Virulence of a Malaria Parasite, *Plasmodium mexicanum*, for Its Sand Fly Vectors, *Lutzomyia vexator* and *Lutzomyia stewarti* (Diptera: Psychodidae)

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ABSTRACT Evolutionary theory predicts that virulence of parasites for mobile vector insects will be low for natural parasite-host associations that have coevolved. I determined virulence of the malaria parasite of lizards, *Plasmodium mexicanum*, for its vectors, two species of sand fly (Diptera: Psychodidae), *Lutzomyia vexator* (Coquillett 1907) and *Lutzomyia stewarti* (Mangabeira Fo & Galindo 1944), by measuring several life history traits. Developmental rate from egg to eclosion differed for the two species when noninfected. For both sand fly species, developmental rate for each stage (egg to larval hatching, larval period, pupal period) and life span were not altered by infection. Infected sand flies, however, produced fewer eggs. This reduction in fecundity may be a result of lower quality of the blood meal taken from infected lizards (lower concentration of hemoglobin). This report is the first measure of virulence of *Plasmodium* for an insect vector other than a mosquito and concords with both expectations of theory and previous studies on natural parasite-host associations that revealed low virulence.

KEY WORDS *Plasmodium*, sand flies, parasite virulence

The theory on evolution of parasite virulence is fecund, with a large literature presenting diverse verbal and mathematical models to account for the observed variation in the harm caused to hosts by their parasites (Bull 1995, Frank 1996, Schall 2002). One view, the "mobility hypothesis" of Ewald (1994, 1995), posits that directly transmitted parasites will evolve low costs of infection because virulent genotypes will disable the host, hindering its mobility and thus reducing the number of transmission opportunities. In contrast, parasites that cycle between a large (typically vertebrate) host and a small arthropod (vector) can evolve to a virulent life history in the vertebrate because efficient transmission is possible even if the host is immobile (immobility may actually make the host more attractive to the vectors) (Ewald 1994, 1995). Broad comparisons among human parasites support the dichotomy in virulence between directly transmitted and vector-transmitted parasites (Ewald 1983). The mobility hypothesis leads also to a corollary prediction concerning the vectors: the vectors must remain highly mobile (flight in mosquitoes, for example), and selection should favor low virulence for the arthropod to ensure successful transmission (Ewald and Shulbert 1989).

The malaria parasites (*Plasmodium* spp.) offer an important system to test the mobility hypothesis. These parasites are known to often (but not always) result in severe pathology for their vertebrate hosts, including humans, rodents, birds, and lizards (Schall 2002, Atkinson and Van Riper 1991), as predicted by the hypothesis. Although epidemiological models for *Plasmodium* typically assume the parasite is benign for the insect host (Ferguson and Read 2002a), the issue of virulence for vectors in fact remains unresolved. Ferguson and Read (2002b) reviewed the literature on virulence of malaria parasites for mosquitoes and concluded that the results are equivocal. About half of the reports find that *Plasmodium* is harmful to the vectors, and the other half find no negative consequences of infection. One trend emerges: measurable virulence is associated with unnatural parasite-host associations, but when coevolved natural systems are examined, the cost to the vector is nil. For example, Marrelli et al. (2007) detected a cost to *Anopheles stephensi* mosquitoes infected with *Plasmodium berghei*, but the insects were from long-term laboratory cultures and were not the natural vector species. In contrast, Yan et al. (1997) examined fitness costs of infection with *Plasmodium gallinaceum* for its natural mosquito vector, *Aedes aegypti*, and found only a reduction in egg production for infected mosquitoes. This loss to fitness was associated strictly with the lower hemoglobin concentration in the blood of infected birds, so it was not a direct effect of the parasite

Use of vertebrate animals followed a protocol issued by the University of Vermont Institutional Animal Care and Use Committee.

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on the insect. These results nicely support the hypothesis but are confounded by other studies finding genetic variation within *Plasmodium* for complex effects on the vector (Ferguson and Read 2002, Ferguson et al. 2003).

