ gametocytemia$^{1/3}$, and number of donor infections (experimental infections only) as predictor variables and ranked the resulting models based on their F-statistic to determine the best combination(s) of predictor variables (Gotelli and Ellison 2004). The LMC model predicts a specific direction for the relationship between clonal diversity and gametocyte sex ratio and between gametocyte density and sex ratio (positive for proportion of male gametocytes vs. clonal diversity and negative for gametocyte density), so one-tailed significance tests were reported when these variables were included in the best model.

To determine whether the clonal diversity of infections affected the sex ratio (and not just the absolute number of clones), we ranked the infections based on the clonal diversity measure and divided them into thirds. We compared the sex ratios of infections with the lowest third versus highest third of clonal diversity using a t-test. The middle third of infections were excluded for two reasons: First, electropherogram peak heights are related to the relative abundance of alleles, but not with great precision (Ford et al. 2010 provide confidence intervals), so removing the middle third ensures that there are true differences in diversity between the groups being compared. Second, the effect of selfing on sex ratios for a *Plasmodium* species with low male fecundity should be obvious only for the highest diversity infections (Fig. 1, esp. $c = 1.2$).

Results

NATURAL INFECTIONS

Of 223 infected lizards sampled over three seasons, 194 were successfully genotyped to determine number of clones present and their relative proportions. We were unable to genotype the remaining 28 infections for at least one of the loci (and thus had incomplete data), so these infections were excluded from analysis. These were primarily infections with very low parasitemia, and thus polymerase chain reaction (PCR) amplification did not yield product that could be successfully run on the analyzer for one or more loci. Of the 194, we were able to obtain sex ratio counts of at least 50 mature gametocytes for 184 infections, and the remaining 10 infections were also excluded from analysis. Summary data on sample sizes, sex ratio, clone numbers, clonal diversity, and gametocytemia are presented in Table 1.

Sex ratios ranged from 0.25 to 0.61 proportion males (median 0.45, mean 0.44, Table 1, Fig. 2A). The sex ratio of single-clone infections differed significantly from the value of 0.25 predicted for a male gametocyte fecundity of $c = 3$, but not from the 0.45 predicted if $c = 1.2$ (mean = 0.429, 95% CI 0.419–0.459). The multiple regression model with the highest F included only transformed gametocytemia ($F = 3.75$, $P_{\text{model}} = 0.054$), although infections with higher gametocytemia tended to have less female-biased sex ratio, contrary to the predictions of Fertility Insurance ($\beta_1 = 0.010$, $t = 1.937$, $P_{t<0} = 0.973$). Gametocytemia was negatively correlated with clonal diversity contrary to prediction ($t = -2.026$, $P = 0.0442$, regression). Analysis with clonal diversity confirmed the lack of significant relationship between clones and sex ratio (Fig. 3A and B; mean sex ratios: high diversity = 0.436, low diversity = 0.439; $t = -0.203$, $P_{t>0} = 0.580$; diversity cutoffs: high > 0.448, low $= 0$). In summary, the factors predicted to be important for gametocyte sex ratio—clone number, clonal diversity, and gametocyte density—did not explain the variation in sex ratio for natural infections.

EXPERIMENT I

Of 143 infections initiated for this experiment, blood smears and dots made 11 weeks postinoculation were available for

Table 1. Summary statistics for each of the three datasets examined in this article.

