Sex-specific effects of an avian malaria parasite on an insect vector: support for the resource limitation hypothesis

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Abstract. Many parasites, such as those that cause malaria, depend on an insect vector for transmission between vertebrate hosts. Theory predicts that parasites should have little or no effect on the transmission ability of vectors, e.g., parasites should not reduce vector life span as this will limit the temporal window of opportunity for transmission. However, if the parasite and vector compete for limited resources, there may be an unavoidable physiological cost to the vector (resource limitation hypothesis). If this cost reduces vector fitness, then the effect should be on reproduction, not survival. Moreover, in cases where both sexes act as vectors, the effect should be greater on females than males because of the greater cost of reproduction for females. We tested these predictions using *Haemoproteus columbae*, a malaria parasite of Rock Pigeons (*Columba livia*) that is vectored by both sexes of the hippoboscid fly *Pseudolynchia canariensis*. Hippoboscids belong to a group of insects (Hippoboscoidea) with unusually high female reproductive investment; eggs hatch in utero, and each larva progresses through three stages, feeding from internal "milk" glands in the female, followed by deposition as a large puparium. We compared fitness components for flies feeding on malaria-infected vs. uninfected Rock Pigeons. Survival of female flies decreased significantly when they fed on infected birds, while survival of male flies was unaffected. Our results were contrary to the overall prediction that malaria parasites should have no effect on vector survival, but consistent with the prediction that an effect, if present, would be greater on females. As predicted, females feeding on malaria-infected birds produced fewer offspring, but there was no effect on the quality of offspring. A separate short-term feeding experiment confirmed that female flies are unable to compensate for resource limitation by altering blood meal size. The unanticipated effect on female survival may be explained by the fact that *H. columbae* also has the option of using male flies as vectors.

Key words: blood feeding; coevolution; Columba livia; Haemoproteus columbae; Pseudolynchia canariensis; *malaria; sex-specific effects; transmission; virulence.*

Introduction

Many infectious diseases are caused by pathogens that are vectored by arthropods (Jones et al. 2008, Colwell et al. 2011). The evolution of arthropod-transmitted parasites, such as those that cause malaria, is shaped by interactions with both the vertebrate and arthropod hosts. Although the virulence of parasites in vertebrate hosts has been well studied, the virulence of these same parasites in their vectors is relatively unknown (Ferguson and Read 2002, Hurd 2003). Hence, in many arthropod-borne disease systems there is a significant gap in knowledge concerning effects of parasites on vectors. Understanding the effects of parasites on vectors is important for understanding the effects of parasites on vertebrate hosts. Selection for avirulence in vectors (to facilitate transmission) could have correlated

effects on the evolution of virulence in the vertebrate host (Ewald 1994, Schmid-Hempel 2011).

Coevolutionary theory predicts that malaria parasites should have little or no effect on vector survival because such effects would decrease the probability of transmission to the vertebrate host (Dye and Williams 1995, Frank and Schmid-Hempel 2008). However, there may be unavoidable physiological costs of infection due to competition for limited resources between parasites and their insect hosts (Smith 2007). One way to test this resource limitation hypothesis is to examine a system where two sexes are used as vectors. In cases where breeding females experience a greater energetic cost of reproduction than males, the effect of malaria parasites on the vector should be greater for females than males. Furthermore, malaria parasites should reduce vector fecundity, rather than vector survival, because reduced fecundity will not hinder transmission. This pattern has been reported for malaria parasites in mosquitoes and sand flies, but never for a malaria parasite vectored by both sexes (Ferguson and Read 2002, Hurd et al. 2005, Schall 2011). In this study we compared the survival and

reproductive success of male and female vectors feeding on malaria-infected and uninfected hosts.