To address these issues, the virulence of *Plasmodium mexicanum*, a parasite of fence lizards, *Sceloporus occidentalis* in northern California, was measured for its vectors, two species of Psychodid sand flies, *Lutzomyia vexator* and *L. stewarti*. This is the first study of virulence of a *Plasmodium* parasite for a vector other than a mosquito. *P. mexicanum* falls within the clade of *Plasmodium* that exploits bird and lizard hosts (Martinsen et al. 2008) and has a life cycle typical for *Plasmodium*, with the exception of exploiting the sand flies as the insect hosts (Fialho and Schall 1995, Schall 2000). The goals of the study were to test the prediction leading from the mobility hypothesis that virulence of *P. mexicanum* for its sand fly vectors will be low, and to determine whether results resemble those reported for other *Plasmodium*-vector studies.

Materials and Methods

Parasite and Host Natural History. The field work was conducted at the University of California Hopland Research and Extension Center in Mendocino County, California. *P. mexicanum* and its vertebrate and insect hosts have been under study there for more than three decades (Schall 1996, 2002; Vardo and Schall 2007). The sand flies *L. vexator* and *L. stewarti* at Hopland Research and Extension Center sequester during daylight hours in the burrows of ground squirrels, *Spermophilis beecheyi*, emerging at night to take blood meals from the lizards (Fialho and Schall 1995). They return to burrows to produce eggs that are deposited on the feces of the rodents. After taking a blood meal from an infected fence lizard, oocysts appear on the insect's midgut 9 d later at the preferred temperature of the insect; typically ≈60 oocysts are seen (Fialho and Schall 1995, Schall 2000, Vardo-Zalik 2009). The parasite has a substantial cost for the lizard, including hematological, physiological, reproductive, and behavioral deficits (review in Schall 2002). Previously, the only information on the effect of *P. mexicanum* on the sand fly hosts showed infection leads to a shift in the thermal preference of the insects, and this behavioral fever that increases the developmental rate of the parasite (Fialho and Schall 1995).

Collection of Lizards and Sand Flies. Lizards were collected at the site by noosing, and taken to the field laboratory where a toe clip supplied drops of blood to make a blood smear for later staining (Giemsa stain). Stained smears were examined under ×1,000 magnification to identify infected animals and to determine parasitemia of gametocytes (as gametocytes per 1,000 erythrocytes). Several other blood parasites are present in the lizard population (*Schellackia* sp., a haemogregarine, and *Trypanosoma* sp.), and, to prevent any possible interaction between parasites, only lizards carrying *P. mexicanum* were used for the infected lizard trials. To obtain control noninfected lizards, animals were collected from sites where malaria has been absent in the lizard population for decades of study (Vardo-Zalik and Schall 2009). Blood smears from these animals were also scanned, with none found infected. Polymerase chain reaction demonstrates that very low-level infections, not detected by such scanning, are rare at the field station (Perkins et al. 1998).

Two groups of sand flies were used in the experiments. The first were wild caught. Sand flies were collected from funnel traps set over the burrows of the ground squirrels (Chaniotis and Anderson 1968, Fialho and Schall 1995). The insects were taken to the field laboratory, where they were kept in cloth cages 22 cm^3 and at 75% RH. These flies are almost all newly eclosed individuals that have not yet taken a blood meal (Chaniotis and Anderson 1968), but the group may also contain some previously fed individuals that could have been already infected with *P. mexicanum*. The second group was laboratory-raised sand flies from single-species colonies previously established in the laboratory; identification of sand flies to species used the criteria of Young and Perkins (1984). Both wild and laboratory sand flies were used in the experiments because wild mosquitoes are known to be healthier than those raised in the laboratory (Huhu et al. 2007), so results were compared for the two groups in case this is true also for sand flies.

Feeding Sand Flies. Sand flies were fed on infected lizards with at least 20 gametocytes per 1,000 erythrocytes, which assures very high rates of infection (>90%) of the vectors (Fialho and Schall 1995, Schall 2000). The control group of sand flies was fed on noninfected lizards. A lizard was placed into the cloth cage with a soft cotton mask to prevent it from eating the insects and kept at ambient room temperature. The sand flies would alight on the lizard and take a blood meal, and the abdomen became expanded and turned bright red. When an insect left the lizard, it was removed and placed into an individual plastic vial (250 ml) with a layer of plaster on the bottom that was kept moist, a gauze cloth top, and a cotton ball soaked in fructose solution placed on the top gauze for a food source. The vials were placed in an incubator set at 26°C and 75% RH, the optimal conditions for parasite development, which results in mature oocysts in 9 d (Fialho and Schall 1995, Schall 2000). The sand flies digested the blood meal, with the abdomen turning brown, then shrinking, and finally produced eggs that could be readily counted on the white plaster background. The time in days to laying post feeding was recorded. After eggs were laid, a layer of finely ground larval diet was placed into the vial (Young et al. 1981). The adult female sand fly was maintained in the vial, with the fructose-water source, and observed until death, and the time in days was recorded (postfeed). The wild-caught sand flies were then dissected to determine species. However, infection status of sand flies could not be reliably determined after they died in the vials because the insects often died overnight and were in poor condition.