Dataset	Group	*N*	Clones	Diversity	Sex Ratio	Gametocytes
Natural	2007	36	2.64 (2, 1–5)	0.44 (0.47, 0–0.74)	0.46 (0.47, 0.30–0.57)	14.7 (6, 1–148)
Natural	2008	53	1.76 (2, 1–4)	0.27 (0.31, 0–0.72)	0.41 (0.39, 0.25–0.58)	16.6 (8, 0–205)
Natural	2009	95	1.71 (2, 1–4)	0.21 (0.16, 0–0.64)	0.46 (0.46, 0.33–0.61)	16.8 (7, 0–169)
Natural	All	184	1.90 (2, 1–5)	0.27 (0.31, 0–0.74)	0.44 (0.45, 0.25–0.61)	16.3 (7, 0–205)
Exp. I	s/s	26	1 (1, 1–1)	0 (0, 0–0)	0.43 (0.44, 0.29–0.55)	29.0 (22.5, 2–117)
Exp. I	s/m	21	2.10 (2, 2–3)	0.48 (0.48, 0.35–0.64)	0.44 (0.45, 0.35–0.57)	33.6 (23, 5–112)
Exp. I	m/m	30	4.40 (4, 3–5)	0.60 (0.66, 0.13–0.76)	0.48 (0.48, 0.39–0.56)	35.0 (33.5, 1–91)
Exp. I	All	77	2.62 (2, 1–5)	0.36 (0.46, 0–0.76)	0.45 (0.46, 0.29–0.57)	32.6 (26, 1–117)
Exp. II	s/s	15	1 (1, 1–1)	0 (0, 0–0)	0.39 (0.39, 0.30–0.52)	13.3 (9, 1–47)
Exp. II	s/m	11	2 (2, 2–2)	0.32 (0.46, 0.10–0.49)	0.41 (0.41, 0.32–0.49)	23.2 (16, 1–78)
Exp. II	m/m	37	4.1 (4, 2–6)	0.53 (0.56, 0.13–0.77)	0.40 (0.41, 0.22–0.53)	23.8 (12, 0–156)
Exp. II	All	63	2.98 (2, 1–6)	0.37 (0.46, 0–0.77)	0.40 (0.40, 0.22–0.53)	21.2 (12, 0–156)

The three datasets are natural infections and experimentally induced infections initiated for two previous studies. Group indicates the year samples were taken for natural infections and the number of donors and clones for experimental infections (s/s = single-donor, single-clone infections; s/m = single-donor, multiclone; m/m = multidonor, multiclone). The calculation of clone number and clonal diversity are explained in the Methods Section. Sex ratio estimates are recorded as the proportion of mature gametocytes counted that were male based on at least 50 mature gametocytes per infection. Gametocytes indicate the number of gametocytes (mature and immature) counted in a sample of 1000 erythrocytes. Measures are reported as mean (median, minimum–maximum).

86 infections. Of these, we were unable to either successfully genotype or obtain sex ratio counts with at least 50 gametocytes for nine infections, bringing the total number of infections included in the analysis to 77. Summary data on these 77 infections are provided in Table 1.

Sex ratios ranged from 0.29 to 0.57 proportion male (mean = 0.45, median = 0.46, Fig. 2B). Sex ratios of single clone infections were significantly different from 0.25 predicted for $c = 3$ but not from 0.45 for $c = 1.2$ (mean = 0.428, 95% CI 0.418–0.457). The two multiple regression models with the highest F statistics each contained a single predictor variable: number of clones ($F = 11.34$) and number of donors (single vs. multiple, $F = 10.93$). Both models showed a relationship in the direction predicted by LMC: there was a negative relationship between the inverse clone number and sex ratio ($t = -3.368$, $P_{t<0} = 0.006$), and a positive relationship between donor number and sex ratio (i.e., multidonor infections had higher sex ratios, $t = 3.306$, $P_{t>0} = 0.0007$). All other models had $F<6.5$. Comparing infections with high versus low clonal diversity confirmed this pattern: high diversity infections (diversity > 0.528) had significantly higher sex ratios than low diversity infections (Fig. 3C and D; diversity = 0; mean sex ratios: high = 0.49, low = 0.43; $t = 3.56$, $P_{t>0} = 0.0004$). In contrast to a prediction of Fertility Insurance, sex ratio and gametocytemia were not correlated, nor was gametocytemia significantly correlated with clonal diversity ($t = 0.738$, $P = 0.463$, regression).

EXPERIMENT II

Sixty-three of the 69 infections initiated for this experiment were successfully genotyped and 50 mature gametocytes counted for samples taken 11 weeks after the infections were initiated. Table 1 provides summary data.

