

Manipulation of the Vertebrate Host's Testosterone Does Not Affect Gametocyte Sex Ratio of a Malaria Parasite

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ABSTRACT: Gametocyte sex ratio of the malaria parasite *Plasmodium mexicanum* is variable in its host, the western fence lizard (*Sceloporus occidentalis*), both among infections and within infections over time. We sought to determine the effect of host physiological quality on the gametocyte sex ratio in experimentally induced infections of *P. mexicanum*. Adult male lizards were assigned to 4 treatment groups: castrated, castrated + testosterone implant, sham implant, and unmanipulated control. No significant difference in gametocyte sex ratio was found among the 4 treatment groups. Two other analyses were performed. A surgery stress analysis compared infection sex ratio of castrated, castrated + testosterone implant, and sham implant groups with the unmanipulated control group. A testosterone alteration analysis compared infection sex ratio of the castrated and castrated + testosterone implant groups with the sham implant and unmanipulated control groups. Again, no significant difference was observed for these 2 comparisons. Thus, physiological changes expected for experimentally induced variation in host testosterone and the stress of surgery were not associated with any change in the gametocyte sex ratio. Also, the experiment suggests testosterone is not a cue for shaping the sex ratio of gametocytes in *P. mexicanum*. These results are related to the evolutionary theory of sex ratios as applied to malaria parasites.

The evolutionary theory of sex ratios posits that the proportion of males to females in a population is shaped by natural selection acting on individuals (Charnov, 1982). In genetically diverse populations, with low levels of selfing among reproducing individuals, the equilibrium sex ratio will be 50:50. Any deviation from equal proportions of the sexes will result in higher fitness for the less common sex and thus selection for females to produce offspring of the uncommon gender (Fisher, 1930). For species existing in structured populations, however, selfing could be locally common and local mate competition (LMC) (Hamilton, 1967) would have a role in controlling sex ratio. Under such circumstances, the sex ratio should often favor females. For example, when only 1 genotype is breeding, and selfing is complete, reproducing individuals will experience highest fitness when they produce just the sufficient number of sons to mate with all their daughters (Hamilton, 1967). LMC is readily applicable to malaria parasites (*Plasmodium* and related genera). Malaria infections within the vertebrate host could consist of 1 clone (complete selfing of gametocytes in the vector) to many clones (low selfing) (Anderson et al., 2000). Thus, the optimal sex ratio should be 1 microgametocyte:K macrogametocytes when only 1 clone is present (K being the number of gametes produced by the male gametocyte), and tend toward an equal proportion of male and female gametocytes for multiclonal infections.

Under the LMC model, host quality (physiological state, gender, age, etc.) would play no role in shaping the gametocyte sex ratio unless changes in host quality reduced the number of gametocytes below the minimum needed for the male and female gametes to meet in the vector ("fertility insurance" of West et al., 2002) or there is some influence of host quality on the selfing rate or fecundity of male gametocytes (K). A possible example of this latter phenomenon was described by Paul et al. (1999, 2000). Over the duration of *Plasmodium gallinaceum* infections in chickens, the host mounts an immune response against the parasite, and antibodies carried into the vector may interfere with the function of male gametes, i.e., the transmission-blocking immunity of Carter and Graves (1988). Thus, the host immune response reduces the fecundity of the male gametocytes by reducing K and favors a shift toward a higher proportion of males. This shift in sex ratio has been

observed over time in both bird (Paul et al., 1999, 2000) and lizard (Osgood et al., 2002) malaria infections.

These issues were pursued in an experiment that manipulated testosterone levels of male western fence lizards (*Sceloporus occidentalis*) experimentally infected with the malaria parasite *Plasmodium mexicanum*. Major alteration of testosterone levels should induce multiple-system physiological changes in the lizard host and thus provide a test to determine the role, if any, of physiological variation of the vertebrate host on gametocyte sex ratio. In particular, testosterone down-regulates the vertebrate immune system (Folstad and Karter, 1992), so the effect noted by Paul et al. (1999, 2000) should be reduced or eliminated in lizards experiencing elevated testosterone levels.