Determining the effect of a parasite on an insect host is challenging because individual insects are difficult to monitor in the field or rear in captivity (Cohuet et al. 2006, Tripet 2009). Some of the insect–parasite associations that have been used as captive models do not occur in nature (Ferguson and Read 2002). Unnatural insect–parasite interactions can have very different outcomes than interactions between insect hosts and the parasites with which they have coevolved (Randolph and Nuttall 1994, Cohuet et al. 2006).

We studied a natural assemblage consisting of wild-caught Rock Pigeon (*Columba livia*), the pigeon malaria parasite *Haemoproteus columbae*, and a hippoboscid fly vector (*Pseudolynchia canariensis*) that feeds on pigeon blood. *H. columbae* has a chronic effect on wild pigeons, leading to a gradual reduction in survival (Sol et al. 2003). However, symptoms of infection tend to be mild under captive conditions (Acton and Knowles 1914, Coatney 1933). Both male and female flies feed on birds, and *H. columbae* can complete its life cycle in either sex (Adie 1915). *P. canariensis* feeds for 20- to 80-minute bouts twice a day (Coatney 1931, Arcoverde et al. 2009).

At least 238 species of *Haemoproteus* are known to infect birds worldwide, yet relatively little is known about their effect on vectors (Valkiūnas 2005). *H. columbae* reproduces asexually in the avian host, and sexually in the vector, where gametocytes form a zygote, immediately followed by an oocyst. The oocyst attaches to the midgut wall of the vector, moving through the wall to the outside of the gut where it grows. After ~10 days, mature oocysts burst and the parasites (now called sporozoites) migrate to the salivary glands of the fly where they are transmitted with saliva during blood meals (Adie 1924). The sporozoites can cause physical damage to the salivary glands, especially in high numbers (Klei and DeGiusti 1973).

The life history of *P. canariensis* has features that make it amenable to study in the lab (see Plate 1). Eggs hatch in utero in the female fly, and then three stages of larvae feed from "milk" glands in the female fly (Harwood and James 1979). The larvae pupate and female flies deposit puparia in the substrate in or around pigeon nests (Bishopp 1929). The flies will reproduce on captive birds, depositing puparia under layers of newspaper lining pigeon cages. Female *P. canariensis* produce their first puparium six days after their first blood meal; they produce one puparium about every two days thereafter (Herath 1966, Klei 1971).

We tested the resource limitation hypothesis by quantifying the effect of *H. columbae* on the fitness of vector populations on pigeons with and without malaria infections. We predicted that malaria parasites would decrease the fitness of female flies more than male flies. In particular, parasites should decrease female reproduction rather than female survival, because reduced vector fecundity is not expected to hinder parasite transmission. We measured both fly survival and reproductive success, and we assessed offspring quality. In a separate feeding experiment, we also quantified the amount of blood taken by male and female flies on birds with and without malaria. The feeding experiment allowed us to test whether infected females are able to compensate for lost resources by feeding more.

METHODS

Pilot experiment

The purpose of this pilot experiment was to develop a protocol for infecting pigeons with *H. columbae* for use in our main experiment. Unlike *Plasmodium, Haemoproteus*-infected bird blood does not contain parasite stages that can infect another bird. Only the mature *Haemoproteus* sporozoites from the insect host can infect a new bird. Briefly, we fed flies on wild-caught pigeons that were naturally infected with *H. columbae*, allowed the parasites to mature in the flies, and then injected infective sporozoites from the flies into bird muscle tissue to generate new infections. Our methods were based on those of Ahmed and Mohammed (1977) and Atkinson and Forrester (1988).

Pigeon blood was drawn and blood smears were prepared and scanned to monitor the infection status of pigeons. Only naturally infected birds with more than two gametocytes observed per microscopy field at 1000× (~150 blood cells) were used to infect flies. Newly eclosed flies were allowed to feed on a single infected pigeon for an average of 12 days (range 10–13 days), sufficient time for malaria parasites to reach the sporozoite stage in 100% of flies (Adie 1915). After the feeding period, flies were removed from the pigeon and 25 flies were macerated in 1.5 mL of cold PBS (phosphate-buffered saline). Within 30 minutes of maceration, the resulting supernatant was injected into the pectoral muscle of a captive-bred pigeon that had not been exposed to *Haemoproteus* (confirmed by examining its blood for parasites prior to injection).