Sex ratios ranged from 0.22 to 0.53 proportion male (mean/median = 0.40, Fig. 2C). Single clone infections had sex ratios significantly higher than 0.25 and lower than 0.45 (mean = 0.392, 95% CI 0.360–0.424). The multiple regression model with the highest value contained only gametocytemia as a predictor variable ($F = 3.09$, $P_{\text{model}} = 0.084$), but the relationship was positive, not negative as predicted by Fertility Insurance ($\beta_1 = 0.01$, $t = 1.76$, $P_{t<0} = 0.958$). Gametocytemia is not significantly associated with clonal diversity ($t = 0.512$, $P = 0.611$, regression). For all other models, F was less than 1.7. Clonal diversity was significantly related to gametocyte sex ratio with higher sex ratios seen in more clonally diverse infections (Fig. 3E and F; mean sex ratios: high = 0.42, low = 0.39; $t = 1.85$, $P_{t>0} = 0.036$; diversity cutoffs: high > 0.53, low < 0.143).

COMPARISON OF NATURAL VERSUS EXPERIMENTAL INFECTIONS

Natural infections had a lower clonal diversity than the experimental infections (medians: 0.30 natural, 0.46 experimental, $P = 0.0005$, Kruskal–Wallis), although the trend was opposite if multidonor infections were excluded (medians: 0.30 natural, 0 experimental, $P = 0.007$). Gametocytemia was higher in experimental infections whether multiple-donor infections were included (medians: seven natural, 17 experimental, $P < 0.0001$). Sex ratio tended to be lower in experimental infections (means: 0.443 natural, 0.428 experimental, $P = 0.057$), especially if multidonor infections were excluded (means: 0.443 natural, 0.422 experimental, $P = 0.025$).

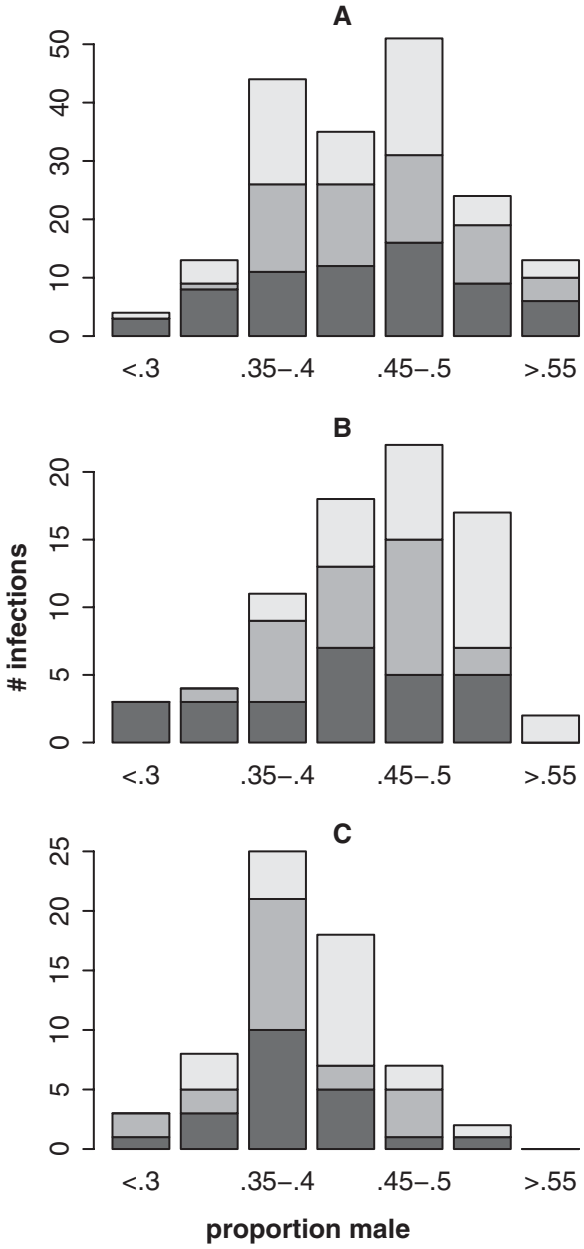

Figure 2. **Distribution of sex ratios observed in natural (A) and experimental ([B] Experiment I, [C] Experiment II) infections of the lizard malaria parasite *Plasmodium mexicanum*. Shading indicates the relative clonal diversity of the infections: dark gray is low clonal diversity, medium gray is medium clonal diversity, and light gray is high clonal diversity.**