Table 1. Life history traits for wild-caught sand flies, *Lutzomyia vexator* and *Lutzomyia stewarti*, after taking a blood meal from a fence lizard, *Sceloporus occidentalis*, not infected with any protist blood parasite

	Lutzomyia stewarti	*Lutzomyia vexator*	*P* value
Days from egg to larvae	6 (1–20); $N = 78$	7 (2–26); $N = 91$	0.0080
Days larvae to pupae	23 (18–31); $N = 70$	27 (21–37); $N = 86$	<0.0001
Days pupae to adult	10 (4–14); $N = 69$	10 (8–15); $N = 83$	0.0001
Days total development	39 (34–55); $N = 69$	44 (38–65); $N = 83$	<0.0001
Days survival	5 (0–28); $N = 43$	2 (0–16); $N = 50$	<0.05

Given are medians (to nearest whole integer), ranges, sample sizes, and *P* value for Mann-Whitney *U* test.

The group fed on infected lizards was assumed to be primarily (>90%) infected based on previous studies (above), and the group fed on noninfected lizard was assumed to be free of *P. mexicanum.* However, each group must have been imperfect, with some infected insects actually free of the parasite and some of the noninfected wild-caught insects being already infected. With such possible errors, the tolerable type I statistical error for comparisons between infected and noninfected groups must be adjusted (Gotelli and Ellison 2004). In this study, I use a cutoff of $P = 0.10$ for determining significance for such comparisons. Because the distribution of data was not normal, I used nonparametric tests throughout. These have a lower power than parametric tests, and, thus, the adjusted cutoff value for statistical significance was more likely to detect differences between groups. Also, the size of blood meals taken by each sand fly was not determined, so any variation could increase the error term for each sample.

Determining Virulence. The measures of virulence directly relevant for the mobility hypothesis would be the sand fly's life span and ability to fly and find a blood meal (the lizard). However, these are not possible to measure in natural environments. Instead, several surrogates for virulence were studied that should be correlated with life span and mobility in nature. First, wild-caught sand flies of both species were fed on noninfected lizards to determine whether various life history traits of the vectors differed by species. Recorded were days from laying of the eggs to appearance of larvae, days from appearance of larvae until pupation, and pupation period in days. Also recorded was the number of days that a sand fly survived after laying eggs. Second, wild-caught sand flies of both species were fed on naturally infected and noninfected lizards, and days from egg to larvae, days larvae to pupae, and days to emergence of adults from pupae were recorded, as well as clutch size, number of days to produce eggs after feeding on blood, and the survival in days from feeding. Third, laboratory-cultured sand flies of both species were also fed on infected and noninfected lizards, and clutch size and days survival time after feeding were recorded. In addition to acting as surrogates for overall virulence, these indirect measures of virulence are of interest for consideration of the population ecology of the parasite-host system, including prevalence.

Results

Data on the suite of life history traits for both sand fly species fed on noninfected lizards are given in Table 1. Each life history trait differed for the two species, with *L. stewarti* development averaging 5 d shorter than *L. vexator* (39 d versus 44 d) (Table 1). Wild-caught sand flies fed on either infected or noninfected lizards did not differ in measures of developmental rate from time to produce eggs to timing when eclosing adults appeared, but again, *L. stewarti* experienced more rapid development than *L. vexator* (Table 2). However, for both species, sand flies fed on

Table 2. Life history traits of wild-caught sand flies of two species, *Lutzomyia vexator* and *Lutzomyia stewarti*, after taking a blood meal from western fence lizards, *Sceloporus occidentalis*, either infected or noninfected with *Plasmodium mexicanum*