Immature forms of *H. columbae* (trophozoites) were visible in blood smears made 24 days post injection (dpi) and stained with Giemsa (diluted with buffer 1:10, pH 7.0, 50 min). Immature gametocyte stages were visible in the host blood at 27 dpi, and mature gametocytes were visible in the blood by 29 dpi. Parasitemia was high, with more than 10 parasites visible in each field of nonoverlapping RBCs at 1000×. Based on the positive results of this pilot experiment, we injected the supernatant from 15 infected flies into each of the pigeons in the main experiment.

Main experiment

We compared the fitness of flies feeding on pigeons with malaria to that of flies feeding on control pigeons. We trapped feral Rock Pigeons using walk-in traps in Salt Lake City, Utah, USA. Birds were housed individually for 8–24 months in wire mesh cages (30 × 30 × 56 cm) in fly-free animal rooms, and were fed ad
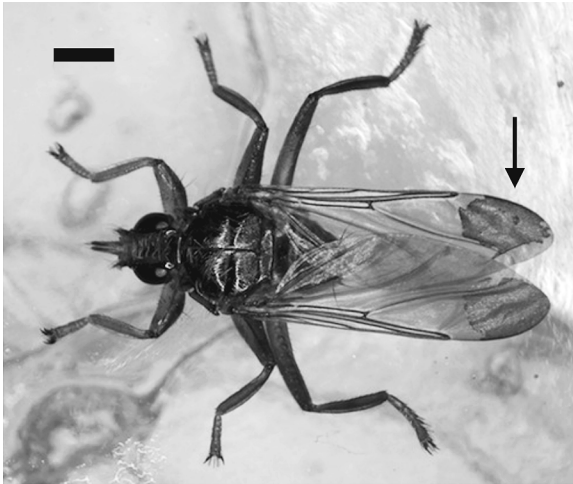
FIG. 1. Pigeon fly (*Pseudolynchia canariensis*) with color marks applied on each wing tip (arrow) for individual identification. The scale bar in the upper left corner is 1 mm.

libitum food, water, and grit. In the six months prior to the start of the main experiment, blood samples were checked at least three times per bird to confirm the absence of parasites (examination of blood smears for 20 minutes at 1000× magnification).

Twenty-eight uninfected pigeons were divided into 14 same-sex pairs (nine male, five female). One member of each pair was infected with *H. columbae* as previously described. The other (control) member was injected with the supernatant from an equal number of unfed, uninfected flies. *H. columbae* is not transmitted transovarially, so unfed flies cannot be infected with *H. columbae*.

Pigeons typically experience an acute infection lasting ~20 days after the onset of *H. columbae* infection in the peripheral blood; the parasite can persist at chronic levels for up to one year (Ahmed and Mohammed 1978). Two pigeons (one male, one female) in the experimentally infected treatment did not survive the initial phase of malaria infection. The two pigeons died at 24 and 28 dpi, which is when *H. columbae* is most abundant in the lungs and is just beginning to infect the peripheral blood. The two control birds paired with the birds that died were also removed from the experiment, leaving 12 pairs of same sex birds.

Prior to being exposed to flies, pigeons were fitted with "bits" to prevent them from preening off flies. Bits are small C-shaped pieces of plastic that are inserted into the nares (nostrils) of a bird; bits create a 1.0–3.0 mm gap between the mandibles, preventing full occlusion of the bill. They do not impair feeding or alter pigeon behavior (Clayton and Tompkins 1995).

Pigeons were housed in cages surrounded by fine netting (wedding veil) to prevent flies from moving between cages. We added 10 male and 10 female flies (≤2 days old and unfed) to each cage. Flies found dead in the bottom of the cage within 12 hours of starting the

experiment were sexed and replaced with a same sex fly that day. At no other point were flies replaced.