Discussion

Ronald Ross, one of the discoverers of the *Plasmodium* life cycle, witnessed the toll the parasite exacted from human populations, and balefully referred to malaria as the "million murdering death." Today, a century later, hundreds of millions of people still are infected each year, with perhaps a million dying (Murray et al. 2012). Understanding the factors shaping gametocyte sex ratios should cast light on aspects of the parasite's life history of public health significance, including clonal diversity, how those clones interact within an infected host, gametocyte production, sexual recombination, and ultimate transmission success. Thus, this is a striking case of sex ratio theory having significance for human health and economic welfare. To date, though, few studies have explicitly tested the predictions of LMC and Fertility Insurance for individual infections using direct measures of the relevant variables, and none for a natural parasite–host system that has not been altered by intense public health measures, such as drug treatment. This study therefore offers a valuable complement to the experimental studies on a laboratory model system (rodent malaria in laboratory mice), experiments for natural lizard and chicken malaria, as well as broad comparisons for natural malaria systems. We present results for a large sample of natural infections of a malaria parasite of lizards over a three-year period and two experimental studies that manipulated the number of clones within infected lizards. We had available data on the relevant biological features needed to test the theory: reliable counts of gametocytes, number of clones, their relative abundance, male gametocyte fecundity, and gametocyte density. The results match the prediction of greater production of males with a lower chance of selfing for the experimental infections, but not for the natural infections. Gametocyte sex ratio was consistent with the low observed male fecundity for single-clone infections. However, sex ratio did not show the negative association with gametocytemia predicted by Fertility Insurance, and in fact showed the opposite tendency in two of the three studies.

LOCAL MATE COMPETITION

LMC predicts that gametocyte sex ratios will be less female-biased when the probability of mating with closely related individuals is lower (Hamilton 1967). This shift in sex ratio could result through local adaptation, that is, all parasites in an area may produce a sex ratio that is adaptive based on the prevailing degree of clonal diversity (and other factors, such as those implicated in Fertility Insurance), or via phenotypic plasticity, that is, parasites in individual infections may be able to detect relevant cues (presence/abundance of coinfecting clones, etc.) and shift their sex ratio accordingly. Here, we focused on the latter strategy: our goal was to determine whether *P. mexicanum* is able to facultatively shift its sex ratio in response to the number and relative abundance of coinfecting clones, which we measured using four microsatellite loci. Two testable predictions emerge from theory. First, in single-clone infections, a specific sex ratio is predicted based on the number of gametes produced by the male gametocyte. Male fecundity for *P. mexicanum* has been estimated as 1.2–3, so the predicted sex ratio in single-clone infections should be 0.25–0.45 proportion male. Second, the overall trend should