	Noninfected	Infected	*P* value
Lutzomyia vexator			
Clutch size	54 (28–91); $N = 27$	49 (12–80); $N = 32$	0.073
Days to lay eggs	15 (8–30); $N = 20$	12 (8–27); $N = 33$	0.128
Days alive after feeding	18 (9–33); $N = 27$	15 (9–29); $N = 31$	0.127
Days from egg to larvae	8 (5–10); $N = 29$	8 (4–12); $N = 33$	0.397
Days larvae to pupae	29 (15–35); $N = 20$	28 (18–37); $N = 33$	0.281
Days pupae to adult	11 (5–18); $N = 28$	11 (3–22); $N = 33$	0.524
Lutzomyia stewarti			
Clutch size	66 (18–97); $N = 32$	54 (17–73); $N = 22$	0.034
Days to lay eggs	16 (7–45); $N = 34$	12 (8–43); $N = 21$	0.135
Days alive after feeding	21 (10–63); $N = 34$	20 (9–50); $N = 20$	0.597
Days from egg to larvae	7 (4–24); $N = 34$	7 (5–16); $N = 22$	0.534
Days larvae to pupae	27 (17–58); $N = 34$	28 (18–33); $N = 22$	0.756
Days pupae to adult	10 (3–43); $N = 34$	10 (2–15); $N = 22$	0.980

Given are medians (to nearest whole integer), ranges, sample sizes, and *P* value for Mann-Whitney *U* test, with a cutoff value of 0.10 considered significant.

Table 3. Two life history traits of two species of sand fly, *Lutzomyia vexator* and *Lutzomyia stewarti*, fed on western fence lizards, *Sceloporus occidentalis*, that were either infected or noninfected with the malaria parasite *Plasmodium mexicanum*

	Noninfected	Infected	*P* value
Lutzomyia vexator			
Clutch size	61 (6–116); $N = 43$	42 (2–103); $N = 24$	0.002
Days alive after feeding	16 (5–44); $N = 43$	22 (9–42); $N = 24$	0.050
Lutzomyia stewarti			
Clutch size	47 (8–107); $N = 40$	45 (4–92); $N = 17$	0.448
Days alive after feeding	20 (8–40); $N = 32$	17 (11–35); $N = 17$	0.270

Sand flies were from a laboratory culture. Given are medians to nearest integer, ranges, and sample sizes, as well as *P* value for Mann-Whitney *U* test.

infected lizards produced fewer eggs than those fed on noninfected lizards (Table 2). Reduction in clutch size was 9% for *L. vexator* and 18% for *L. stewarti.* Life span after feeding on blood did not differ for sand flies fed on infected and noninfected lizards, but *L. stewarti* lived longer (median of 21 d after feeding on blood compared with 18 d for *L. vexator*). Sand flies did not live long after laying eggs in laboratory conditions, but again, *L. stewarti* lived longer (median of 5 d compared with 2 d for *L. vexator;* Mann-Whitney *U* test, $P = 0.036$). Laboratory-raised sand flies were scored for clutch size and life span after taking a blood meal. Life span did not differ by blood source for either species; clutch size was smaller for *L. vexator* fed on infected lizards, but no effect by source of blood meal was seen for *L. stewarti* (Table 3).

Discussion

The issue of parasite virulence for insect vectors is of theoretical interest (to test evolutionary theory), but also is of substantial medical importance. For example, efforts to manipulate the transmission success of malaria parasites in their mosquito hosts by release of vectors' resistance to a parasite will be productive only if the parasite reduces the fitness of its insect host, thus favoring the resistant genotypes in the environment (Yan et al. 1997). Any cost of infection to the mobility of the vector would also influence transmission efficiency and competitive ability of resistant and normal vectors. Therefore, information on the effect of malaria parasites across species will cast light on both theoretical and applied questions in vector biology. This study sought to explore how a malaria parasite affects a vector other than a mosquito; *P. mexicanum* may be unique in exploiting sand flies as the vectors.