The experiment was terminated after five weeks to avoid the possibility of counting mature F1 flies when estimating the survival of the original (parental) population. It takes about a week for female flies to produce their first puparium, and four weeks for puparia to eclose (Herath 1966). Hipposboscid flies do not normally survive for more than five weeks after they eclose (Klei 1971, Arcoverde et al. 2007).

An experienced observer removed dead flies and puparia weekly from the bottom of each cage. A second experienced observer reexamined all cage material to make sure that nothing was overlooked. Rarely did flies escape from cages, but when this happened they were recaptured and returned to the appropriate cage or, if this was not possible, their escape was noted. The number of fly escapees did not differ significantly between treatments (three flies from control cages, five from experimental cages; Fisher's exact, $P = 0.724$). The number and sex of dead flies, as well as the number of puparia in each cage, were recorded weekly.

To test for an effect of treatment on offspring quality, we weighed haphazard subsamples of puparia from individual cages. Puparia were then placed in vials in an incubator set at 26°C and 20% relative humidity and were allowed to eclose. We calculated the average F1 survival time in vials after eclosion. Eclosed F1 flies were also sexed.

The number of puparia per female was calculated by dividing the number of puparia produced each week by the number of live female flies at the start of that week. Live female flies were estimated by subtracting the cumulative number of dead female flies from the initial starting number ($N = 10$) on each pigeon.

Feeding experiment

To determine whether *Haemoproteus* affects the feeding and off-host survival of flies, we conducted a second experiment. We cooled young flies (≤2 days old and unfed) on ice to immobilize them, and then marked their wings with a unique colored pattern using a permanent marker pen (Fig. 1). Each fly was weighed (within ±0.001 mg) and sexed, and was then placed on a pigeon (10 flies per bird). Male and female flies did not differ significantly in body mass prior to feeding (unfed male 4.80 ± 0.13 mg (mean ± SE); unfed female 5.03 ± 0.94 mg; *t* test, $t = 1.45$, $P = 0.15$, df = 149).

Populations of 10 flies were added to each of nine infected and nine uninfected pigeons, all of which were fitted with plastic bits and housed individually in cages surrounded by fine netting (as just described). Flies were allowed to remain on birds for 72 hours, providing ample time for them to feed.

Flies were weighed immediately after being removed from pigeons by ruffling their feathers while the bird was still inside a netted cage. Blood meal size was measured by comparing the mass of each fly before and after the
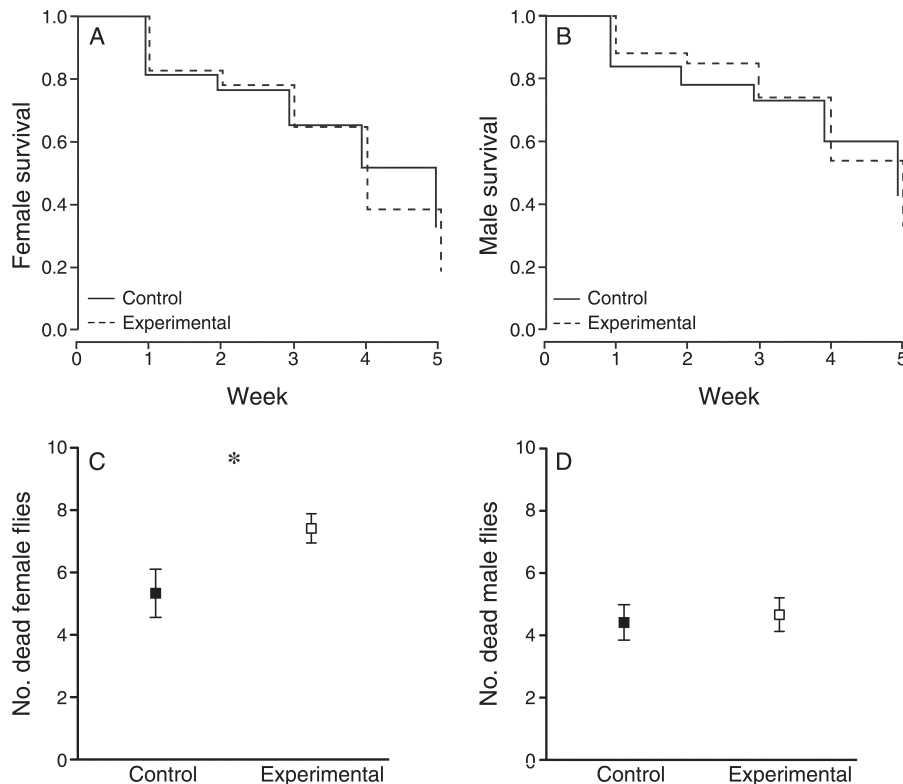
FIG. 2. (A, B) Proportion of flies surviving over time, by sex. Survival on experimental (infected) birds was less than that on control (uninfected) birds for female flies (A), but not male flies (B); see text. Cumulative (C) female and (D) male fly mortality (mean ± SE) at the end of the five-week experiment. The asterisk indicates a statistically significant difference ($P < 0.05$).