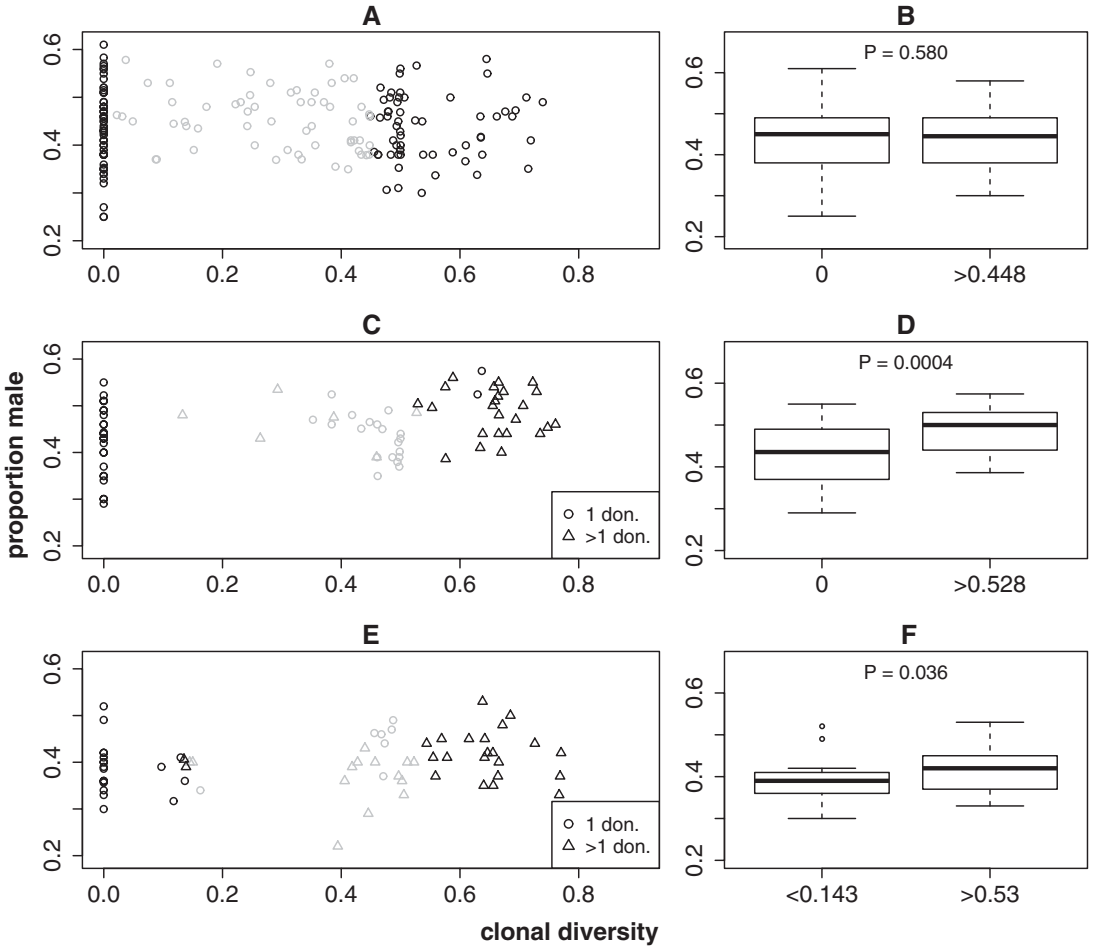

Figure 3. **Relationship between clonal diversity and sex ratio in natural and experimental infections of *Plasmodium mexicanum*. The first column of graphs shows individual data points. The shape of the point indicates number of donors (experimental infections only; see legend). Black points indicate infections with the highest and lowest third of clonal diversity measures; gray points are the middle third and were excluded from the diversity analysis. The second column of graphs shows a comparison of the sex ratios of infections with high (highest 1/3) and low (lowest 1/3) clonal diversity. (A) and (B) are data from natural infections collected over three years. (C) and (D) are data from experimental infections from Experiment I and (E) and (F) are data from experimental infections from Experiment II.**

be a decrease in female bias as clonal diversity increases (increase in number of clones and/or more equal relative proportions).

The sex ratio for single-clone infections for both natural and experimental infections was only slightly female biased. A measure of male fecundity based on number of gametes produced per male gametocyte ($c = 3$) predicts a significantly more female-biased sex ratio than observed (means for the three studies: 0.440, 0.428, 0.392). However, if developmental success of gametes is about 40% as determined for *Plasmodium falciparum* (Sinden 1983) and indirectly estimated for *P. mexicanum* (Neal 2011), male fecundity would be 1.2, and this predicts a sex ratio of 45% males, close to the observed value for all three studies.