The life cycle of *P. mexicanum* is similar to other *Plasmodium* species. In the sand fly midgut, male and female gametocytes produce gametes that mate and eventually yield oocysts on the midgut wall (Fialho and Schall 1995, Vardo-Zalik 2009). These oocysts are large, and >200 may cover the entire midgut (mean ≈60) 9 d after a blood meal is taken from an infected lizard (Fialho and Schall 1995, Schall 2000, Vardo-Zalik 2009). A high proportion (>90%) of sand flies becomes infected after feeding on the blood of an infected lizard (if at least 20 gametocytes/1,000 erythrocytes are present) (Schall 2000, Vardo-Zalik 2009). Although oocysts must exact a cost to the sand fly during their development, no effect on life span was observed from presumed infection (feeding on an infected lizard). Also, eggs produced from sand flies that fed on infected lizards must have been normally provisioned because they experienced equal hatching success, and the offspring completed development at the same rate as those produced by sand flies that had fed on noninfected lizards. The only consequence of feeding on an infected lizard was a reduction in clutch size for both species of sand flies collected from the wild, and only for *L. vexator* for laboratory-raised sand flies. Although wild mosquitoes are known to be healthier than laboratory-raised insects (Huhu et al. 2007), results in this study were similar for wild and cultured sand flies.

The results presented in this work agree with the general pattern noted by Ferguson and Read (2002b), that natural *Plasmodium*-vector systems displayed little or no virulence for the insect host, as predicted by the mobility hypothesis of Ewald and Shulbert (1989). Modern theory on parasite-host coevolution regards virulence as a life history trait, and high versus low virulence will be favored based on the parasite's transmission biology (Schall 2002). For vector-borne parasites, high virulence is predicted for the vertebrate host, and low virulence (for mobility and life span) for the vector. Laboratory model systems may join a parasite and vector that are not found in nature, and thus not coevolved; the parasite therefore would not express adaptive phenotypes to reduce its harm to the host. The sand fly-*P. mexicanum* association, in contrast, is an ancient and highly coevolved system. For example, the parasite appears to manipulate the sand fly's thermoregulation to favor transmission efficiency (Fialho and Schall 1995). Such a parasite-host association should reveal the natural, coevolved phenotype.

The results for the lizard malaria system are similar to those reported for *P. gallinaceum* and its natural mosquito host, *Aedes aegypti.* Yan et al. (1997) detected no cost of infection for the mosquito, except for a reduction in clutch size. They noted that birds infected with *P. gallinaceum* suffer a reduction in blood hemoglobin, and this would account for the smaller clutch size produced by vectors feeding on infected birds. That is, the quality of the blood meal would be reduced for mosquitoes feeding on infected birds, with a reduced concentration of protein required for successful production of eggs. Infection with *P. mexi-*

canum also results in reduced hemoglobin levels in its lizard host, averaging 20% (Schall 2002), which would readily explain the reduction in number of eggs produced by infected sand flies. It is also possible that the vectors take a smaller blood meal from infected vertebrate hosts, thus reducing resources available for production of eggs. However, there is no evidence of this effect for sand flies feeding on fence lizards, nor for the bird malaria system. Therefore, the Yan et al. (1997) study and the results presented in this work suggest that the cost to the vector's fecundity, one component of its fitness, is not a direct effect of parasite virulence on the insect, but of its cost to the vertebrate host. Studies on the rodent malaria parasite, *Plasmodium chabaudi*, and *Plasmodium falciparum* in humans, however, lead to a more complex conclusion. Effect on mosquito hosts of *P. chabaudi* was a product of the specific parasite genotype, and not level of anemia in the mouse, nor parasite virulence for the mouse (Ferguson and Read 2002a, Ferguson et al. 2003). Mosquitoes fed on human blood containing *P. falciparum* that did not themselves become infected experience higher fecundity than mosquitoes fed on blood from noninfected human subjects (Ferguson et al. 2005). Thus, just the presence of parasites in the blood meal altered the fecundity of the potential host.

The laboratory results show *L. stewarti* has a 5-d (12%) shorter development time than *L. vexator.* Development time in insects is readily altered in selection experiments. For example, under strong selection, *Drosophila melanogaster* development time was reduced by 20%, but this took 600 generations (Burke et al. 2010). At the Hopland, California, site, *L. vexator* is far more common than *L. stewarti* in sampling studies (10-fold, Fialho and Schall 1995; 4-fold, Vardo-Zalik 2009). Although both species of sand fly are competent vectors for *P. mexicanum* (Schall 2000), *L. vexator* may be the more important insect host because of its relatively higher abundance. Both sand fly species use rodent burrows for laying eggs and for larvae to develop, so the shorter development time for *L. stewarti* may allow this species to produce one more generation during the warm season than *L. vexator.* Therefore, why *L. stewarti* is much less common presents a vexing question.

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