72-hour period on a pigeon. The rate of blood digestion was calculated by weighing flies again 24 hours after removal from the bird. Flies were monitored daily in vials in the incubator to determine longevity off the host.

Statistical analysis

Simple statistical analyses were carried out using Prism v. 5.0d (GraphPad Software 2010). Linear mixed-effects models were tested in R version 2.13.0 (R Development Core Team 2011) with the lme4 package (Bates et al. 2011). Survival analysis using a Cox proportional hazards model was also run in R with the survival package (Therneau and Lumley 2011) The Cox PH (proportional hazards) model is the most widely used regression model for survival data. It estimates the instantaneous risk of death for all times of death without making assumptions about the shape of the baseline hazard function (Crawley 2007).

RESULTS

Main experiment

Overall, fly survival was significantly affected by treatment and sex (Cox proportional hazards test (where exp(coef) is the exponential of the coefficient): for treatment, $P = 0.018$, exp(coef) = 1.34; for sex, $P = 0.011$, exp(coef) = 0.726) (Fig. 2A, B). Mortality of

experimental females (Fig. 2C; 7.42 ± 0.47, mean ± SE) was higher than that of control females (5.33 ± 0.77) (paired $t = 2.97$, $P = 0.012$, df = 11). In contrast, mortality of experimental male flies (Fig. 2D; 4.67 ± 0.54) did not differ significantly from that of control males (4.42 ± 0.57) (paired $t = 0.36$, $P = 0.72$, df = 11).