A male fecundity as low as 1.2 would make it difficult to observe an effect of increasing clonal diversity on sex ratio, necessitating quite high clonal diversity for any shift in sex ratio to be predicted (Fig. 1). Data for natural infections of *P. mexicanum* showed no effect of the number of clones or clonal diversity on sex ratio. In contrast, results from two experimental studies showed a significant effect of clonal diversity on gametocyte sex ratio in the direction predicted by the LMC model. The experimental infections were generated by two researchers with somewhat different experimental designs conducted several years apart, and despite the only slight female-bias seen in infections of *P. mexicanum* (medians about 0.45 males), the overall trend supporting the model appeared for both experimental studies. What accounts for the conflict between natural and experimental infections? Three possible differences between natural and experimental infections could drive the disparate results.

First, the low male fecundity estimated for *P. mexicanum* would require a high clonal diversity, and thus low selfing, for a shift in gametocyte sex ratios to be predicted. The natural

infections had a lower clonal diversity (median 0.31) than the experimental infections (median 0.46), which may have obscured effects predicted by LMC.

Second, in the experimental infections, all clones entered a host simultaneously, and we have a complete record of the history of the infection. Natural infections were gathered over the entire course of the warm season over three years, and thus clones may have been entering and leaving, which would be undetectable with only a single sample taken. Also, the microsatellite markers do not differentiate between asexual parasite stages (the replication stages) and the gametocytes. If clones are at different developmental stages, the relative proportions of all parasites and only gametocytes may differ, causing our measure of clonal diversity not to reflect the true genetic diversity of the mating population.

Third, experimental infections may create combinations of clones in the vertebrate host that do not exist naturally. *Plasmodium mexicanum* shows signs of genetic differentiation at sites only 0.5 km distant (Fricke et al. 2010), and the donors used to initiate the experimental infections were often collected from different sites. Clones naturally found together may often be found together (because they readily transmit together to the insect host [Vardo-Zalik 2009]), and could cooperate to reach the overall infection sex ratio (all clones producing a female-biased sex ratio should increase zygote production and thus transmission success) or have a higher degree of relatedness than microsatellites would suggest. Thus, combining clones from different sites in experimental infections may increase the likelihood that clones are more diverse, increasing the likelihood of detecting a pattern.

FERTILITY INSURANCE

In addition to testing the predictions of LMC, this study tested one prediction of Fertility Insurance for *P. mexicanum*. Fertility Insurance predicts that factors reducing the probability of male gametes encountering and combining with female gametes should lead to an increased investment in males (up to a 1:1 sex ratio). These factors include low gametocyte density, host anemia, small vector blood meal size, a quickly clotting blood meal, and a strong immune response. We tested if gametocyte sex ratio was more female-biased in infections with higher gametocyte density. In 2 of the 3 studies, gametocytemia was a predictor of sex ratio in the multiple regression model, but in both studies the trend was the opposite of that predicted by Fertility Insurance: both showed a positive rather than negative trend. *Plasmodium mexicanum* in general has high gametocytemia relative to malaria parasites for which a negative association between gametocytemia and sex ratio has been observed previously (Bromwich and Schall 1986; Taylor and Read 1997; Reece et al. 2008 [*P. chabaudi*]; Robert et al. 2003 and Sowunmi et al. 2009 [*P. falciparum*]), so it is quite possible that the lowest gametocytemias for *P. mexicanum* are not low enough to reduce fertilization rates and thus favor a higher production of males.

Positive associations between gametocytemia and sex ratio have been observed previously for *Plasmodium tropiduri* (Pickering et al. 2000) and *P. mexicanum* (Schall 2000). Pickering et al. (2000) attributed this positive association to infections with more clones likely having both higher gametocytemia and less biased sex ratios, although they did not measure clone numbers. This does not appear to be the case here, as gametocytemia is not positively associated with clonal diversity for any of the datasets.