The overall biology of the *P. mexicanum* parasite–host association has been under study for many years at a field site in southeastern Mendocino County, California (Hopland Research and Extension Center [HREC]) (Schall, 1996). Gametocyte sex ratios of *P. mexicanum* infections vary both among infections and over the course of individual infections (Schall, 1989, 1996, 2000; Osgood et al., 2002).

The experimental design was described previously (Eisen and DeNardo, 2000). In brief, 1 naturally infected donor lizard served to initiate infection in 125 uninfected adult male lizards. Recipient lizards were collected from sites where malarial prevalence has been low for many years (Schall and Marghoob, 1995). Thin blood smears were made from these lizards, stained with Giemsa (pH 7.0, 50 min) and examined at $\times 1,000$ magnification to confirm the recipients were not infected. Subpatent, i.e., very weak infections, are rare at the HREC (Perkins et al., 1998). Recipient lizards were randomly assigned to 1 of 4 treatment groups. To minimize testosterone levels, lizards in 1 group were castrated (n = 33). Lizards in a second group were castrated and given a testosterone implant (n = 22). Implants were inserted intraperitoneally with testosterone in a 3-mm piece of silastic tube (0.078 mm inside diameter) sealed at both ends with silicon glue. This method increases testosterone levels up to 24-fold over ambient in the lizards (Klukowski et al., 1998). A sham implant group (n = 29) remained gonadally intact but received a subcutaneous saline-filled implant. Last, an unmanipulated control group did not undergo any surgical procedure (n = 41). Lizards were maintained by treatment group in large, vector-proof outdoor enclosures. Two weeks after surgery, lizards were inoculated intraperitoneally with 2×10^5 asexual stages of *P. mexicanum*. Each week, for 18 wk, thin blood smears were made and stained with Giemsa. Each smear was examined at $\times 1,000$ magnification, and parasitemia was determined by counting the number of parasites in 1,000 erythrocytes.

The sex ratio of recipient infections was determined for the date of maximal gametocytemia. Some infections never produced gametocytes, or the lizards died before the infection produced gametocytes, thus reducing the total number of infections available in the study from 125 to 96. For each count, 100 mature gametocytes were scored as male or female on the basis of staining color and size of the nucleus (Schall, 1989). Some infections produced only low gametocyte numbers, not allowing a count of 100 mature gametocytes. These were also eliminated from the analysis, reducing the final number of infections studied to 70 (castrated n = 19, castrated + testosterone implant n = 10, sham implant n = 21, and unmanipulated control n = 20). Sex ratio is defined as the percentage of male gametocytes observed in the sample. To determine the repeatability of the sex ratio counts, 5 smears were scanned twice on different days and gametocytes were scored; the counts did not differ significantly for the 2 scans (paired t-test, $t = 0.564$, $P = 0.6031$).

Testosterone levels were not measured for the recipient lizards. How-

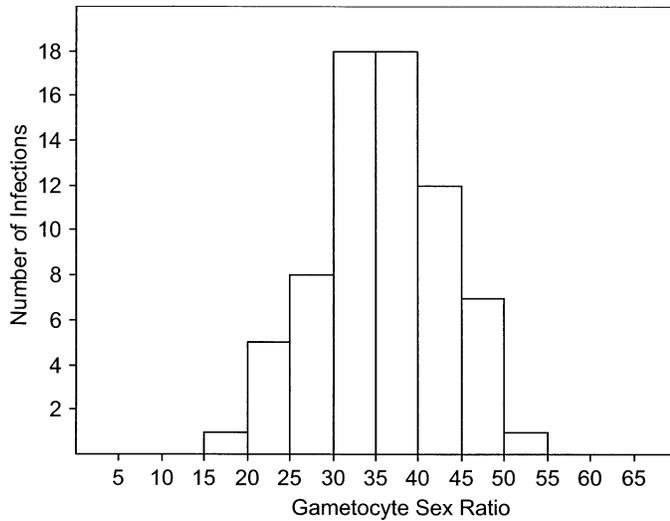


FIGURE 1. Distribution of gametocyte sex ratios (percentage male gametocytes) for experimental infections of *Plasmodium* in the western fence lizard *Sceloporus occidentalis*. Data for all 70 induced infections of the 4 treatment groups are combined.