We compared the number of puparia for experimental and control treatments using a repeated-measures ANOVA (Fig. 3A); there was a significant effect of time ($F_{4,88} = 38.30$, $P < 0.0001$) and a marginally nonsignificant effect of treatment ($F_{1,22} = 3.52$, $P = 0.07$). There was no significant interaction between time and treatment ($F_{4,88} = 1.46$, $P = 0.22$). Given the lack of significant interaction, we reanalyzed the data without an interaction term, which required a linear mixed-effects models approach (repeated-measures ANOVA automatically generates an interaction term). We omitted Week 1 from the analysis because flies reproduced so little at the start of the experiment, regardless of treatment; this had the advantage of linearizing the data (Fig. 3B, C). We analyzed the data using a linear mixed-effects model with the interaction term present, and then analyzed the data with the interaction term removed. In the first case, as expected, the interaction term was not significant (time × treatment model: time × treatment, $P = 0.18$). With the interaction term removed, there were highly significant effects of both time and treatment
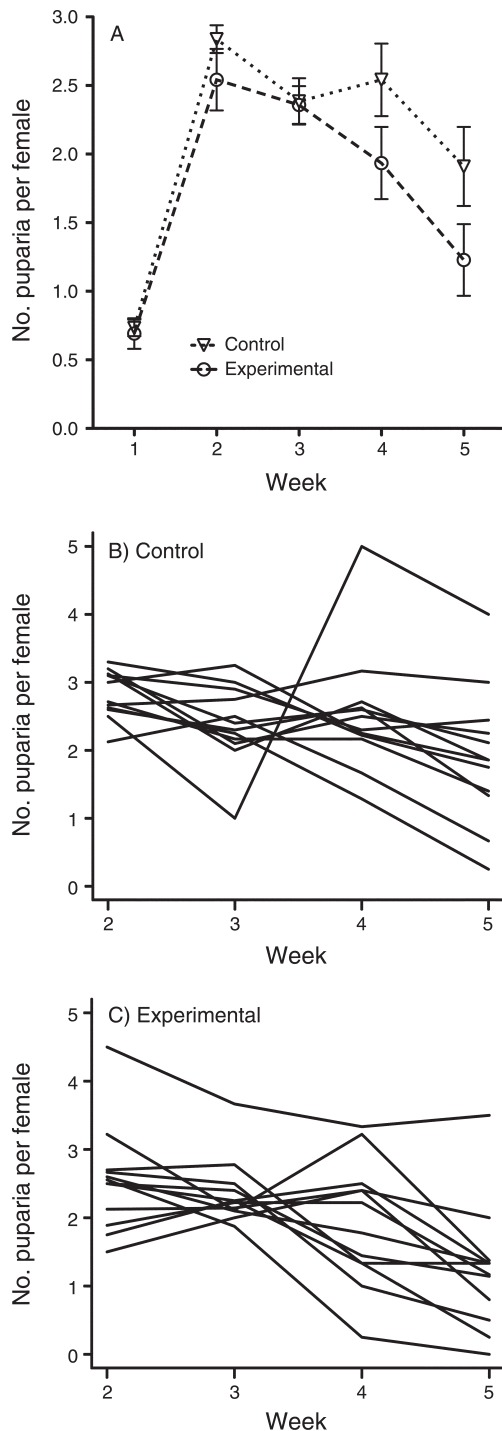
FIG. 3. Comparative reproductive success of flies on experimental (infected) and control (uninfected) birds: (A) Number of puparia deposited by female flies (mean ± SE), pooled for the 12 birds in each treatment. For individual birds, the mean numbers of puparia per week are shown for (B) control and (C) experimental birds. Flies on experimental birds produced significantly fewer puparia than flies on control birds.

PLATE 1. The hippoboscid fly, *Pseudolynchia canariensis*. Left to right: puparium, adult male fly, adult female fly. Photo credit: A. R. Henry.

(time + treatment model: time $P < 0.001$, treatment $P = 0.007$).

F1 offspring from experimental and control flies did not differ in sex ratio, body mass, or off-host survival (Table 1).

Feeding experiment

At the end of the experiment, 176 of 180 flies were recovered from pigeons (two flies from each treatment were missing, possibly because they were ingested by bitted birds). Treatment had no effect on fly mortality; 12 of 88 flies from infected birds died during the 72-hour experiment, compared to eight of 88 flies on uninfected birds (Fisher's exact test, $P = 0.35$). Treatment also had no effect on fly feeding (72/76 flies fed on infected birds, whereas 79/80 flies fed on uninfected birds; $P = 0.62$). The few flies that either died or were assumed not to have fed (no mass gain) were excluded from further analyses.

There was no effect of treatment on blood meal size (Fig. 4A; two-way ANOVA: for treatment, $F_{1,32} = 0.27$, $P = 0.61$). In contrast, blood meal size was strongly influenced by sex ($F_{1,32} = 12.46$, $P = 0.001$), with females ingesting larger meals than males, but there was no significant interaction between treatment and sex ($F_{1,32} = 0.21$, $P = 0.65$).