Although our results do not suggest that *P. mexicanum* has low enough gametocyte density to require production of additional males, other factors requiring sex ratio adjustment for Fertility Insurance that we did not measure directly may affect sex ratio evolution for this species. One example is rapid clotting of blood within the insect host. Although millions of erythrocytes may be taken in a blood meal, the meal may be rapidly divided into very small breeding groups if the blood quickly forms clots. Anecdotal evidence suggests that lizard blood clots more quickly than blood from mammals or birds (Neal and Schall 2010), as does blood taken from infected lizards relative to not infected animals (Vardo-Zalik and Schall 2008). Another example is vector size: sand flies, the vectors of *P. mexicanum*, take smaller blood meals and thus consume fewer parasites than the mosquitoes and black flies that transmit mammalian and some avian malaria parasites (Shutler and Read 1998). Blood clotting and vector size were not considered in detail here because they are more likely to explain variation among species than within species, but they may well account in part for the only slight female bias often observed for *P. mexicanum*.

Additionally, anemia and strength of immune response are two factors that may affect selection on sex ratios within a species. Although neither anemia nor immunity have been investigated in detail in relation to sex ratio for *P. mexicanum*, both have been associated with increasing sex ratio over time in other species (e.g., *Plasmodium gallinaceum* [Paul et al. 1999]; *P. gallinaceum* and *Plasmodium vinckei* [Paul 2000]), and *P. mexicanum* infections followed over time show no consistent shift in sex ratio (Schall 1989; Osgood and Schall 2004; Neal and Schall 2010). Also, a previous study attempted to downregulate the lizard host's immune system using testosterone, but no effect on sex ratio was observed (Osgood et al. 2003). Fertility Insurance does not appear to explain variation among infections in *P. mexicanum*, but may play a role in this species' relatively high sex ratios compared with other malaria parasites.

Conclusions

Data on gametocyte sex ratio for malaria parasites are still sparse, with less than a dozen species surveyed (reviewed in Schall 2009).

Results show, however, that malaria parasites display a great diversity of sex ratios ranging from strongly female-biased to close to 1:1, with even a few reports of male-biased sex ratios. Previous laboratory studies of both a rodent malaria parasite in a model host and a chicken malaria parasite found evidence of facultative shifts in sex ratio in response to environmental factors relevant to LMC and Fertility Insurance. Our study examined a natural *Plasmodium* system (the parasite in its natural vertebrate host), both in natural infections and infections with experimentally manipulated clonal diversity. Again, results concord broadly with the LMC model. The best studied *Plasmodium* species—*P. falciparum* of humans, *P. chabaudi* of rodents, *P. gallinaceum* of chickens, and *P. mexicanum* of lizards—are not closely related (Martinsen et al. 2008). Thus, overall, these studies argue that malaria parasites are able to assess the functional male gametocyte fecundity and detect the diversity of genetic clones within the infection to alter sex ratio as expected by the LMC model.

Our study of a natural *Plasmodium* system highlights several important issues. First, the male gametocyte fecundity is a life-history trait that is central for an understanding of the variation in sex ratio. This is because low male fecundity can limit the range of selfing values over which an effect on sex ratio is predicted. Paul et al. (1999) suspected the importance of fecundity when following increases over time in the gametocyte sex ratio of *P. gallinaceum* in chickens, as expected if the functional male fecundity is reduced by action of the host immune system. Fertility Insurance also highlights the importance of any reduction in functional male fecundity (Gardner et al. 2003). Second, the clonal diversity within infections may be low for some species, even when parasite prevalence is high. Read et al. (1995) and Shutler et al. (1995) used prevalence as a proxy for clonal diversity within infections, but prevalence and clonal diversity may not be strongly related (Vardo and Schall 2007). Last, the Fertility Insurance addition to the LMC model notes that low gametocyte density would favor a less female-biased sex ratio. This would be critical for species that produce few gametocytes in the vertebrate host blood (*P. falciparum*, e.g., [Taylor and Read 1997]), but not for other species that show high density of gametocytes such as we found for *P. mexicanum*.

When the LMC model was first applied to malaria parasites, Read et al. (1992) noted that traits related to sex ratio evolution, such as the interaction of parasite clones within the host and ultimate transmission success, could also be important for public health efforts to control one of the most important infectious diseases of human populations. Sex ratio theory has a central place in evolutionary biology, and its application to malaria parasites demonstrates both its generality (for even protists) and significance for applied biology as well as evolutionary theory.

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DATA ARCHIVING

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