ever, 3 kinds of evidence suggest there were physiological changes induced by the surgery and experimental alteration of testosterone. First, previous studies on lizards have shown that castration significantly reduces plasma testosterone levels and testosterone implants significantly elevate plasma testosterone levels (Moore, 1987; DeNardo and Licht, 1993). Second, ventral scales of the treatment groups differed in brightness of sexually dimorphic colors, and more aggressive behavior displays were observed in the testosterone-implanted group (Eisen and DeNardo, 2000). Last, mortality was not independent of treatment group. The castrated + testosterone implant group suffered 50% mortality before parasites developed in the blood, whereas 36% of the castrated group and none of the sham-implanted and unmanipulated control lizards died before the infection became patent in the blood (χ^2 test, $P < 0.0001$).

The distribution of gametocyte sex ratio for the total of 70 studied induced infections was not significantly different from a normal distribution, both within treatments and overall for all induced infections (Shapiro-Wilk W tests, $W = 0.975$, $P = 0.40$), and variances among treatment groups were homogeneous (O'Brien test, $P > 0.05$) (Fig. 1). However, because sample sizes were small within treatments (10–21), nonparametric tests were performed to compare treatment groups. No significant difference in sex ratio was found among the treatment groups (Fig. 2; Kruskal-Wallis test, $P = 0.43$). Two additional comparisons were made: (1) a “surgery stress” group of castrated, castrated + testosterone implant, and sham implant treatment lizards ($n = 50$) that had all experienced surgery against the unmanipulated control group, which had not been altered in any way ($n = 20$); and (2) a “testosterone alteration” group of castrated and castrated + testosterone implant lizards ($n = 29$) that had their testosterone levels altered against the “no testosterone alteration” group pooling sham implant and unmanipulated control lizards ($n = 41$). No significant difference was observed for these 2 comparisons (Mann-Whitney tests, $P = 0.24$ and 0.93). A retrospective power analysis was performed, and the least significant number (sample size) was determined. The analysis concluded that for a data set with the same distribution and differences among treatments, 181 infections would have been needed for the results comparing the 4 treatment groups to be statistically significant.

Variation in gametocyte sex ratio is always observed for natural *Plasmodium* infections (Schall, 1989; Read et al., 1992; Pickering et al., 2000) as well as for experimentally induced infections, as seen here (Fig. 1). A previous experiment (Osgood et al., 2002) initiated induced infections from 6 donor infections. A small, but statistically significant, portion of the overall variation in sex ratio among recipients was accounted for by the donor, indicating some genetic basis for the variation in sex ratio. The present experiment sought to determine whether host