Neither treatment nor sex significantly influenced blood meal digestion over 24 hours (Fig. 4B; two-way ANOVA: for treatment, $F_{1,32} = 0.56$, $P = 0.46$; for sex, $F_{1,32} = 2.71$, $P = 0.11$), nor was there a significant interaction (treatment × sex, $F_{1,32} = 0.43$, $P = 0.52$).

Females survived off the host longer than did males (Fig. 4C; two-way ANOVA: $F_{1,32} = 5.51$, $P = 0.025$), but

TABLE 1. Sex ratio, mass, and off-host survival of the offspring of pigeon flies (*Pseudolynchia canariensis*) on experimental (infected) vs. control (uninfected) birds in the main experiment.

Parameter	Experimental ($N = 475$)	Control ($N = 587$)	P	Test statistic
Percentage male	47.9%	50.1%	0.53	†
Mass (mg)	8.45 ± 0.29	8.91 ± 0.25	0.31	$t = 1.09$, df = 7‡
Survival (days)	3.48 ± 0.14	3.32 ± 0.08	0.28	$t = 1.17$, df = 7‡

Notes: Survival was measured as the number of days that newly eclosed flies survived once removed from the host. For mass and survival, vales are given as mean ± SE.
† Fisher's exact test.
‡ Paired *t* test.

there was no effect of treatment on off-host survival ($F_{1,32} = 0.004$, $P = 0.95$), nor was there a significant interaction (treatment × sex, $F_{1,32} = 0.03$, $P = 0.87$).

DISCUSSION

Malaria parasites reduced the fitness of female vectors more than male vectors, as predicted by the resource limitation hypothesis. Parasites reduced female survival but had no effect on male survival. Malaria parasites had a marginally nonsignificant effect on female reproduction; however, after removing the nonsignificant interaction term, there was a highly significant effect of treatment on reproduction. There was no significant difference in either the size or survival of offspring produced by flies with and without exposure to malaria parasites, suggesting that females did not reduce investment in the offspring that they managed to produce.

Breeding female *P. canariensis* invest considerably more in reproduction than males. Females produce one large puparium at a time. Closely related flies, such as sheep keds and tsetse flies, also breed slowly, producing one offspring at a time (Askew 1971, Harwood and James 1979). Under the resource limitation hypothesis, females investing in energetically expensive reproduction may not be able to sustain the additional energetic cost of malaria infection without fitness consequences. Our results are consistent with this hypothesis: malaria parasites decreased female survival, but not male survival.

In cases in which infection causes increased mortality, infected females may be under selection to produce offspring as rapidly as possible (fecundity compensation; Schmid-Hempel 2011). Indeed, one study found that two species of mosquitoes exposed to malaria parasites produced significantly more eggs than mosquitoes that were unexposed (Ferguson et al. 2005). We found no evidence for fecundity compensation in our system. It might not be possible for female *P. canariensis* to compensate by increasing the number of offspring, given their slow reproductive strategy.