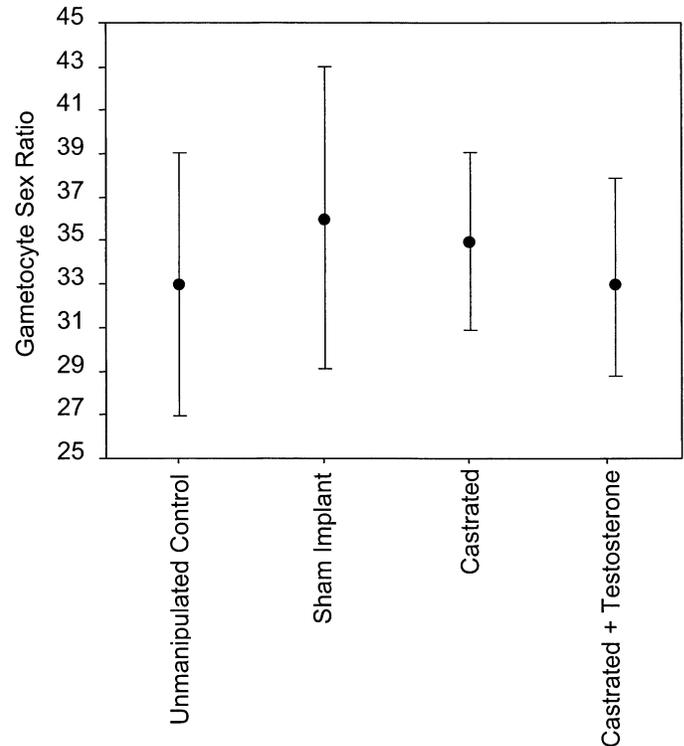


FIGURE 2. Median (points) and median absolute deviation (MAD) (vertical lines) for gametocyte sex ratios (percentage male gametocytes) of recipient infections of *Plasmodium mexicanum* in 4 treatment groups.

physiological quality influences the gametocyte sex ratio. The mean gametocyte sex ratios for all treatment groups were marginally female-biased and did not differ significantly. Thus, major physiological differences among hosts expected for animals experiencing surgery and substantial changes in testosterone levels did not influence the ratio of male to female gametocytes in the recipient infections.

Testosterone typically down-regulates the vertebrate immune system (Folstad and Karter, 1992), so immune system activity could have differed among the lizards in the treatment groups (especially reduced testosterone from castration against elevated testosterone from the implants). Although no information is available on the effects of testosterone on the fence lizard's immune system, a sister species, *Sceloporus undulatus*, experienced reduced parasite resistance when testosterone levels were experimentally increased (Klukowski and Nelson, 2001). Additionally, Salvador et al. (1996) reported a significant increase in tick infestation in the lizard *Psammotromus algeris* when testosterone was experimentally elevated.

If the immune system of the fence lizards was altered by the manipulation of the animals' testosterone levels, why was there no difference in gametocyte sex ratios as expected on the basis of the experiments of Paul et al. (2000)? That is, gametocyte sex ratios should have been more strongly female-biased in the castrated lizards and less female-biased in the lizards experiencing elevated testosterone levels. The results of Paul et al. (1999, 2000) show that *P. gallinaceum* uses a cue that is not part of the immune system (erythropoietin production) to signal when to produce more male gametocytes. Thus, the mounting immune attack may not directly signal the parasite to change its life history. It is possible that the appropriate cue was not provided to the *P. mexicanum* infections in the current study. Alternatively, the reptilian immune system may not mount the same kind of attack against infection by a malaria parasite as observed for mammal or bird hosts. Experimental manipulation of testosterone levels also does not influence the growth rate or peak parasitemia of *P. mexicanum* infections (Eisen and DeNardo, 2000). This would explain the ability of *P. mexicanum* infections to remain at very high parasitemias and continue asexual rep-

lication for several months in natural infections (Bromwich and Schall, 1986; Eisen, 2000).

Treatment did not influence the gametocyte sex ratio of recipient infections; thus, the explanation of the variation from 15 to 55% male in these infections remains unanswered. Recipients were infected with a large number of asexual parasites (2×10^5), presumably bringing in all the parasite genotypes contained within the donor lizard. However, experimental error or random sampling of the original infection may have resulted in the inoculation of different relative abundances of those genotypes, and thus different levels of selfing. The relative proportion of the clones in a recipient lizard could also shift after establishment of the infection. These possible changes in clonal structure may drive an infection to seek some new optimal sex ratio.

Sex ratio theory applied to *Plasmodium* is important for medical and evolutionary studies on population genetics, virulence, and transmission. Therefore, empirical tests of the theory are vital for progress in understanding the ecological and evolutionary factors that shape life-history traits of infections.

We thank the staff of the Hopland Research and Extension Center for logistical support. A. Chatfield assisted in the field and laboratory. Funding was provided by grants from the U.S. NSF, NSF EPSCoR, and the American Museum of Natural History.

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