We also conducted an experiment to test the hypothesis that infected females can compensate for resources lost to malaria parasites by increasing their rate of feeding. Interestingly, increased feeding could conceivably also be adaptive for malaria parasites if it increases the rate of transmission to the vertebrate host (Koella et al. 1998). Blood parasites have been shown to manipulate vector feeding by changing the characteristics of the vertebrate host's blood, and/or by altering the vector's salivary glands (Klei and DeGiusti 1973, Rossignol et al. 1986). Alternatively, if flies are capable of detecting malaria parasites in bird blood, they might reduce blood meal size to limit their intake of parasites (Parker et al. 2011). Our feeding experiment allowed us to test for cumulative effects of malaria parasites on the feeding ecology of flies over a 72-hour period. However, our design did not test for shorter term effects on feeding (*P. canariensis* requires just 20–80 minutes to feed to repletion; Arcoverde et al. 2009). Our design also could
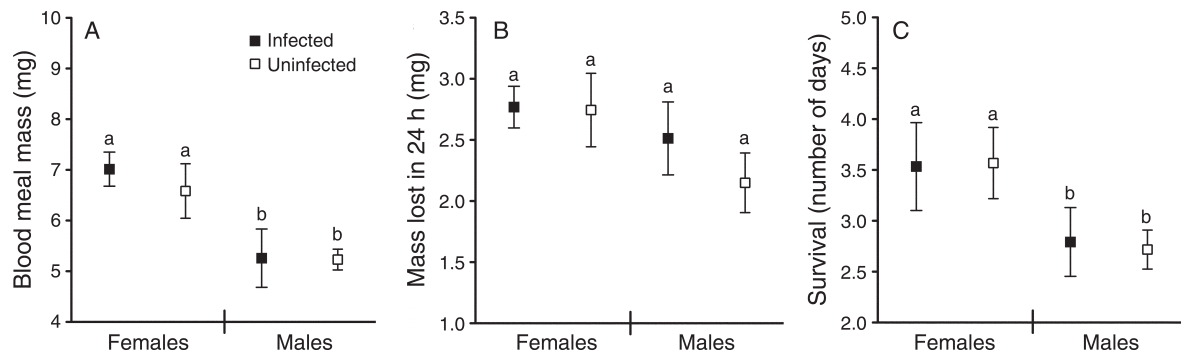

FIG. 4. Feeding experiment results (mean ± SE): (A) blood meal size of flies on infected and uninfected birds; (B) amount of blood digested (measured as mass loss) over 24 hours; (C) number of days flies survived after being removed from hosts. Different lowercase letters indicate significant differences ($P < 0.05$).

not test whether flies prefer to feed on uninfected hosts, because they were given no choice of host. These are two areas for future work.

In the feeding experiment, we did not find a significant effect of malaria parasite treatment on blood meal size, nor was there an interaction of treatment and sex on blood meal size. The digestion rate of blood meals (estimated by mass loss over 24 hours) did not differ between treatments. We infer from these results that the decrease in female survival in our main experiment was not due to interference of parasites with feeding. Off-host survival of flies in the feeding experiment also did not differ significantly for flies that fed on birds with and without malaria; therefore, we have no reason to think that the quality of blood meals differed between treatments. In our feeding experiment, female flies took meals that were up to 40% larger than those of males. Females may lose more energy to malaria parasites than do males if they ingest more parasites in these larger blood meals. It is conceivable that larger blood meals might also expose females to more immunological defenses (Owen et al. 2009), placing them under even greater stress when feeding on malaria-infected blood.

The relative importance of differential reproductive costs vs. differential parasite ingestion to female fly survival could be tested with an experiment in which virgin and mated females are placed on infected and uninfected hosts, with their survival compared over time. If the mortality of mated females were higher than that of virgin females on infected hosts, this would provide further support for the resource limitation hypothesis.

Studies of natural vector–malaria parasite associations are rare (Ferguson and Read 2002). Studies of systems in which parasites are vectored exclusively by female flies tend to find effects on reproduction, but not survival (reviewed by Hurd et al. 2005). For example, in sand flies that vector lizard malaria parasites, only reproduction is affected, not mortality (Schall 2011). Indeed, in nearly every natural malaria parasite study that shows a cost of infection to the vector, the effect has been on vector fecundity; significant effects on survival have seldom been found (Ferguson and Read 2002). Our results provide an interesting exception in a natural malaria parasite system in which both insect sexes are vectors. Perhaps when the parasite has an alternate vector (males, in this case), there is reduced selection for avirulence in the more resource-limited vector (females), so long as the less resource-limited vectors (males) provide adequate transmission opportunities. Future work to compare the malaria parasite transmission efficiency of male and female insects is needed for this system and others in which both insect sexes can transmit parasites. Other factors, such as how often male flies move between pigeons, relative to how often females move, may also influence parasite transmission and would be interesting to study.

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